CONTUSIVE SPINAL CORD INJURY:
ENDOGENOUS RESPONSES OF DESCENDING SYSTEMS AND EFFECTS OF
ACUTE TRANSPLANTION OF GLIAL RESTRICTED PRECURSOR CELLS

DISSERTATION

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By
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ABSTRACT

Contusive spinal cord injury (SCI) in the rat mimics many of the aspects of human injury. It provides a good model for examination of endogenous responses to injury, and alterations that occur following therapeutic manipulations. Damage to the spinal cord, results in cell death at the injury site and the development of a chronic cystic lesion cavity, separated into chambers by tissue bridges (called trabeculae), and from the spared white matter by a glial and molecular scar. Transplantation of stem cells and immature cells can ameliorate tissue damage, induce axonal regeneration, and improve locomotion. However, unless these cells are pushed down a neuronal lineage, the majority of cells become glia; suggesting that the alterations observed are potentially glially mediated. Glial restricted precursor (GRP) cells— a precursor cell population restricted to oligodendrocyte and astrocyte lineages— offer a novel way to examine the effects of glial cells after injury.

The studies performed within, examine the endogenous responses of descending axons from the cortex and brainstem to SCI, and how they respond to acute transplants of GRP cells. The survival and differentiation of GRP cells, and their ability to modulate the development of the lesion is also examined. GRP cells isolated from a transgenic rat that ubiquitously expresses human placental alkaline phosphatase (hPLAP) were used to specifically and robustly detect transplanted cells. Following transplantation into acute
SCI lesions, GRP cells retained their differentiation potential and appeared to differentiate in a site-specific manner into astrocytes and oligodendrocytes. Transplanted GRP cells altered the lesion environment, reducing astrocytic scarring and the expression of inhibitory chondroitin sulphate proteoglycans (CSPGs). Normally after injury descending systems from the cortex and brainstem initiated endogenous sprouting responses after injury, but these responses were limited and delayed. Transplanted GRP cells supported axonal growth from corticospinal tract (CST) and raphespinal axons, and altered CST retraction bulb morphology towards that of growth cones. However, this did not result in long distance axonal regeneration or locomotor recovery 6 weeks after injury. Acute transplantation of GRP cells can produce a growth promoting environment, similar to that produced by immature astrocytes, that alters the axonal response by six weeks. By differentiating into both astrocytes and oligodendrocytes, GRP cell-derived-oligodendrocytes could potentially mediate recovery after SCI by either promoting axonal growth or remyelinating spared demyelinated axons around the lesion. Further experiments are required to determine if the axonal changes observed at six weeks correspond to long-distance regeneration at later time points, or if GRP cells can affect recovery through remyelination.
Dedicated to my Nana
I would like to thank all the members of the B&B lab for their assistance, advice and technical expertise, without which these experiments would not have been possible. In particular, I gratefully acknowledge: Drs. Gerlina Hermann and Gregory Holmes for teaching me the fluoro-ruby injections; Amy Tovar, (whose outstanding technical skills and dedication did not go unnoticed) for teaching me the many aspects of animal surgery and care and for taking care of the GRP cells at Ohio State; Tina Van Meter, for sharing her histological and immunohistological expertise; John Komon for his graphical advice and poster making and for teaching me the in’s and out’s of Photoshop; and John Gensel and Fang Sun for their assistance and contributions.

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I would like to thank my family for teaching me to be independent and self-confident, thus giving me the foundation to succeed. Especially, I would like to thank my mom for reminding me that ‘life is not fair’ but that hard work and perseverance pay off; and my dad for teaching me to pay attention to detail, and to know the difference between ‘careless and careful’. Thank you, Susannah and Michael for being both siblings
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CHAPTER 1

INTRODUCTION

Spinal cord injury (SCI) can result in a permanent loss of motor and sensory function below the level of the lesion. Initial mechanical damage at the impact site is followed by expansion of the injury by secondary events. The net result is the formation of a cystic cavity surrounded by a small spared rim of white matter. Many therapeutic interventions aimed at treating SCI target acute or sub-acute events after injury, to prevent cavity expansion and increase white matter sparing, in hopes of reducing the underlying pathology and increasing spared function. However, even with methylprednisolone, the only current therapeutic treatment for humans, spinal cord injury results in paralysis. With over 250,000 Americans living with chronic SCI, and 11,000 new cases each year the need for new, effective therapies that prevent tissue damage and initiate reparative mechanisms is paramount.

Damage to the spinal cord results in a loss of target neurons at the level of the injury, and a loss of input to neurons distal to the injury. Once axons are severed and primary and secondary damage has occurred, recovery from spinal cord injury requires that target neurons regain lost synaptic input. And, in the case of injuries to the cervical and lumbar enlargements, lost neurons may need to be replaced. The spinal cord is able
to initiate endogenous repair responses, and some function is regained after injury by remyelination of spared fibers, and reorganization of distal circuits by sprouting of spared fibers. Function can be further improved by locomotor training, which likely further and reorganizes distal circuits. Endogenous repair responses indicate that the spinal cord is quite plastic and amenable to change. However, endogenous responses are insufficient for behavioral recovery, in part due to the limited regeneration of axons after injury in the adult CNS.

It is well established that at least some injured adult neurons retain their intrinsic ability to sprout and regrow after axotomy (Beattie et al., 1997; Li and Raisman, 1995; Cajal S.R., 1928). Despite the intrinsic repair that occurs after injury and the innate ability of axons to sprout, most axons fail to traverse the lesion site. A number of possible mechanisms have been postulated for the lack of axonal regeneration following SCI, including, a lack of substrate, insufficient growth promoting molecules, the presence of inhibitory molecules, and the formation of an impenetrable physical and molecular lesion scar (reviewed in (Stichel and Muller, 1998)). The environment in which the axons must travel is a major cause for the lack of axonal extension and the failure of axons to reestablish synaptic connections after injury.

Cellular transplantation is one of many avenues of research used to explore the changes that occur after injury, in an attempt to alter the final outcome. The cell type transplanted dictates in part how the transplant will act. Cells from a variety of source have been transplanted into the CNS: peripheral nerves (Cheng et al., 1996; David and Aguayo, 1981); Schwann cells (Li and Raisman, 1997; Xu et al., 1995a; Xu et al., 1995b;
Paino et al., 1994); olfactory ensheathing glia (OEG) (Ramon-Cueto et al., 2000; Ramon-Cueto et al., 1998; Li et al., 1997); macrophages (Rapalino et al., 1998); microglia (Rabchevsky and Streit, 1997); fibroblasts (Liu et al., 1999b; McTigue et al., 1998; Blesch and Tuszyński, 1997); fetal cells (Coumans et al., 2001; Bregman et al., 1997; Jakeman and Reier, 1991); stem cells (Cao et al., 2001; McDonald et al., 1999); precursor cells (neural progenitor cells, glial progenitor cells) (Herrera et al., 2001; Yang et al., 2000; Olby and Blakemore, 1996; Warrington et al., 1993; Vignais et al., 1993). Some transplanted cells have been modified to secrete neurotrophins to supplement axonal growth (Liu et al., 1999a; Liu et al., 1999b; McTigue et al., 1998; Menei et al., 1998; Blesch and Tuszyński, 1997). Generally transplanted cells are used to replace lost cells, act as a relay, form a tissue bridge, supplement the environment with growth promoting molecules, or alter the production of growth inhibitory molecules.

Transplantation experiments have resolved a number of basic biological issues. Using peripheral nerve grafts Aguayo and colleagues (1981) demonstrated that adult axons are capable of long-distance regeneration. Others have shown that transplanted cells can integrate into the host tissue and form functioning cells. For example, transplanted Schwann cells, OPCs (oligodendrocyte progenitor cells), and ES-derived oligospheres form myelin (Herrera et al., 2001; Liu et al., 2000; Paino et al., 1994; Groves et al., 1993) and improve axonal conductance (Kocsis, 1999), and fetal tissue can form synapses within the host and the transplant (Bregman et al., 1993; Jakeman and Reier, 1991). Rarely have these proof-of-principle experiments corresponded to functional changes after injury. Instead, cellular transplantation usually results in increased sprouting and
limited behavioral improvement. Increasingly, transplantation is focusing on the use of immature cells (stem cells, precursor cells, fetal cells) or is being mixed with other growth promoting factors either through exogenous application of growth promoting molecules or through transplantation of genetically modified cells (Coumans et al., 2001; Menei et al., 1998; Grill et al., 1997; Xu et al., 1995a). Recently there have been reports of behavioral recovery following transplantation of stem cells (McDonald et al., 1999), suggesting that immature cells that can differentiate into multiple phenotypes may be beneficial. As well, the use of myelinating or ensheathing cells has shown promise for both regeneration and functional recovery (Takami et al., 2002b; Ramon-Cueto et al., 2000; Ramon-Cueto et al., 1998; Li et al., 1997). How stem cells improve functional recovery after SCI remains to be established. The relatively small number of neurons formed following transplantation suggests that it is unlikely due to neuronal replacement. More likely, stem cells improve function through remyelination, regeneration or reduced environmental inhibition.

A number of factors present after injury inhibit axon growth and contribute to regeneration failure. Following injury, astrocytes hypertrophy and wall off the lesion site. Their interaction with infiltrating cells results in the formation of a glia limitans. Together with the molecules expressed within, the glia limitans is thought to form an impenetrable barrier to axonal growth (reviewed in Hoke and Silver, 1996; also see Fawcett and Asher, 1999; Stichel and Muller, 1998; Canning et al., 1996; McKeon et al., 1991). There is an increasing list of molecules present after injury that inhibit axonal growth including, NOGO and MAG (myelin associated glycoprotein) two molecules expressed by myelin
(McKerracher et al., 1994; Cadelli et al., 1992), proteoglycans (Hoke and Silver, 1996), semaphorins (De Winter et al., 2002), and matrix-metalloproteinases (deCastro RC. et al., 2000). Modest improvement of axonal regeneration can be achieved by removing any given inhibitory molecule through neutralization or knocking out (Noble et al., 2002; Merkler et al., 2001; Stichel and Muller, 1998; Schafer et al., 1996; Schnell and Schwab, 1990). Recently, digestion of CSPGs resulted in increased axonal regeneration (Bradbury et al., 2002; Moon et al., 2001) and improved behavior, as did neutralization of matrix-metalloproteinases (Noble et al., 2002) or NOGO (Merkler et al., 2001) supporting the idea that environmental inhibition can be overcome to increase axonal growth (Schnell and Schwab, 1990). However, this increase is usually limited to a small percentage of fibers, suggesting that multiple mechanisms will be required to encourage axons to grow across the lesion site.

The complexity of changes within the lesion environment following CNS trauma is one of the major inherent difficulties in studying CNS regeneration in vivo. This is further complicated in many injury models by partial tissue sparing. However, the development and characterization of reliable and reproducible injury models, and subsequent standardized behavioral tests has greatly enhanced our ability to assess cellular changes after injury and ensuing recovery.

This study uses the contusive spinal cord injury model that mimics many of the properties of human spinal cord injury, to look at the endogenous changes that occur after injury and how these changes are affected by transplantation of spinal cord precursor
cells. Endogenous responses of descending axons from the cortex and the brainstem are characterized from 1 day to 8 months after injury (Chapter 2). The remaining chapters focus on transplantation of glial restricted precursor (GRP) cells. GRP cells are multipotent cells that can differentiate into oligodendrocytes and astrocytes in vitro and in vivo and are a novel cell type for transplantation in that they can potentially mediate recovery after spinal cord injury through remyelination of axons or by providing a substrate for axonal growth. The survival and differentiation of transplanted GRP cells after spinal cord injury is assessed (Chapter 3), and their ability to modulate the injury response at 8 days (Chapter 4) and 6 weeks (Chapter 5) is discussed in the context of glial scarring and axonal growth.

These studies add to the characterization of contusion injury and also to the growing literature on the effects of cellular transplantation after SCI. They reveal that following contusive SCI descending axons produce limited and delayed sprouting response, and persist at the lesion margin for a very long time after injury. Therefore, they may be amenable to either acute or chronic manipulations. Using GRP cells derived from transgenic rats, transplanted cells were specifically and robustly detected after transplantation, overcoming a persistent problem for transplantation studies. This is the first study to show graft survival and differentiation using transgenic PLAP cells after CNS injury; a tool that will be very useful in future transplant survival, migration and differentiation studies. Transplanted GRP cells retained their bipotentiality after injury, and were able to alter the formation of the glial scar, and the deposition of inhibitory
molecules, resulting in modest axonal sprouting. Further studies are required to ascertain, the long-term functionality of these cells, and the optimal transplantation time.
A number of studies have examined the responses of identified descending spinal axons to injury. The purpose of many of these studies has been to determine whether descending axons have the capacity to regenerate or sprout either without treatment or after some intervention. It is becoming increasingly clear through studies using the hemisection and transection models of spinal cord injury that descending axons retain their ability to regenerate, with different axonal populations responding to specific exogenous treatments. For example, application of antibodies that block inhibitory molecules in the cord, or application of neurotrophins can increase sprouting and regeneration of corticospinal tract (CST) axons (Bregman et al., 1995; Schnell et al., 1994; Schnell and Schwab, 1993; Schnell and Schwab, 1990), while transplantation of Schwann cells (Menei et al., 1998; Chen et al., 1996; Xu et al., 1995b) or olfactory ensheathing glia (Ramon-Cueto et al., 2000; Ramon-Cueto et al., 1998; Ramon-Cueto and Avila, 1998) into a complete transection can increase sprouting and elongation of propriospinal and brainstem spinal axons. In general, it has been shown that axotomy of descending tracts is followed by a period of die-back, and abortive attempts at sprouting
and regeneration, similar to that described by Ramon y Cajal in unidentified axons (Cajal S.R., 1928). Experiments using transection and hemisection models of SCI have provided an important baseline for analysis of the effects of treatments on regeneration and sprouting of specific axonal systems, along with functional indices of recovery (see Schwab and Bartholdi, 1996 for a review).

Recently, there has been a renewed interest in studying contusion or compression injuries of the cord as a model of human SCI. We and others (Rosenberg and Wrathall, 1997; Basso et al., 1996; Martin et al., 1996; Guizar-Sahagun et al., 1994; Bresnahan et al., 1991; Noble and Wrathall, 1989; Iizuka et al., 1987; Noyes, 1987; Noble and Wrathall, 1985; Bresnahan, 1978; Balentine, 1978a; Balentine, 1978b) have described the development of the contusion lesion cavity over time. Initially, at the injury site, petechial hemorrhages occur in the gray matter and are followed by a rapid centripetal and rostro-caudal development of frank necrotic damage and the eventual development of a cystic cavity. The secondary expansion of the cavity is associated with the invasion of peripheral immune cells as well as the activation of resident microglia (Beattie et al., 1997; Popovich et al., 1997; Shuman et al., 1997; Blight, 1992). Axonal destruction and die-back occur during this same time. Chronic contusion cavities are occupied by axons as well as tissue bridges or trabeculae, some of which arise from the ependymal zone and dorsal root entry zones. The source of the axons in the cavity tissue is not known although many of them are thought to arise from the dorsal roots (McTigue et al., 1998). Although a number of detailed histological studies have been done that describe the contusion injury lesion (Rosenberg and Wrathall, 1997; Basso et al., 1996; Martin et al.,
1996; Guizar-Sahagun et al., 1994; Bresnahan et al., 1991; Noble and Wrathall, 1989; Iizuka et al., 1987; Noyes, 1987; Noble and Wrathall, 1985; Bresnahan, 1978; Balentine, 1978a; Balentine, 1978b), there is little information about the effects of contusion injuries on identified descending or ascending spinal cord axons.

The purposes of the present study were 1) to describe the initial axonal response to contusion injury in an identified axonal population, 2) to determine whether and when sprouts grow in the face of the expanding contusion cavity, and 3) in the long term, to see whether any of these sprouts might contribute to the axonal bundles that have been seen within the chronic contusion lesion cavity (Beattie et al., 1997; Quencer and Bunge, 1996; Bunge et al., 1993). The design of the experiment also allowed us to further characterize the development of the lesion cavity after injury.

The CST was chosen for study as this tract is completely obliterated even by mild contusion injuries (Basso et al., 1994) since most of its axons occupy the region of the spinal cord just above the central canal in the rat. We were thus able to examine the initial destruction, the degree of sprouting and regeneration, and the possible contribution of this tract to axons within the contusion lesion cavity. We also looked to see whether reticulospinal tract (RST) axons from the gigantocellular-lateral paragigantocellular reticular nucleus (Gi-LPGi) contribute fibers to the cavity trabeculae as well. These axons tend to run in a peripheral position in the lateral and ventral white matter where sparing is prevalent especially after mild-moderate contusion lesions. We observed considerable sprouting of the CST proximal to the contusion lesion and a very small number of axons entering the trabeculae especially at long survival times. RST axons were considerably
more likely to grow along the trabeculae into the lesion cavity. These data suggest that descending fibers from the cortex and the Gi-LPGi do contribute axons to the trabeculae, although in a limited and delayed manner, and that endogenous repair continues for a long time after contusive SCI.

MATERIALS AND METHODS

Subjects. Thirty-three adult Long Evans female rats (Simonsen Laboratories) were used for this study. Animals received one of three contusion injuries as described below, 6.25 mm (n=4), 12.5 mm (n=26) or 25 mm (n=3) impact. The cortex (n=23) or the brainstem (n=10) was injected with fluoro-ruby (FR; 1% w/vol; Molecular Probes, Eugene, OR) to label axons descending through the spinal cord. All procedures were carried out using aseptic technique and in compliance with NIH guidelines and were approved by the Institutional Laboratory Animal Care and Use Committee. Animals were housed in standard plastic cages and were given food and water ad libitum.

Experiment 1. To examine the direct effect of contusion spinal cord injury (SCI) on a select population of fibers, the corticospinal tract (CST) was labeled 1 month prior to SCI (n=8). The rats were sacrificed at 1,3,8, or 21 days (n=2/time point) after a moderate (12.5mm) contusion injury made with the NYU device (Gruner, 1992).

Experiment 2. To examine the long-term effect of SCI on CST axons, animals sustained a SCI and were allowed to recover for either 14 weeks (n=7; 12.5mm injury) or 6.5-8 months (n=8; 12.5 mm injury, n=4; 6.25mm injury, n=4) before sacrificing. One-
month prior to sacrifice the CST was labeled. These cases were used to evaluate the status of CST axons at the lesion interface and beyond after chronic SCI.

**Experiment 3.** To examine the long term effects of SCI on a reticulospinal tract (RST), brainstem-spinal axons were labeled with FR one month prior to sacrifice at 14 weeks (n=7; 12.5mm injury; 4/7 had good labeling and were analyzed) or 5.5-7 months (n=3; 25mm injury) after SCI.

**Spinal Cord Injury Procedures:** Rats were anesthetized with 40-60 mg/kg of pentobarbital i.p. (Abbott Laboratories, Chicago, IL). Using aseptic technique a laminectomy was performed to expose the dura of the spinal cord at T 9-10. A contusion injury was induced using the NYU/MASCIS device by dropping a 10g weight from 6.25 mm (n=4), 12.5 mm (n=18), or 25 mm (n=3) onto the exposed dura of the spinal cord (Constantini and Young, 1994; Gruner, 1992). The wound was closed in anatomical layers and rats were placed singly in incubators overnight. Subsequently, they were housed in pairs in standard plastic cages and given food and water *ad libitum*. Keflin (25 mg/kg) (Lilly, Indianapolis, IN) was given before surgery and twice a day for the first week after surgery to prevent infection. Bladders were expressed twice a day until reflex urination returned.

**Labeling Procedures:** Rats were anesthetized with 40-60 mg/kg of pentobarbital (Abbott Laboratories, Chicago, IL), given 4 mg/kg of dexamethesone (American Regent Laboratories, Inc., Shirley, NJ) s.c. to minimize cerebral edema and 1mg/kg s.c. of atropine (Fujisawa USA, Inc., Deerfield, IL) to maintain airway patency. Twenty-five mg/kg s.c. of keflin antibiotic (Lilly, Indianapolis IN) was administered as above for SCI.
surgery. The rats were placed in the stereotaxic device and the scalp was incised to expose Bregma and Lambda. Burr holes over the intended injection sites were made with a number 9 dental burr and enlarged with rongeurs. One percent (w/v) FR (25mg/2.5ml) was pressure injected through a pulled glass pipette (Radnoti star-bore tubing: Radnoti, Monrovia, CA) with a beveled tip (20-40 µm in diameter). Following surgery the rats were housed singly in incubators overnight. The rats were housed in standard plastic cages singly until incisions healed and doubly thereafter.

For labeling CST axons, 3 injections (100 nl each) of FR were made into the sensorimotor cortex (anterior-posterior (AP) -0.7 mm, -1.4 mm, -2.3/-2.5 mm, medial-lateral (ML) +/-2.3 mm, depth (D) –1.5 mm) on each side of the brain, using Bregma as a landmark for AP and ML and the dura as a landmark for depth. For labeling RST axons, bilateral injections (200 nl each) of FR were made into the Gi-LPGi (AP –11.3 mm, ML +/-1.3 mm, D –9 mm), according to the rat brain atlas by Paxinos and Watson (Paxinos and Watson, 1998).

Perfusion procedures: Animals in Experiment 1 were perfused 1, 3, 8 or 21 days after SCI, while those in Experiments 2&3 were perfused 3.5-8 months after SCI. All rats were terminally anesthetized with 80 mg/kg of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 10 mg/kg of xylazine (Vedco, Inc, St. Joseph, MO) and transcardially perfused with 0.9% saline; 0.5 ml of lidocane (Elkins-Sinn, Inc, Cherry Hill, NJ) and 0.1 ml of heparin (Elkins-Sinn, Inc, St. Joseph, MO) were injected into the line at the initiation of perfusion to dilate vessels and inhibit clotting. The saline was
followed by 1 L of 4% paraformaldehyde (Fisher Scientific). The brain and spinal cord were then removed and stored in 4% paraformaldehyde.

**Histological Procedures:** A 10-15 mm block containing the lesion center was excised, placed in 30% sucrose in PBS overnight, and then cut horizontally on a cryostat. In Experiment 1 horizontal sections were cut at 20 µm, whereas in Experiments 2&3 they were cut at 30 µm. Sections were mounted on glass slides coated with gelatin, dehydrated and coverslipped with entellan mountant (Electron Microscopy Science, Fort Washington, PA). To verify the accuracy of the location of the FR injections, the injection sites were prepared for histological analysis. The brains were placed in 30% sucrose overnight and cut transversely at 60 µm on either a freezing microtome or cryostat. Sections were mounted on glass slides coated with gelatin, dehydrated and coverslipped with entellan. Alternate brain sections were taken for staining with cresyl violet to evaluate the location of the injection sites.

**Data Analysis**

*Analysis of injection sites:* The locations of the injection sites were evaluated to ensure consistency between the animals. Tissue slices containing the injection sites were drawn using a trisimplex projector (Bausch & Lomb, Rochester NY); the location of the injection and the spread of the FR were mapped onto the drawings. Alternatively, a fluorescent stereo microscope was used to evaluate the injection sites. Images captured using a digital camera were directly imported into a Pentium II computer (Dell, www.dell.com) and serial composites were arranged using Photoshop (Adobe Systems Inc., San Jose, CA) and then compared.
Analysis of axon distribution: A fluorescent stereo microscope (Zeiss Ste SV11) with the FITC (575 nm) filter set was used to show red blood cells (RBCs) and the lesion cavity to advantage at low magnification. For microscopic examination, the spinal cord tissue was viewed through a Nikon fluorescent microscope using a rhodamine filter cube (480 – 535 nm wavelength). A detailed description of the appearance of fluorescently labeled axons around the lesion cavity was made by assessing the distribution of labeled axons around the cavity in each tissue section, the extent of sprouting, the location and extent of retraction bulbs, and the relationship of the fibers with the lesion interface. One cord from Experiment 2 (6.25 mm injury; 8 months survival case) was used to make detailed tracings of the distribution of labeled CST axons around the cavity (see Fig. 2.7C). The tissue image was projected from the fluorescent microscope onto a monitor. Fluoro-ruby labeled axons, as seen on the monitor with the 16X objective (and doubled checked through the eyepiece), were drawn onto acetate sheets to produce a serial montage of axons around the lesion cavity. Every other tissue section was drawn through the dorsal CST and ventral CST.

Axon counts: To quantify the number of axons around the cavity, axons were counted in 5 tissue slices per cord for CST cases (Fig. 2.1) and on 8 tissue slices per cord for RST cases. Counts were made at -1.5, -0.5, 0, 0.5, 1.0, 1.5, 2.0 mm from the rostral-most part of the lesion cavity at the level of the central canal. Axon counts were made on all CST cases (n=23), and on 2 RST cases (5.5–7 months survival). All axons were counted from the monitor image and double-checked for accuracy through the
microscope eyepiece with the 16X objective (Nikon Microphot FXA microscope: Tokyo, Japan), or 20X objective (Axioplan2, Carl Zeiss, Hallergmoos, Germany).

*Cavity length:* The length of the cavity was measured in the section from the level of the central canal off the low power images taken with the fluorescent stereo microscope (Zeiss STE SV11).

**RESULTS**

*Development of the Cavity After Contusion Injury*

Most cases (26/33) received a 12.5 mm injury and were sacrificed at a range of times from 1 day to 8 months. Therefore, this group of animals provided the opportunity to observe the progression of cavitation over time in cases with a consistent injury.

*The Acute/Subacute Contusion Injury Cavity.* Figure 2.2 shows longitudinal sections through the center of the injury site in representative cases from 1 day to 14 weeks after a 12.5 mm contusion injury. One day after this injury, obvious signs of tissue damage were observed in the impact epicenter as well as areas distal to it. The impact site was hemorrhagic, but no cavitation was present (Fig. 2.2A 1d, 2B). Red blood cells appeared as small, round, extremely autofluorescent cells with concave centers (Fig. 2.2B&C, arrow). These characteristics made the extensions of the hemorrhage easy to detect. The hemorrhage was concentrated within a 5 mm long central core area (Fig. 2.2A 1d), predominantly in the gray matter, but also appeared in the white matter in patches intermixed with areas of severe axonal damage in the white matter (Fig. 2.2C). Hemorrhagic areas were surrounded by normal looking tissue, especially in lateral and
ventral areas of the cord. Extensions of the hemorrhage were observed at the interfaces of gray and white matter up to 5 mm rostral to the impact site especially at the base of the dorsal funiculus (Fig. 2.2A 1d (arrow)).

The boundaries of the injured zone were detectable by three days after injury (Fig. 2.2A 3d). The core of the impact site was filled with blood cells intermixed with damaged axons (Fig. 2.2D). Fewer RBCs were present than at 1 day post injury (dpi) and the damaged zones appeared to be coalescing (Fig. 2.2A 3d). Macrophages could be observed to infiltrate the rim of spared white matter and the cavity laterally (Fig. 2.2D, E). RBCs and the newly infiltrating macrophages no longer tightly abut the spared lateral white matter (Fig. 2.2E), permitting lesion boundary demarcation (Fig. 2.2A 3d). The long extension of RBCs in the dorsal columns was maintained, but extensions of RBCs along the gray-white interface were less apparent than at 1 dpi.

Eight days after injury, the injury site was filled with macrophages and the borders of the lesion were more definable (Fig. 2.2A 8d). The lesion had enlarged somewhat with maximal extension occurring at the base of the dorsal funiculus rostrally and caudally. A massive number of densely packed macrophages were observed in the middle of the injured zone (Fig. 2.2F) replacing the RBCs observed at 1 and 3 dpi. Some macrophages were seen intermixed with fibers in the spared rim of white matter. At the gray white interface, patches of densely packed macrophages could sometimes be observed directly apposed to regions with few macrophages. Whereas, in other regions macrophages and white matter had separated leaving open spaces (Fig. 2.2G, small arrow). Non-fluorescent cells within the cavity aggregated as scattered bands (Fig. 2.2G,
block arrow); a prominent band was observed around the area of the central canal. These bands of cells appeared to be the first signs of the forming trabeculae or tissue bridges which would later divide the cavity (Beattie et al, 1997).

By twenty-one days, a clearly defined cavity was present (Fig. 2.2A 21d) surrounded by a thin rim of spared white matter. Macrophages were still present within the lesion (Fig. 2.2H, I). Macrophages had retracted away from the edges of the cavity in several locations, while maintaining contact with the edge of the cavity in other regions (Fig. 2.2A 21d). The beginnings of trabeculae (Fig. 2.2H, arrow) were clearly observed between clusters of macrophages (Fig. 2.2H, block arrows). Isolated groups of unattached macrophages, were present in the lesion cavity (Moriarty et al., 1998) (Fig. 2.2I, block arrows). Cords of cells connected to the surviving tissue were prominent around the edges of the cavity and around the area of the central canal, which had expanded. Cells in these trabeculae were elongated and appeared similar to the ependymal cells.

**The Chronic Contusion Injury Cavity.** The chronic lesion cavity was fully developed at fourteen weeks after injury, and persisted without change over the 8 months examined (Fig. 2.2A 14w; Fig. 2.7 F-H). The maximal extensions of the cavity occurred in the dorsal funiculus rostrally and caudally. The walls of the cavity were smooth, and separated the fluid filled cavity from the surrounding spared white matter. A cellular infiltrate was located within the cavity and was attached by trabeculae to the edges of the cavity at various points (Fig. 2.2A 14w). Auto-fluorescent macrophages were intermixed with other cells in the cellular infiltrate and in the trabeculae (Fig. 2.2J, K). Trabeculae appeared either fibrous (Fig. 2.2J) or cellular (Fig. 2.2K) and were likely formed by
Schwann cells, fibroblasts, astrocytic extensions, collagen and ependymal cells (Guizar-Sahagun et al., 1994). Macrophages were present in the tissue at the rostral and caudal edges of the cavity. In addition, the rostral tip of the cavity contained a cellular infiltrate (Fig. 2.2L) that extended at the base of the dorsal columns at least 5 mm (the maximum length of the tissue section).

Location and distribution of labeled descending CST fibers

Experiment 1

Following injection of FR into the sensorimotor cortex, an area of intense fluorescence surrounded by a halo of decreased fluorescence extended 3-4 mm in the cortex dorsal to the corpus callosum (data not shown). In the spinal cord rostral to the lesion, the location of FR labeled axons within the CST was similar in our study to those described previously (Brosamle and Schwab, 1997; Schreyer and Jones, 1983). The majority of labeled axons in the thoracic cord were observed at the base of the dorsal funiculus in the dorsal CST (Fig. 2.3A); several labeled axons (less than 5 per cord) were observed in the ventral funiculus adjacent to the ventral median fissure, and in the dorso-lateral funiculi (5 to 20 labeled axons per cord).

FR-labeled CST axons in the intact portion of the spinal cord appeared slightly wavy and extended for long distances as lines of uninterrupted of fluorescence at the base of the dorsal funiculus in horizontal sections (Fig. 2.3B, C). Thin branches of CST axons collateralized in the gray matter and terminated as fine varicose fibers in the neurophil at the base of the dorsal horn (Fig. 2.3D, E).
Degeneration of the CST. CST fibers labeled prior to SCI, retained the fluorescent tracer in both the proximal and distal axonal segments after injury. Surprisingly, no labeled CST fibers were observed within the lesion cavity in any of the cases, suggesting that the CST was completely severed immediately after moderate contusive SCI. CST axons were still found rostral and caudal to the impact site, predominantly within the dorsal funiculus. The proximal segments of CST fibers rostral to the cavity were thick and wavy and terminated in retraction bulbs (Fig. 2.3F) as far as 5 mm rostral to the lesion area; the majority of bulbs however, occurred within 1-2 mm of the leading edge of the developing cavity. Occasionally labeled fibers were observed as punctate fragments (Fig. 2.3G, arrow), suggesting that these fibers were undergoing degeneration.

Injured CST fibers terminated in retraction bulbs rostral and caudal to the lesion cavity and had a variety of morphologies (Fig. 2.4). Some fibers ended in a round bulb either completely filled with dye (Fig. 2.4D, F, G, L, M) or with a dye halo surrounding a non-fluorescent core (Fig. 2.4A, C). Other fibers had longer terminal expansions that were completely filled with dye and extended over a greater distance (Fig. 2.4B, E, H, K, N). Retraction bulbs presumably formed by retrograde transport in the distal stumps of the injured axons (Bresnahan, 1978) (Fig. 2.3J, K, L and Fig. 2.4K-N) were morphologically similar to those found on the proximal CST stumps, but they tended to be smaller (compare sizes in Fig. 2.4A-J vs K-N).

Caudal to the lesion cavity, distal portions of the severed CST fibers were visible away from the major area of tissue damage. Other interrupted fibers were observed lateral to the lesion area (Fig. 2.3J, K, L). CST fibers caudal to the injury persisted for the 21
days examined (Fig. 2.3J, K, L), but decreased in number, as they became progressively fragmented, and underwent Wallerian degeneration (Fig. 2.3M-P).

Rostrally, CST fibers were interrupted but persisted proximal to the major area of tissue damage throughout the 21 days examined (Fig. 2.5 A, D, G). At 1 and 3 days after injury, some CST fibers were pushed laterally by infiltrating RBCs (Fig. 2.5B), and others were observed to terminate within the hemorrhagic extensions in the dorsal funiculus rostral to the impact site (Fig. 2.5C). At eight days after injury, CST fibers were present at the proximal end of the lesion area (Fig. 2.5E), and in one case, a few fibers were interspersed with the macrophages at the rostral edge of the forming cavity (Fig. 2.5F). At twenty-one days, CST axons persisted rostral to the cavity intermixed with both a cellular infiltrate (Fig. 2.5H) and macrophages. Quantitative analysis of the number of axons present at the rostral edge of the lesion area/cavity indicates that a considerable number of CST axons were present within 1.5 mm of the rostral cavity edge. Fewer labeled CST fibers were present at 0.5 mm and almost no axons were even with the cavity (Fig. 2.6). No CST fibers were found to extend past the rostral edge of the lesion cavity, with the exception of one fiber that was observed at 0.5 mm past the rostral tip of the cavity one day after injury in one case.

Twenty-one days after injury was the first time point after injury that CST fibers were observed in the gray matter rostral to the impact site. Some thin, varicose fibers could be followed for long distances (Fig. 2.5I) and appeared to be collateralizing in the gray matter. They projected out of the CST at an angle and arborized in the gray matter, sometimes appearing as a tangle of fibers.
Location and distribution of descending CST fibers labeled in chronic injuries

Experiment 2

Most CST fibers at 14 weeks and 6.5-8 months after injury remained confined to the dorsal funiculus until they approached the lesion cavity (Fig. 2.7A, B, C) where the tract splayed. The axons either terminated in retraction bulbs or sprouted out of the normally tight CST in all directions. The retraction bulbs appeared similar to those observed at earlier post-injury time points (Fig. 2.4I, J). The morphology of the fibers also appeared similar to those previously described; either thick and wavy, or thin and varicose (Fig. 2.3H, I). Apparent collateral sprouts off the CST axons began as far as 5 mm rostral to the lesion interface and increased in frequency closer to the cavity. Collaterals were found dorsally within the white matter of the dorsal columns and laterally throughout the dorsal horns and central gray (Fig. 2.7D). Less frequently, axons were found to enter the lateral funiculus and the dorsal portion of the ventral horns. Sprouting CST axons had multiple morphologies: masses of very thin axons which formed a tangle (Fig. 2.8A, B); slightly thicker axons that were twisted, changed directions and had multiple bulbs (the typical appearance of lost axons (Li and Raisman, 1995) (Fig. 2.8C); or relatively straight branches that could extend through the gray matter into the white matter. Some CST axons were observed to extend to the cavity edge (Fig. 2.8D), and were sometimes intermixed with macrophages (Fig. 2.8E) while others collateralized proximal to the cavity and were able to extend short distances past the rostral tip of the lesion cavity in the spared rim of tissue (Fig. 2.8F, G).
Despite the extensive sprouting observed 14 weeks after injury, no labeled CST axons were found within the lesion cavity at this time point. However, in 2/7 14-week cases, axons were found at the tip of the lesion cavity and possibly in tissue bridges at the very tip of the cavity (Fig. 2.7E). Fibers observed at this interface at 14 weeks were thin and varicose with multiple varicosities (Fig. 2.7E), and followed a tortuous route. These fibers were much thinner than most of the fibers found within the cavity at 6.5-8 months (Fig. 2.7I, J, K). In addition to the numerous sprouts of CST fibers proximal to the lesion that were observed in all long-term cases 6.5-8 months after injury, CST axons were found in the tissue inside the lesion cavity (Fig. 2.7F-K) in 3/8 cases. Axons within the lesion cavity appeared as short segments at least 100 µm in length and were either thick and wavy or thin and varicose. Labeled fibers were followed over adjacent sections to ensure their identification as axons.

Quantitation showed that the number of CST fibers approaching the lesion cavity was relatively constant until 0.5 mm rostral to the edge, at which point the number of fibers decreased (Fig. 2.6). Despite the rapid decline, a number of sampled axons were counted at the edge of the lesion cavity (mean 16.3 +/- SEM 5.8, n=7) at 14 weeks; similar axon numbers were counted 6.5 – 8 months after injury (17.8 +/- SEM 5.2, n=8). The number of axons that extended past the lesion cavity was minimal at both 14 weeks and 6.5-8 months. However, some of the axon collaterals elongated into the spared rim of tissue and extended caudally past the rostral tip of the cavity as described above. A few CST fibers were counted 0.5 mm past the tip at 14 week and at 6.5-8 months after moderate injury. Very few fibers extended beyond 1 mm in the spared rim of tissue. No
CST axons were counted beyond 2.0 mm from the rostral tip of the lesion cavity. The distribution of long-term CST axons was similar between the 6.25 and 12.5 mm injuries 6.5-8 months after injury, so these data were grouped for axon count analysis (Fig. 2.6). Measurements of the cavity length at the level of the central canal revealed a constant cavity size (mean +/- SEM: 1dpi 5.05 +/- 0.08; 3dpi 4.18 +/- 0.20; 8dpi 4.14 +/- 0.29; 21 dpi 3.94 +/- 0.32; 14 weeks 4.25 +/- 0.17; 6.5-8 months 3.74 +/- 0.16), indicating that the change in axon counts is not due to a time dependent increase in cavity size.

**Distribution of Gi-LPGi axons after long-term SCI**

**Experiment 3**

Following injection of FR into the area of Gi-LPGi, RST axons were observed almost exclusively in the lateral and ventral funiculi (Fig. 2.9A) at thoracic spinal cord levels. In the white matter, RST axons appeared as long straight fluorescent axons that projected right angle collaterals into the dorsal, intermediate and ventral horns at all levels of the spinal cord (Fig. 2.9B). In the gray matter, collaterals of RST axons appeared thin and varicose (Hermann et al., 1998; Hermann et al., 1996).

RST axons around the contusion site had the same two general morphologies [long and straight and thin and varicose (Fig. 2.9C, D), as in undamaged tissue. However, fewer long straight thick axons and more long thin varicose axons were observed in the lateral and ventral funiculi rostral to the injury site and a number of fibers were observed terminating in retraction bulbs. Some RST axons had retraction bulbs rostral to the lesion cavity or adjacent to it in the rostral-most spared rim of white matter. Thin, varicose
axons that branch numerous times in the gray matter were still present in tissue rostral to the lesion cavity in RST cases. Rarely, spared fibers could be observed extending caudal to the cavity through the spared rim of tissue.

Five and a half to seven months (n=3) after a 25mm injury, RST fibers rostral to the cavity were found throughout the dorso-ventral axis of the spinal cord, and were not confined to the ventral two thirds of the cord as they were at 14 weeks. A few RST axons extended past the rostral tip of the lesion cavity, in the spared rim of white matter as much as half the cavity length (3.5 mm beyond the rostral edge of the lesion cavity) at 5.5–7 months. Labeled RST fibers had the same general appearance as they did at 14 weeks, however, the sprouts rostral to the lesion cavity and within the lesion cavity appeared to be thicker than they were at 14 weeks. At 14 weeks, only a small number of very thin varicose fibers were observed in the trabeculae in one well-labeled case. Axons exhibited extensive collateralization, sprouting, and the formation of retraction bulbs. But at 5.5-7 months after injury, numerous thin fibers that had apparently grown in to the trabeculae (Fig. 2.9E, F; 3/3 cases) were observed inside the lesion cavity.

Sample axon counts were also performed for 2/3 of the RST cases 5.5-7 months after a 25 mm injury. Axon counts were made at the same locations along the rostral caudal axis as the CST counts, every 5th section through the cord was used to count Gi-LPGi fibers. Fluoro-ruby labeled Gi-LPGi axons extend past the rostral tip of the lesion cavity in both animals examined (distance 0.5 mm, n=2, mean = 12.0 +/- 0.0 fibers), and fibers were counted as far as 1.5 mm caudal to the rostral cavity edge, both in the spared rim of white matter and the trabeculae within the cavity.
DISCUSSION

The present study was designed to examine the early progression of cavitation in response to SCI and to determine whether descending CNS axons from the brainstem and cortex regenerate into or grow around the cavity following contusive SCI in the rat. To that end, bilateral FR injections into the cerebral cortex or brainstem (gigantocellular-lateral paragigantocellular reticular nucleus (Gi-LPGi) were performed to anterogradely label descending fibers as they encountered the lesion site. A range of injury severities was used (10 g weight dropped 6.25, 12.5, 25 mm with the NYU impactor). We examined various time points (1, 3, 8, 21 days, 14 weeks and 5.5-8 months) to 1) look at the progression of cavitation, 2) assess the die-back and sprouting of descending fibers in relation to the developing contusion cavity and 3) determine whether descending fibers contribute to the population of fibers found in the trabecucale within the lesion cavity (Beattie et al., 1997). As well, we quantitatively assessed the extent of axonal growth in and around the lesion after contusive spinal cord injury

Progression of cavitation. The chronic lesion cavity consists of a central fluid filled cavity separated into chambers by cellular tissue bridges surrounded by a circumferential zone of partially demyelinated axons and an outer zone of spared fibers (Bunge et al., 1994; Noble and Wrathall, 1989; Noble and Wrathall, 1985; Bresnahan, 1978; Balentine, 1978a; Balentine, 1978b). The lesion cavity is a dynamic structure and progresses over time, with the final stable chronic lesion cavity evolving through the interaction of degenerative and regenerative processes (Beattie and Bresnahan, 2000). In
this study, the development of the lesion cavity was examined in conjunction with the
degeneration and sprouting of the corticospinal tract and the sprouting of the
reticulospinal tract following contusion of the rat spinal cord. One day after injury,
extensive infiltration of blood components was observed throughout the gray matter
around the impact site, and in the white matter of the dorsal funiculus rostral and caudal
to the impact site. In the white matter, regions of blood cell infiltration were intermixed
with patches of intact and severely damaged axons. By 3 days after injury, the blood cell
infiltrate was confined predominantly to the impact area, with the exception of the base
of the dorsal funiculus. By eight days after injury, extensive macrophage invasion had
occurred which replaced the earlier red blood cell infiltrate. Macrophages persisted
throughout the rest of the time points studied but at chronic times were confined
predominately to the trabeculae (Beattie et al., 1997; Puckett et al., 1997) (which form
within the cavity by 21 days) and the base of the dorsal funiculus rostrally. By 21 days
after injury, the cavity walls appeared smooth and the cavity was clearly defined, as the
spared white matter became walled off from the fluid filled cavity by astrocytes (Brook et
al., 1998; Martin et al., 1996; Bunge et al., 1994). The cavity remained in this state
throughout the 8 months examined.

Relationship of descending axons with the lesion cavity. Following contusion,
transection, or hemi-section injury to the spinal cord the proximal portion of damaged
axons terminate in retraction bulbs and retract away from the injury site before mounting
a sprouting response, while the distal portions of the axons undergo progressive
fragmentation and Wallerian degeneration (Bresnahan, 1978). By labeling the CST fibers
prior to injury in experiment 1, we were able to assess the sparing of the CST after contusive SCI as well as examine the degeneration and sprouting responses of CST axons and their relationship with the developing lesion cavity. No short-term CST fibers were found within the confines of the lesion cavity at any of the early time points (1, 3, 8 and 21 days). Instead, labeled fibers within the CST were observed to terminate in retraction bulbs rostral and caudal to the impact site, often intermixed with the infiltrating blood cells that extended for long distances in the dorsal funiculus. The lack of labeled CST fibers within the lesion cavity 24 hours after injury suggests that axotomy of the CST fibers is complete and occurs rapidly after injury. It is possible that the mechanical damage at the impact site causes complete severance of the CST fibers or that secondary mechanisms initiated early after injury results in destruction of axons. Distal to the lesion, spared, but disconnected fibers were found up to 21 days after injury distal to the lesion.

CST axons have been shown to progressively retract away from the site of transection. The extent of retraction has been reported to be between 1 mm and 2.5 mm 8–10 weeks after hemi-section (von Meyenburg et al., 1998) and transection (Oudega et al., 1999; Pallini et al., 1988). Following contusion injury CST fibers were observed to terminate in retraction bulbs as far as 5 mm rostral to the cavity, with the majority of retraction bulbs within 1-2 mm of the leading edge of the cavity. The presence of retraction bulbs 5 mm from the lesion cavity suggests that dieback of the CST may be more extensive following contusive injury than after transection injury.

Sprouting of CST fibers has been observed as early as 2 weeks after injury in some models (Li and Raisman, 1995). Some reports indicate that the sprouts are
maintained with little change for 9.5–13 weeks (Li and Raisman, 1995) whereas others (Li et al., 1994) suggest that sprouting of injured fibers increases for up to 2 months but decreases thereafter if the target tissue is not found. In the present study, the first sprouting of the CST following a moderate contusion injury was observed in the 21-day post injury cases. It was limited to a small number of fibers that entered the gray matter just rostral to the lesion cavity. Robust sprouting of the CST was observed at 14 weeks. At 21 dpi Wallerian degeneration was still observed in the CST distal to the impact site. Rostral to the injury, dorsal column fibers, although unlabeled in this study, undergo Wallerian degeneration within a similar time frame (Shuman et al., 1997; Bresnahan et al., 1976). The presence of CST sprouting at a time when Wallerian degeneration is being observed in a closely adjacent tract suggests that corticospinal fibers are able to sprout even though degenerating axons and myelin fragments are present. However, since more extensive sprouting is observed at later time points, the extent of sprouting might be regulated by the extent of myelin removal. Fourteen weeks after injury, labeled CST fibers exhibited substantial sprouting and collateralization into both the gray and white matter rostral to the lesion cavity. Numerous masses of very thin labeled axons were present in the gray matter, and other sprouts were found in the lateral funiculus, the white matter dorsal to the CST and within the CST. Sprouts were also observed to penetrate and meander among the macrophages rostral to the cavity similar to that observed by Li and Raisman (Li and Raisman, 1995). Extensive sprouting of the CST persisted up to 8 months after injury (the longest time examined) without any apparent decrement. This suggests that despite the lack of proper distal target reinnervation, the sprouts persisted,
perhaps in a dormant state or through reinnervation of the local gray matter. The maintenance of both retraction bulbs and sprouts at long survival times could potentially be advantageous if a method to increase their elongation could be established. Why some axons sprout while others maintain dormant retraction bulbs, is unclear.

*Labeled axons within the lesion cavity.* The presence of axons within the trabecular tissue bridges that form inside of the lesion cavity has previously been described (Beattie et al., 1997; Quencer and Bunge, 1996). The tissue within the trabeculae has a very cellular appearance, unlike the spared rim of white matter tissue to which it is attached. Trabeculae are thought to be formed by infiltrating cells after injury, which include Schwann cells, fibroblasts, astrocytic extensions, ependymal zone cells and collagen (Beattie et al., 1997; Guizar-Sahagun et al., 1994).

It has been postulated that the trabeculae may serve to guide fibers from the CNS into the lesion cavity (Beattie et al., 1997). In experiments 2 and 3 of this study, the contribution of descending systems from the cortex and brainstem were assessed at long times (14 weeks and 5.5 to 8 months) after injury to determine whether central axons are present in the lesion cavity trabeculae. Anterogradely-labeled axons from both the cortex and brainstem were found within the lesion cavity trabeculae 5.5–8 months after contusion injury, at all 3 injury levels (6.25 mm, 12.5, 25 mm). A few thin Gi-LPGi fibers were observed in the trabeculae within the lesion cavity close to where they attach to the spared rim of tissue in 1 of 4 well-labeled cases at 14 weeks and in all (3/3) cases 5.5 – 7 months after injury. Labeled CST fibers were observed within the lesion cavity in 3/8 cases 6.5–8 months after injury. No descending fibers from the cortex were observed.
within the cavity earlier than 6.5-8 months, although in 2/7 14 week CST cases, axons were found at the very tip of the cavity, in an extension of the spared rim of tissue.

The presence of labeled central axons in the trabeculae within chronic contusion cavities more than five months after injury suggests that central axons can penetrate into the lesion cavity as part of the endogenous repair response following SCI. Further, the data suggest that the penetration of CST and brainstem axons into the lesion matrix occurs over an even longer time course than the collateralization of the CST into the gray matter proximal to the lesion, which occurs between 3 weeks and 3 months. This is in contrast to primary afferent axons that can be observed to enter the lesion cavity as early as two weeks after injury, escorted by proliferating Schwann cells (Beattie et al., 1997; Bunge et al., 1994; Bresnahan, 1978). Although descending systems contribute fibers to the trabeculae after injury, it appears that the CST plays a limited and delayed role. This is in agreement with previous reports of a poor CST regenerative response after injury with and without therapeutic intervention (Oudega et al., 1999; Weidner et al., 1999; Menei et al., 1998; von Meyenburg et al., 1998; Blesch and Tuszynski, 1997; Bregman et al., 1997; Grill et al., 1997; Ye and Houle, 1997; Guest et al., 1997a; Chen et al., 1996; Martin et al., 1996; Xu et al., 1995a; Xu et al., 1995b; Paino et al., 1994; Schnell et al., 1994; Schnell and Schwab, 1993; Schnell and Schwab, 1990; Pallini et al., 1988; David and Aguayo, 1981). It is also possible that despite the ability of the corticospinal tract to undergo extensive sprouting after contusion injury, local sprouting proximal to the lesion may preempt long distance regeneration past the lesion cavity.
Brainstem-spinal axons, including those from the reticular nuclei, have an increased propensity to regrow after axotomy compared to CST axons, especially when provided with a favorable growth environment. Brainstem-spinal axons, but not corticospinal axons, have been shown to grow into peripheral nerve grafts (Cheng et al., 1996; David and Aguayo, 1981) and Schwann cell grafts with exogenous factors added. The addition of either methylprednisolone (MP), or specific neurotrophins (brain derived neurotrophic factor and neurotrophin-3) to Schwann cell grafts results in brainstem-spinal axons penetrating into the graft (Chen et al., 1996; Xu et al., 1995a). However, brainstem-spinal axons stop at the edge of Schwann cell grafts when no other treatment is applied (Xu et al., 1995b), suggesting that Schwann cells alone are insufficient to elicit brainstem spinal axons regrowth. The presence of a small number of reticulo-spinal fibers within the trabeculae after contusive SCI suggests that trabeculae offer an environment conducive to axonal growth, but not an environment sufficiently enriched for substantial growth of reticular fibers. Increasing evidence suggests that propriospinal and vestibulospinal axons initiate robust regenerative responses after injury (Menei et al., 1998; Guest et al., 1997b; Chen et al., 1996; Xu et al., 1995a; Xu et al., 1995b). Whether these latter fibers contribute to the population of fibers observed within the trabeculae remains to be determined.

We observed limited growth from the two descending pathways studied here. It is likely that other descending tracts may also make a contribution to the fibers within the trabeculae, in addition to the contribution of ascending sensory fibers from the dorsal root (McTigue et al., 1998). The presence of labeled fibers within the chronic lesion cavity
suggests that descending fibers can grow into the cavity at very long time-points after injury, despite the presence of the inhibitory glial scar. To be certain that fibers within the cavity were regenerating fibers and not spared fibers, the CST was pre-labeled prior to contusion and the distribution of labeled axons was assessed 1, 3, 8, and 21 days after injury. No labeled fibers were found in any of the cases where the CST was pre-labeled prior to injury, nor in any of the CST cases 14 weeks after injury. The failure to detect any labeled CST fibers within the lesion cavity at any time points other than after 6.5-8 months supports the notion that CST fibers present within the cavity are regenerating fibers.

Quantifying axons around contusion cavities. In the present experiment, the number of CST fibers in the region of the lesion cavity were quantified after contusive spinal cord injury. This method can be used to look at different populations of neurons and how far they extend their axons after injury. The number of spared fibers following hemi-section and compression injuries has been assessed using retrograde tracers (Grill et al., 1997; Fehlings and Tator, 1995), and the distribution of CST fibers has been assessed following hemi-section injury (Schnell and Schwab, 1990) and transection (Oudega et al., 1999), but to our knowledge no one has quantified the number of selected populations of fibers that are spared or regenerate after contusive spinal cord injury.

Axon counts in a sample of tissue slices were used to estimate the number of anterogradely labeled fibers that approached and extended past the lesion cavity from the CST at early (1, 3, 8, 21 days) time points and from both the CST and Gi-LPGi at late (14 weeks and 6.5-8 months) time points after injury. Since the cavity extends variable
distances in the dorsal ventral axis it is necessary to maintain a systematic approach to counting axons in the contusion injury model. Therefore, axon counts were made relative to position of the lesion cavity at the level of the central canal. By using this site to put count in register, a conservative estimate of the number of axons that extend past the cavity was made because the cavity typically extends further rostral in the white matter of the dorsal funiculus than in the central gray matter. This method overcomes the inherent variability of the lesion extension at the base of the dorsal funiculus. The consistency between axon counts across time points (Fig. 2.6) using this sampling method supports it's usefulness in quantitatively examining the extension of axons following contusive spinal cord injury. Quantitative analysis of the number of fibers from specific descending tracts after contusive spinal cord injury can be used for examining the sprouting and elongation of specific fiber populations and evaluating treatments. It would be expected that treatments that successfully increase sprouting will result in an increase in the fiber number counts, while treatments that increase elongation will result in a shift to the right, with more axons being counted at more distal sample sites. Thus this method allows two different steps in successful regeneration to be separated and compared between treatments.

In summary, labeling of descending axons before, and after, contusion injuries to the cord have shown 1) substantial die-back of CST axons, which then sprout and grow back to the lesion margin, 2) long-term confined growth of CST axons with sprouting and regeneration extending past an injury and 3) the presence of a few regenerating descending axons within the lesion cavity at long times after injury. These findings add to
the evidence that limited endogenous repair occurs after SCI in adult mammals and may continue for an extended period where it could potentially be recruited to repair chronic injuries. Further understanding of this repair process should be useful in designing therapeutic approaches to enhancing recovery after SCI.
Figure 2.1: Location of CST axon counts. To quantify the number of axons around the lesion cavity 5 tissue slices per cord were selected (A), and labeled axons were counted at 0.5 mm intervals proximal and distal to the cavity edge (B).
Figure 2.2: Appearance of impact site after a 12.5 mm contusion injury 1 dpi (A, B, C), 3 dpi (A, D, E), 8 dpi (A, F, G) 21 dpi (A, H, I) and 14 weeks (A, J, K, L) after injury. (A) The progression of cavitation. At 1d, the impact site is hemorrhagic but no cavitation present, RBCs extend up to an additional 5 mm rostral (arrow), between 3d, 8d and 21d the injury site becomes progressively more defined, it appears as a dark region in low magnification at 8 dpi and is clearly defined at 21d. At 21d and 14w a cellular mass is present within the cavity attached to the spared rim of white matter at several points via trabeculae (arrow). One dpi (B) RBCs, and (C) damaged axons at the impact site. Three dpi (D) damaged axons (small arrow) intermixed with blood cell infiltrate (large arrow) at the impact site and (E) some macrophages (large arrowhead) have infiltrated the rim of spared white matter (small arrows). Eight dpi (F) macrophages are densely packed at the center of the cavity while (G) open spaces (small arrow) are present between groups of macrophages and other non-fluorescently labeled cells (large arrowhead) that appear in scattered bands within the cavity. Twenty-one dpi (H) trabeculae, thin tissue bridges with regions of no tissue beside them, are beginning to form (small arrow), and macrophages (large arrowhead) are still present within the cavity in association with the trabeculae or as (I) macrophage rafts. Fourteen weeks after injury (J, K, L) autofluorescing macrophages (large arrowhead) are present within (J) fibrous trabeculae and (K) cellular trabeculae as well as (L) the cellular infiltrate rostral to the cavity. Scale in A is 1 mm, all other scales are 100 µm. C, E, F are same magnification. B, D, G-L are same magnification.
Figure 2.3: Location and appearance of intact and degenerating corticospinal fibers. (A) The majority of CST fibers are located in the base of the dorsal funiculus, several labeled fibers were also observed in the ventral and dorsolateral funiculi. (B) Intact fibers within the CST are (C) wavy and extend for long distances (arrow) at the base of the dorsal funiculus and (D) collateralize into the gray matter (arrow). (E) within the gray matter fibers appear thin and varicose (arrow) and branch many times. After injury (F) rostral fibers appear thick and straight and terminate in retraction bulbs (arrow) at 1 dpi, at later time points (G) some punctate fragments appear, while other fibers maintain their pre-injury appearance (H) thick and wavy in the white matter, (I) thin and varicose in the gray matter. Caudal to the injury (J-L) fibers undergo Wallerian degeneration, some fibers terminate in retraction bulbs (long thin arrow), some intact fibers persist throughout the 21 days examined (large arrow) but progressively decrease in number over time. (J) 1 dpi caudal fibers are pushed laterally by infiltrating blood cells (arrowhead). (K) 8 dpi and (L) 21 dpi fibers are interspersed with macrophages (arrowheads) at the lesion interface. (M) 1 dpi fibers are dystrophic but remain intact (arrows). (N) 3 dpi is the first appearance of fragmented fibers. (O) 8 dpi the fibers are increasingly fragmented. (P) 21 dpi primarily small segments of labeled fibers still remain. B magnification 100 µm, C-E same magnification 100 µm, F-I same magnification 50 µm, J-L same magnification 100 µm, M-P same magnification 50 µm.
Figure 2.4: Morphology of retraction bulbs rostrally at 1 dpi (A, B), 3 dpi (C, D), 8 dpi (E, F), 21 dpi (G, H), 14 wpi (I, J), and caudal (K-N). Retraction bulbs had 3 general appearances, they were round and completely filled with dye (D, F, G, J, L, M), or round and surrounded by a dye halo (A, C) or they were completely filled with dye and extended over greater distances (B, E, H, I, K, N). Retraction bulbs caudal (K-L) to the lesion cavity appeared similar to those rostral to the cavity but smaller. Examples are shown of small bulbs filled with dye (M, L) and fibers with long thick dye extensions (K, N). Scale bar = 50 µm.
Figure 2.5: Distribution of CST fibers rostral to the cavity at acute/subacute (1, 8, 21 dpi) times after injury. (A, D, G) CST fibers terminate proximal to the major area of tissue damage at all short-term time points (boxed area of inset represents area depicted). 1 dpi (B) fibers (arrows) are pushed laterally by infiltrating blood cells or (C) terminate within areas of blood cell infiltration distal to the impact site. 8 dpi (E) CST fibers (arrows) are observed terminating at the lesion interface, (the expansion of the cavity at the base of the dorsal funiculus (arrowhead) can also be observed), (F) a few fibers (arrows) were observed interspersed with macrophages that had infiltrated the base of the dorsal funiculus. 21 dpi (H) fibers (arrows) rostral to the cavity are found within the tube of cells observed at the base of the dorsal funiculus (I) thin and varicose fibers (arrow) the first signs of sprouting of the CST into the gray matter rostral to the cavity is observed. All scale bars are 100 µm. A, D, G same magnification, B-E, H, I same magnification.
Figure 2.6: Number of labeled axons sampled around the lesion cavity at subacute (1, 3, 8, 21 dpi) and chronic (14 weeks and 6.5-8 months) times after injury. Imaginary lines through 5 tissue slices were used to count the number of labeled axons around the cavity. Displayed is the mean of the total number of sampled axons counted per animal (see Fig. 2.1 for sample sites). No fibers extended past the cavity at subacute times, while fibers were frequently observed past the lesion cavity at chronic times. This quantitatively depicts the observed sprouting and elongation of fibers observed to extend past the edge of the lesion cavity, predominantly in the spared rim of tissue.
Figure 2.7: Sprouting and elongation of fibers in and around the chronic lesion cavity. The majority of labeled CST fibers were confined to the base of the dorsal funiculus 14 weeks (A) and 6.5-8 months (B, C) after injury. Axons terminated in retraction bulb and were observed to sprout out of the CST (best observed in (C) a drawing of all the labeled CST fibers in 1 slice). The location of labeled fibers 14 weeks after injury is depicted in D, the light gray dots represent the location of sprouts, the darker dots and filled areas represent the normal locations of the dorsal CST, the dorso-lateral CST and the ventral CST. Sprouts were observed dorsally within the white matter of the dorsal columns and laterally throughout the dorsal horns and the central gray. 14 weeks after injury labeled fibers (arrows) are present at the lesion interface (E). 6.5-8 months after injury fibers were observed within the cavity and could be followed over serial sections (F- K). Fibers (arrows) appeared thick and short (I, J) and thin and varicose (K).
Figure 2.8: Appearance and location of sprouting CST axons rostral to the lesion cavity 14 weeks after injury. Numerous masses of very thin labeled axons were present in the gray matter (A, B). Other sprouts were found in the lateral funiculus (C), the white matter dorsal to the CST and within the CST (arrow) (D). Sprouts (arrows) were also observed to penetrate and meander amongst the macrophages rostral to the cavity (E). And occasionally sprouts were found that extended past the tip of the cavity in the white matter laterally (F, G). A-B, E-G same magnification 100 μm, D = 100 μm, C = 50 μm.
Figure 2.9: Location, appearance and distribution of labeled RST fibers. (A,B) Normal spinal cord. (A) RST axons in the thoracic cord descend in the lateral and ventral funiculi. (B) RST labeled axons appear as long straight fluorescent axons (large arrow) that project right angle collaterals (arrowhead) into the dorsal, intermediate and ventral horns. After long-term contusion injury RST fibers have the same morphology (C) long and straight (small arrow) and (D) thin and varicose (small arrow). (C) Retraction bulbs are also present (arrowhead). (E) Longitudinal section of spinal cord with trabeculae inside the cavity. (F) RST fibers are found within the trabeculae.
CHAPTER 3

SURVIVAL AND DIFFERENTIATION OF GRP CELLS FOLLOWING TRANSPLANTATION INTO ACUTE SPINAL CORD CONTUSION INJURIES

INTRODUCTION

Immature cells (fetal cells, stem cells, precursor cells) may provide a viable therapy for SCI. They are able to replace lost cells (neurons and/or glia) (McDonald et al., 1999; Whittemore, 1999; Rosenbluth et al., 1997; Giovanini et al., 1997; Privat et al., 1989), provide a substrate permissive for axonal growth (Bernstein-Goral et al., 1997; Schnell and Schwab, 1993; Bregman et al., 1993; Jakeman and Reier, 1991) and alter the inhibitory glial scar (Wang et al., 1995; Smith and Miller, 1991; Houle and Reier, 1988; Reier and Houle, 1988). Recently, transplantation of embryonic stem cells into acute contusion lesions resulted in improved locomotor performance (McDonald et al., 1999). Despite reports of behavioral recovery, the use of stem cells as a potential therapy for spinal cord injury is still in its infancy and relatively little is known about how stem/precursor cell transplants respond to and affect the lesion environment, and whether specific axonal populations can sprout in response to these cells. In the following studies, glial restricted precursor (GRP) cells were transplanted into acute contusion injury sites. Chapter 3 examines the viability and differentiation of GRP cells transplanted immediately following injury. Chapters 4 and 5 report on the effects of transplanted GRP cells 8 days and 6 weeks after injury respectively.
Neural stem cells. Although neural stem cells, which can be derived from a variety of sources (for review see Cao et al., 2002a), can differentiate into both neurons and glia in vitro and in vivo (Chow et al., 2000), when they are transplanted into injury sites the majority of cells differentiate into glia (Cao et al., 2001; McDonald et al., 1999). This suggests that the alterations observed after injury may be mediated by glia derived from transplanted stem cells.

Glial restricted precursor cells. Glial restricted precursor (GRP) cells offer a novel approach for studying the effects of transplanted glial cells following SCI. GRPs are multipotent cells that can differentiate along oligodendrocyte and astrocyte lineages both in vitro and in vivo (Herrera et al., 2001; Rao et al., 1998). They show extensive self renewal in vitro (Rao et al., 1998), and can be isolated from neural stem cells (Rao and Mayer-Proschel, 1997) and embryonic spinal cord (Herrera et al., 2001; Rao et al., 1998) allowing for sufficient cells to be produced for transplantation. GRP cells appear to be an intermediate restricted cell between neural stem cells and the extensively studied O-2A/OPC (oligodendrocyte-type-2-astrocyte and oligodendrocyte progenitor cells) (Gregori et al., 2002; Rao and Mayer-Proschel, 1997). GRPs differ from O-2A/OPCs in that they are able to differentiate into both oligodendrocytes and astrocytes following transplantation (Herrera et al., 2001). Astrocytes have important regulatory roles within the CNS, and are involved in maintenance of the blood-brain-barrier and the ionic environment, normally and after injury. The presence of astrocytes may also be important for the induction of neurogenesis (Song et al., 2002), and remyelination of axons by oligodendrocytes (Franklin and Blakemore, 1998). GRP cells appear to remain restricted to the glial lineage in vivo (Herrera et al., 2001). Transplanted GRP cells differentiate into
astrocytes when transplanted into the corpus callosum in normal rats, and form myelin when transplanted into myelin deficient rats (Herrera et al., 2001). How they respond when transplanted into an injury environment has not been previously examined. This experiment was designed to assess 1) the survival and distribution of GRP cells and 2) the ability of GRP cells to differentiate into astrocytes and oligodendrocytes following transplantation into spinal cord lesions.

Tracking transplanted cells-methods of labeling. The ability to specifically identify transplanted cells is important when addressing migration and differentiation of transplanted cells. Labeling transplanted cells can be problematic, and a clear understanding of the questions to be answered and the advantages and disadvantages of different labeling methods for identifying transplanted cells is essential prior to transplantation (good reviews are available (Cao et al., 2002b; Chandross et al., 2001; Harvey, 2000; Whittemore and Holets, 1995)). Markers that label cells in intact tissue may not be suitable for damaged tissue, and markers that work well for differentiated cells may not be suitable for undifferentiated proliferating cells. Markers used to label cells fall into two broad categories: exogenous markers that are applied to label the cells prior to transplantation, and endogenous markers that are expressed by the transplanted cells and that differ from the environment into which they are being transplanted.

A wide range of exogenous markers can be applied to cells. Generally they fall into one of four categories: (1) cytoplasmic (fluorescent beads, fast blue, RDA, HRP, fluoro-gold, gold/carbon); (2) membrane (DiI, PKH26, Lectins); (3) nuclear (Hoechst, [³H] thymidine, BrdU); and (4) reporter genes (retrovirus/lacZ, adenovirus/lacZ). The two main issues concerning exogenously applied markers are specificity and endurance.
of labeling. Hoechst binds to DNA, and has been used extensively to label transplanted cells. Recently it has been shown to extend beyond the area of transplanted cells, suggesting that the surrounding endogenous cells become labeled (Ruitenberg et al., 2002; Iwashita et al., 2000a). It appears that while Hoechst may be a good marker for finding the general location of a transplant its use for cells that divide and migrate may not be appropriate. The Ruitenberg (2002) study also demonstrates two problems associated with transfecting cells with genes prior to transplantation. First, some cells will not be readily transfected with some vectors, and second, the transgene can decrease expression with time. Decreased expression can be partially ameliorated by FACS sorting to select cells with high transgene expression to increase the likelihood of persistent expression. However, labeling cells ex vivo for transplantation can be difficult, and even once labeled it can be difficult to detect the cells after long periods of time in vivo.

Endogenous markers eliminate the problems of specificity and endurance by using cell specific markers (DNA, mRNA, neurotransmitters, enzymes, receptors) that are expressed in the transplanted cells but not in the graft location. Good examples of endogenous markers used to answer specific questions are: the use of DRG neurons which express CGRP to show long distance regeneration on myelin (Davies et al., 1997); the use of mouse ES cells transplanted into rat contusion sites to show the ability of ES cells to differentiate into astrocytes, oligodendrocytes and neurons after SCI (McDonald et al., 1999); and the use of male cells transplanted into female hosts and then identified by in situ hybridization for the Y chromosome (O'Leary and Blakemore, 1997). Although endogenous markers can be used to address some specific problems, it is difficult to find endogenous markers that can be broadly used for transplantation. Therefore, there is no
single endogenous marker that can be widely used to label transplanted cells following injury.

Cells isolated from transgenic animals can also be considered endogenous markers. Although some of the same problems that arise from *ex vivo* gene application may still apply, transgene expression in transgenic animals is generally more robust. Transplanted transgenic cells can be identified in host animals without prior *ex vivo* cell labeling. Until recently, transplants of transgenic cells from mice have been of limited use for the study of CNS diseases since most of the injury models were developed in rats and xenografts have an increased risk of rejection. New injury models developed in mice are starting to exploit this technology. Recently transgenic rats that ubiquitously express green fluorescent protein (GFP) or human placental alkaline phosphatase (hPLAP) (Kisseberth et al., 1999) have been developed and subsequently demonstrated to stably express the transgene following transplantation (Mujtaba et al., 2002; Hakamata et al., 2001). Transgenic rats will provide an extremely useful and accurate tool for identifying cells following transplantation. In conjunction with immunohistochemistry, transgenic cells will enable identification of transplanted cells and their subsequent survival and differentiation.

This study was designed to assess 1) GRP cell survival 8 days and 6 weeks after transplantation immediately following SCI, and 2) the ability of GRP cells to differentiate into astrocytes and oligodendrocytes 6 weeks after transplantation. Three different cellular markers were assessed for their ability to label glial restricted precursors. We found that only cells derived from the transgenic PLAP rat were able to specifically and robustly label GRP cells. The results of this study show that GRP cells are able to survive
immediate transplantation following spinal cord injury and that they can be detected via their PLAP expression to differentiate into oligodendrocytes and astrocytes within the injured spinal cord. The survival and differentiation of GRP cells, how they alter the lesion environment, and whether they affect recovery from spinal cord injury is addressed in subsequent chapters.

MATERIALS AND METHODS

Subjects.

Fifteen female rats (Long Evans, Simonsen Laboratories; 85-94 days old) received a moderate 12.5 g-cm injury with the MASCIS/NYU impactor. All procedures were carried out using aseptic technique, in compliance with NIH guidelines and were approved by the Institutional Laboratory Animal Care and Use Committee. Animals were housed in standard plastic cages and were given food and water ad libitum.

Experiment 1.

GRP cell survival and migration 8 days following transplantation into a moderate contusive SCI. Nine animals received GRP cell transplants. In experiment 1 GRP cells were isolated either from Fisher 334 rats (n=6) or from Fisher 334 transgenic PLAP rats (n=3) in which the human placental alkaline phosphatase (hPLAP) gene was inserted behind the ubiquitously expressed ROSA26 promotor (Kisseberth et al., 1999). All transplanted cells in experiment 1 were prelabeled with Hoechst 33342 (10µg/ml) prior to injection.
Experiment 2.

GRP cell survival, migration and differentiation 6 weeks following transplantation into a moderate contusive SCI. Injured animals received GRP cells isolated from transgenic PLAP rats (n=6). Cells were isolated by Dr. Chris Proschel (CP) and were shipped to Ohio State live and transplanted into the contusion site the following day shortly after injury.

Surgical Procedures

Rats underwent a single surgery that involved jugular catheterization, spinal cord injury, drug administration and transplantation. Rats were anesthetized with 40-60 mg/kg of pentobarbital i.p. (Abbott Laboratories, Chicago, IL) and given an injection of 25 mg/kg of keflin (Lilly, Indianapolis IN) antibiotic sc. Animals then had a catheter inserted into their jugular vein and received an initial dose of MP (30mg/kg) 5 min following a 12.5 mm injury with the NYU device. This was immediately followed by transplantation of 500,000 GRP cells in 5 µl (within 20 minutes of the spinal cord injury). Two additional doses of MP (30mg/kg) were given at 2 hrs and 4 hrs after injury and cyclosporin was given (10mg/kg) daily throughout the study.

Jugular catheterization: A jugular catheter was inserted to allow intravenous injections of MP. An incision was made through the skin above the jugular vein. The skin, muscle and fat were dissected away from the jugular vein. Two pieces of suture thread were slipped below the jugular vein. The jugular was tied off, and a piece of pulled PE 50 tubing was then inserted into the jugular vein directed caudally and tied down. The distal end of the catheter tubing was threaded subcutaneously around the neck and brought out of the back of the head to allow easy access for injections. The skin above
the jugular was closed with wound clips and an additional wound clip was used at the base of the head to keep the catheter in place and stable.

Spinal cord injury and transplantation: A laminectomy was performed to expose the dura of the spinal cord at T 9-10 and a 12.5 g-cm (n=15) contusion injury was induced using the NYU/MASCIS device by dropping a 10g weight from 12.5 mm onto the exposed dura of the spinal cord (Constantini and Young, 1994; Gruner, 1992). A Hamilton syringe was inserted 1.5-2.5 mm into the center of the impact site with the bevel facing caudally to allow for injection of 500,000 GRP cells suspended in 5 µl of conditioned media. The wound was then closed in anatomical layers and rats were placed singly in temperature-controlled incubators overnight. Subsequently, they were housed in pairs in standard plastic cages and given food and water ad libitum. Keflin (25mg/kg) s.c. (Lilly, Indianapolis, IN) was given daily for the fist 10 days. Cyclosporin (10mg/kg) was given daily (i.p. for the first week and then added to the drinking water for the remainder of the study). Bladders were expressed twice a day until reflex urination returned.

GRP Cell Isolation: GRP cell were isolated as described previously (Herrera et al., 2001; Rao et al., 1998). Briefly, neural cells were isolated from E13.5 embryos. The trunk segments including the last 10 somites were dissected and triturated to remove the neural tube from the somites. The neural tube was dissociated; cells were trypsinized and suspended onto E-NCAM coated dishes (this removes NCAM+ cells and other cells that bind to dish). The supernatant containing the cells of interest was removed and plated onto A2B5 Ab coated dishes to isolate A2B5+ GRP cells. The supernatant was removed, the plate washed and the bound A2B5+ cells were scraped off (>98% purity for A2B5+...
cells). Cells were placed into 80 cm$^2$ tissue culture flask, coated with fibronectin/laminin solution (FN/LN) containing Complete Medium (10mL total/ 80cm$^2$ flask) and grown in an incubator at 37°C, 6% to 7.5% CO$_2$.

Perfusion procedures.

Animals were perfused at either 8 days or 6 weeks after SCI. All rats were terminally anesthetized with 0.8 mg/kg of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 0.5 mg/kg of xylazine (Vedco, Inc, St. Joseph, MO) and transcardially perfused with 0.5 ml of lidocaine (Elkins-Sinn, Inc, Cherry Hill, NJ) and 0.1 ml of heparin (Elkins-Sinn, Inc, St. Joseph, MO) followed by 0.9% saline, and then by 4% paraformaldehyde (Fisher Scientific). The spinal cord was removed, blocked, and post-fixed for 4-8 hours in 4% paraformaldehyde.

Histological Procedures.

The spinal cords were placed in 30% sucrose-PBS overnight, blocked, frozen and stored at –80°C until cutting on a cryostat. After cutting, sections were mounted onto gelatin-coated slides and stored at –80°C until staining. For the 8-day survival study (experiment 1) a 10 mm tissue block centered on the lesion was cut longitudinally at 16 µm (four serial sets of slides per animal) on the cryostat. Two sets were used in this study to identify GRP cells, and the remaining two sets were used to assess how GRP cells modulate the lesion environment acutely (described in Chapter 4). For the 6-week survival cases (experiment 2), a 15 mm section of tissue blocked in 3 mm blocks centered on the lesion was cut coronally at 20 µm (three sets of slides per animal). One set was used to determine the distribution of transplanted cells and the other two were used to identify differentiated GRP cells.
PLAP can be detected either enzymatically with NBT/BCIP (Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt), which detects alkaline phosphatase (AP) activity, or with a human specific antibody (hPLAP). Enzymatic AP staining, produces a purple precipitate, and requires that endogenous AP be denatured or inhibited to ensure only transgenic cells are labeled. This is done by heating the tissue (the hPLAP transgene is heat stable) and by blocking endogenous AP activity with lavamisol. NBT/BCIP staining was used to initially detect the transplants, however, the precipitate did not allow for the detection of fine processes, and the density and cytoplasmic nature of the enzymatic staining made double labeling using transmitted light techniques impractical. The hPLAP antibody was used for double-labeling immunofluorescence to identify differentiated GRP cells following transplantation. The human antigen allowed for specific labeling of the GRP cells within the rat tissue.

**NBT/BCIP staining:** Enzymatic staining was performed as previously reported for cells isolated and transplanted from PLAP transgenic rats (Mujtaba et al., 2002). Tissue was allowed to warm to room temperature and was washed 3 times with PBS before heating in PBS to 60°C for 1 hour. After heating the tissue was washed 3 times with AP buffer (100mM Tris, 100mM NaCl, 50 mM MgCl₂, pH 9.5) and NBT/BCIP solution (Boehringer Mannheim, Germany) (NBT/BCIP stock solution diluted 1/20 in AP buffer containing 5mM lavamisol to block endogenous AP activity) was applied until a visible purple precipitate was observed (usually 1.5 hours). Tissue was rinsed with AP buffer, 50:50 AP buffer:distilled water, dehydrated through a graded alcohol series into xylene and coverslipped with permount. Some sections were counterstained with methyl green prior to dehydration to aid in tissue visualization.
**Immunohistochemistry:** Immunohistochemistry was performed to detect, hPLAP, GFAP, CC1, nestin and neurofilament (see Table 1). All antibody staining was performed by washing the tissue (three times for 10 minutes each) in buffer, blocking the tissue for 1 hour, in either 0.5% Triton 100X, 5% normal goat serum in buffer or 0.1% Tween-20, 10% milk in buffer before applying the primary antibody diluted in the blocking solution. After primary antibody incubation (4°C) the tissue was again washed three times (10 minutes each), before secondary antibody incubation at room temperature. Following secondary antibody incubation the slides were washed, coverslipped with Vectashield (Molecular Probes, Eugene, OR) and stored at 4°C until examined. For double-labeling tissue the process was repeated for the second antigen prior to coverslipping. Tissue stained for CC1 or nestin underwent antigen retrieval via microwaving (2 x 5 minutes in citrate buffer ph 6.0) prior to PLAP staining.

PLAP staining was performed alone (experiments 1&2) or with GFAP, CC1 or nestin (experiment 2). Specific PLAP staining was only obtained when the tissue was first stained for PLAP; if GFAP, CC1 or nestin staining was done prior to PLAP staining, the PLAP antibody appeared to bind to both the PLAP labeled cells and the previously labeled areas. Double-labeled tissue was counterstained with Hoechst 33258 (5 µg/ml) and mounted with Vectashield (Molecular Probes).

In experiment 1 Hoechst was not initially detected in slides dehydrated and coverslipped with Entellan (Electron Microscopy Science, Fort Washington, PA). It was detected, when an additional set of slides was stained for neurofilament and coverslipped with Vectashield (Molecular Probes, Eugene, OR).

*Data Analysis.*
Slides were examined using a Zeiss Axioplan2 microscope (Carl Zeiss, Hallergmoos, Germany) that also has a CARV attachment (Atto instruments, Rockville, MD). Slides were viewed using both standard fluorescence and non-laser confocal fluorescence. Standard fluorescent digital images were taken using an Optronics Dei 470 digital CCD camera (Optronics, Goleta, CA), directly imported into a Pentium II computer (Dell, Round Rock, TX) and overlaid in Photoshop (Adobe, Systems Inc., San Jose, CA). Confocal images were obtained using the CARV attachment with a high-resolution digital CCD camera for confocal imaging (Hamamatsu Photonics, model C4742-95-12, Hamamatsu, City, Japan) using CARVer software version 1.2 (Atto instruments, Rockville, MD). Prior to creating a Z-stack of images, the objectives were calibrated, and the settings for the digital camera were determined. Using the auto-exposure button, the correct exposure for each filter was determined prior to capturing images. A multi-channel Z-stack was captured by determining the upper and lower focal planes for the tissue section and then setting the interval thickness so that between 10-20 sections were obtained (usually 0.5-1 µm). By using the multi-channel function, signals from up to three different wavelengths of light could be detected and imported sequentially for each optical section. Once the z-stack was captured individual channels had their color replaced and were scanned for double labeling.

**Survival and Migration of GRP cells:** Initial assessment of GRP cell survival was based on the presence of Hoechst labeled nuclei within the 8-day post-injury tissue. GRP cells were incubated with Hoechst (10 µg/ml for 30 minutes) prior to transplantation. Tissue slices were assessed for the presence of Hoechst labeled nuclei to determine the location of labeled GRP cells. The number of Hoechst-positive nuclei within the 10 mm
long tissue section was qualitatively assessed, and in one case, the location of labeled cells were plotted from a computer monitor onto camera Lucida tissue section drawings. The specificity of the Hoechst labeling 8 days after injury was determined in a group of 3 animals in which the GRP cells were derived from PLAP rats. The PLAP staining was plotted from tissue sections adjacent to the Hoechst sections and difference in distribution between the two markers was assessed.

The distribution of transplanted cells was assessed 8 days and 6 weeks after injury via enzymatic labeling with NBT/BCIP. To make longitudinal composite figures (Figs. 3.4&3.5), images of tissue sections containing NBT/BCIP labeled cells were imported into Photoshop, outlined, and modified (using the levels, brightness/contrast and magic wand functions) to select the areas containing cells. Areas containing selected labeled cells were filled using the paint function, and overlaid, to produce a schematic representation of the cellular distribution of the transplanted cells.

Differentiation of transplanted GRP cells: To detect GRP cells that had differentiated, double labeling immunohistochemistry was performed to detect either PLAP-positive/GFAP-positive astrocytes or PLAP-positive/CC1-positive oligodendrocytes. Double labeling was performed on all 6-week animals. The presence of transplanted GRP cells that remain undifferentiated at 6 weeks was determined by PLAP/nestin staining. Slides were examined under standard fluorescence to detect possible double-labeled cells; confocal images of these cells were then taken. Using the CARVer software, cells that co-expressed markers could be detected within the stacked images. Only cells, with nuclei counterstained with Hoechst, that expressed PLAP and either CC1, GFAP or nestin were confirmed as double labeled.
RESULTS

Detection of GRP cells following transplantation

Initial results for the distribution of GRP cells 8 days after injury were obtained using GFP transfected GRP cells prelabeled with Hoechst 33342 for nuclear detection. In the 6-week experiment the GRP cells were derived from transgenic rat embryos that express hPLAP (Kisseberth et al., 1999). Three of the 8-day animals also received GRP cells from transgenic rats in order to more accurately determine the survival of GRP cells 8 days after injury.

Transplantation of GFP-transfected/Hoechst-labeled GRP cells immediately after injury resulted in many Hoechst-positive nuclei within the lesion site and in the surrounding gray and white matter rostrally and caudally (Fig. 3.1 A-E). However, no GFP expression was observed in this study. In the white matter Hoechst-positive nuclei were observed in rows similar to the appearance of oligodendrocytes in vivo (Fig. 3.1A,C). In the gray matter Hoechst-positive nuclei had a more irregular distribution (Fig. 3.1D). Hoechst-positive nuclei surrounded NF-positive neuronal cell bodies (Fig. 3.1 E), blood vessels (Fig. 3.1C, arrow), and lined the central canal (Fig 3.1F). When the locations of Hoechst labeled cells were plotted onto the tissue sections, the distribution within the cavity as well as in the gray and white matter could be detected (Fig. 3.2). Hoechst-positive nuclei were observed throughout the gray and white matter up to 5 mm rostral and caudal to the injury site.

Although Hoechst has been used to detect transplanted cells in a number of experiments (Menei et al., 1998; Ramon-Cueto et al., 1998; Harvey and Plant, 1995;
recent experiments have increasingly questioned the specificity of Hoechst as a marker for transplanted cells (Ruitenber et al., 2002; Iwashita et al., 2000a); raising concern that the Hoechst dye may leak out of the transplanted cells and be taken up by the surrounding tissue. To address this issue we transplanted three additional 8-day survival animals with transgenic PLAP cells prelabeled with Hoechst. The transgenic PLAP cells were detected using either AP enzymatic histochemical staining or PLAP immunohistochemistry. Transplanted PLAP-positive cells were detected within the lesion site at 8 days after injury (Figs. 3.3&3.4). Although some Hoechst labeled nuclei corresponded to PLAP-positive cells (Fig. 3.3), many Hoechst+ nuclei were not PLAP-positive (Fig. 3.3F-H), indicating that the Hoechst probably leaked out of the transplanted cells and was taken up by neighboring endogenous cells. This was particularly evident at the central canal where the ependymal cells were all labeled with Hoechst, while none were observed to be PLAP-positive (Fig. 3.3 C-E). Endothelial cells were also non-specifically labeled with Hoechst (Fig. 3.1C, arrow). Preferential uptake of Hoechst dye by endothelial and ependymal cells was also reported by Blakemore’s group (Iwashita et al., 2000a). Eight days after injury the PLAP labeled GRP cells were confined to the lesion site, while the Hoechst labeled nuclei were observed within 10 mm around the injection site (Fig. 3.3A,B). The remainder of the results presented are based on the distribution and differentiation of the transgenic PLAP GRP cells 8 days (n=3) and 6 weeks (n=6).

Survival and Migration of GRP cells following acute transplantation

GRP cells that express PLAP were able to survive immediate transplantation following contusive spinal cord injury and were detected at the lesion center at both 8
days and 6 weeks after injury. PLAP-positive GRP cells were detected with either the hPLAP antibody, or with NBT/BCIP histochemistry. The distribution of PLAP expressing cells detected via enzymatic staining is depicted in longitudinal sections 8 days after injury and in cross-sections 6 weeks after injury (Figs. 3.4&3.5).

Eight days after injury PLAP expression was confined to the lesion center. The intensity of NBT/BCIP staining, and the distance over which the stained cells were found appeared to be lower at 8 days (n=2) than at 6 weeks (n=6).

Six weeks after injury, dense staining was observed within the lesion center in 6/6 cases, indicating that the transplanted cells were able to survive up to 6 weeks following transplantation. Some cystic cavitation was observed in all cases, but there appeared to be good graft-tissue integration rostrally and caudally, as well as, within the trabeculae and within the spared rim of white matter. The majority of the transplanted cells were located within a 2.9 +/- 0.3 mm (mean +/- SEM) region centered at the impact site (max: 3.8; min: 2.0), however PLAP labeled cells were observed with a maximal spread of 13.4 mm within the spinal cord (mean +/- SEM: 6.4 +/- 1.4 mm; maximum: 13.44 mm; minimum: 4.0 mm). Within the core of the transplant, the density of the cells and the cytoplasmic nature of the PLAP staining precluded detection of individual cells. However, in cases where there appeared to be migration the number of GRP cells decreased away from the lesion center making detection of individual cells possible. Away from the transplant core, some GRP cells occurred in small patches, and had profiles similar to oligodendrocytes (Fig. 3.5). The case with the largest distribution of cells contained ramified profiles in the ventral white matter rostrally and caudally.
Differentiation of GRP cells following acute transplantation

Antibodies for nestin, GFAP and CC1 were used to label undifferentiated cells, astrocytes and oligodendrocytes respectively. The antibodies labeled both transplanted and endogenous cells. Double-labeled cells were identified using the antibody to human PLAP.

Nestin. Away from the injury site, nestin labeled the blood vessels, the radial glia, the dorsal and ventral roots and a few ependymal zone cells (Fig. 3.6A-D). At the injury/transplant site, a few scattered nestin-positive cells were found within the transplant (Fig. 3.6E-G), only a small proportion of PLAP cells labeled with nestin within the injury site. In some cases an increase in double-labeled nestin/PLAP cells was found dorsally suggesting that the location of the cells may have influenced their phenotype (Fig 3.6E).

GFAP. GFAP labeling within the transplant appeared more punctate than in uninjured tissue away from the injury site (see Chapter 5, Figs. 5.1&5.2). GFAP labeled cell bodies were observed within the gray and white matter rostral and caudal to the lesion. There was an increase in GFAP labeling at the lesion center that coincided with an increase in PLAP expression. The GFAP expression within the transplant was fine and mesh-like, consisting of small, interwoven processes, suggestive of immature astrocytes (see Chapter 5 for a more complete description). The presence of PLAP ventrally in the spared tissue that contained host astrocytes failed to show an increase in GFAP expression compared to adjacent areas that did not contain PLAP. As well, astrocyte cell bodies appeared to be smaller in the areas containing PLAP. Within the
PLAP labeled area, cells double-labeled for GFAP were observed (Fig. 3.7). Two double-labeled cells with Hoechst counterstained nuclei are observed (Fig 3.7, arrows).

**CC1.** Within the injured tissue, CC1 labeling appeared weaker than in intact control tissue away from the injury site. Oligodendrocyte profiles were observed within the transplanted tissue. Whether these are endogenous oligodendrocytes or GRP cells that have differentiated into oligodendrocytes is difficult to determine due to the weaker nature of the staining. Individual PLAP-positive cells could be distinguished within the spared tissue ventrally. These cells had a ramified appearance and at least a few were observed to double-label with CC1 (Fig. 3.8). CC1 labeling was confined to the small soma surrounding Hoechst counter-stained nuclei, while PLAP was observed both in the soma and in the processes. This is consistent with the CC1 antibody staining of oligodendrocytes described in several other reports (e.g. (Crowe et al., 1997)).

**DISCUSSION**

GRP cells have been shown to survive and differentiate into astrocytes and oligodendrocytes in the immature and mature CNS (Herrera et al., 2001), and to form myelin when transplanted into immature or myelin deficient rats (Herrera et al., 2001). However, their ability to survive and differentiate in an injured environment has not previously been assessed. This experiment was designed to examine 1) the survival and distribution of GRP cells 8 days and 6 weeks after spinal cord injury and transplantation and 2) the ability of GRP cells to differentiate into astrocytes and oligodendrocytes 6 weeks after injury and transplantation. A method to specifically label GRP cells that would last the duration of the transplant was a necessity of this experiment.
**GRP cell identification**

Three different markers were used to identify the transplanted cells: GFP-transfection of cells, nuclear prelabeling with Hoechst, and isolation of cells from transgenic PLAP rats. Only cells from transgenic rats labeled the transplanted cells specifically, and the cells maintained robust labeling for the 6 weeks examined.

*GFP transfected GRP cells.* In the initial 8-day survival experiment GRP cells were isolated and transfected with GFP. Prior to transplantation approximately 30% of the cells expressed GFP. However, 8 days after injury no GFP expression was observed within the injury site, at least as detected by our methods without immunohistochemistry for GFP-containing cells.

Although transfection of GRP cells prior to transplantation has been successfully used to identify cells, we were unable to detect the GFP expression. The lack of GFP expression could be due to lack of cell survival, however, our experiments using cells derived from the PLAP rat indicates that the latter is unlikely. More likely, the low GFP expression prior to injury made subsequent detection difficult. Use of FACS sorting prior to transplantation and use of an antibody to GFP has been used in other studies with transplanted GRP cells (Wu et al., 2002; Herrera et al., 2001) or neuronal restricted precursors (Yang et al., 2000) to detect transplanted cells. However, decreased GFP expression over time following transplantation has been observed in other studies (Ruitenberg et al., 2002; Wu et al., 2002; Yang et al., 2000).

*Hoechst labeled nuclei.* In addition to transfecting the GRP cells with GFP, GRP cells were preincubated with Hoechst 33342 immediately prior to transplantation so that both a cytoplasmic and nuclear label could be used to detect the cells. Although Hoechst
has been used to detect transplanted cells in a number of experiments (Menei et al., 1998; Ramon-Cueto et al., 1998; Harvey and Plant, 1995; Vignais et al., 1993), recent experiments have increasingly questioned the specificity of Hoechst as a marker for transplanted cells (Ruitenberg et al., 2002; Iwashita et al., 2000a), raising the concern that the Hoechst dye may leak out of the transplanted cells and be taken up by the surrounding tissue. In the present experiments, Hoechst labeled nuclei were present throughout the lesion site as well as in the gray and white matter rostrally and caudally. Hoechst did not label neurons, suggesting that it is not taken up by endogenous cells. However, due to our concern about non-specific Hoechst labeling of host cells, we transplanted 3 additional 8-day survival animals with GRP cells isolated from a transgenic PLAP rat and prelabeled then with Hoechst. In these animals, some Hoechst labeled nuclei corresponded to PLAP-positive cells (Fig. 3.3), however, many Hoechst+ nuclei were not PLAP-positive (Fig. 3.3), confirming that the Hoechst leaked out of the transplanted cells and was taken up by neighboring endogenous cells. This adds to the growing evidence that the use of Hoechst to specifically identify transplanted cells may not be appropriate, especially when transplanting cells that are able to proliferate, or when transplanting into an injury environment.

**Transgenic PLAP GRP cells.** The recent generation of a transgenic rat that ubiquitously expresses hPLAP (Kisseberth et al., 1999) provides a new tool for identifying transplanted cells. The advantages of using cells derived from transgenic rats are that all the cells express the gene of interest, and they do not have to be labeled _ex vivo_ prior to transplantation. AP staining can be detected at both the light and electron microscopy level using either histochemistry or immunohistochemistry (Leitner et al.,
2001; Rowden and Dean, 1991; Cordell et al., 1984). PLAP is detected in the cytoplasm and on the cell surface of transgenic cells permitting detection of cellular processes (Mujtaba et al., 2002). Two potential disadvantages of using AP as a reporter gene, are it has been reported to be secreted from cells (Fishman, 1990), and the CNS contains endogenous AP. The potential secretion of PLAP, by cells, means identification of nuclei and co-localization of markers is essential for differentiation studies. The concern of endogenous AP labeling is overcome in histochemical staining, by using a heat stable hPLAP transgene and denaturing or inhibiting the endogenous AP, and in immunohistochemistry by using a human specific antibody.

We show here that cells derived from the hPLAP rat can be detected for up to 6 weeks following transplantation into the rat CNS, and that these cells can be clearly identified and phenotyped. It is likely that transgenic rats will be widely used in the future for identification of transplanted cells. Although cells derived from transgenic animals appear to be very promising, it should be mentioned that the technology is still new, and the long term expression following transplantation of a variety of cell types remains to be demonstrated. Also, it has been recently reported that both bone marrow cells and pluripotent embryonic stem cells can fuse with differentiated cells resulting in tetraploid hybrid cells that adopt the phenotype of the recipient cells (Terada et al., 2002; Ying et al., 2002). Thus, careful consideration must be given to the markers used to label cells for transplantation and the conclusions drawn from these studies.

GRP cell survival

A number of transplantation experiments following SCI have revealed that transplanted cells survive best when transplanted either immediately after injury or after a
delay of at least 8 days (Giovanini et al., 1997; Martin et al., 1996; Theele et al., 1996). We chose to transplant acutely and assess the survival of cells initially at 8 days, and examine their differentiation potential 6 weeks after injury. PLAP labeled GRP cells were observed within the lesion site 8 days and 6 weeks after injury, confirming that GRP cells were able to survive immediate transplantation following SCI. Eight days after injury GRP cells were confined to the lesion site and labeling appeared weaker than at six weeks after injury. The increase in PLAP expression with time suggests that some of the GRP cells may initially die following transplantation, but that the remaining cells may proliferate over time to increase their distribution within the lesion. This has been observed to occur following acute transplantation of fetal tissue into spinal cord injuries (Theele et al., 1996). Although the lesion site contained GRP cells 6 weeks after injury, some cystic cavities were present in all cases. It is possible that delayed transplantation may result in increased survival of GRP cells and filling of the lesion cavity. Delayed transplantation may also be more applicable when considering transplantation as a therapy for human spinal cord injury. Alternatively, transplantation of a larger number of cells may result in better lesion filling (Takami et al., 2002b).

**GRP cell migration**

Eight days after injury PLAP expression was confined to the lesion center intermixed with macrophages. By six weeks, fewer macrophages were present within the lesion, and GRP cells were observed throughout the lesion site. Although transplanted GRP cells were observed in all cases, the extent that GRP cells filled the lesion, the extent of migration of GRP cells, and the presence of GRP cells within the spared white matter varied between animals. Good graft-tissue integration was observed at the rostral
and caudal margins of the injury site, suggesting that the GRP cells were able to integrate with the spared tissue. The majority of the transplant was located within close proximity to the injection site. GRP cells were able to integrate into the trabeculae and within the spared rim of white matter. PLAP-labeled cells extended beyond the main transplant in all cases, suggesting that at least some of the transplanted cells were able to migrate. Indeed in one case labeled cells were observed rostral and caudal of the main transplant area, and were found within a 13.4 mm region of the spinal cord. The greatest extension of GRP cells appeared to be in the white matter, predominantly along the ventral funiculus, but in some cases along the base of the dorsal funiculus. Due to the location of the posterior median spinal vein and attempts to avoid it during transplantation many of the transplants appeared to be concentrated unilaterally in the ventral cord. The difference between the longitudinal and lateral spread of GRP cells suggests that GRP cells migrate preferentially along the length of the white matter tracts rather than across them.

The ability of myelinating cells to migrate is an important issue and of interest to researchers studying demyelinating diseases, such as multiple sclerosis, since the diffuse nature of the demyelinating plaques means that if transplantation is going to be a viable therapeutic option, transplanted cells must be able to find the areas of demyelination. Examinations of the extent of migration of oligodendrocyte precursors and other myelinating cells have shown variable results (Blakemore et al., 2000; Iwashita et al., 2000b; Warrington et al., 1993), some perhaps due to the labels used to examine the cells. Generally, cells early in the oligodendrocyte lineage have a better migratory potential than more differentiated cells (Warrington et al., 1993). GRP cells have been shown to myelinate axons in the dysmyelinated rat and immature CNS (Herrera et al., 2000).
The possibility that GRP cells can migrate further than other cells that myelinate is promising given that A2B5+/O4- cells isolated from postnatal rat brains have been shown to migrate better than A2B5+/O4+ cells or O4+/GalC+ cells when transplanted into myelin-deficient mice brains (Warrington et al., 1993). GRP cells initially are A2B5+/O4- and their expression of oligodendrocyte lineage markers changes as they become more differentiated (Gregori et al., 2002). However, the extent to which GRP cells can migrate within demyelinated lesions remains to be examined.

**GRP cell differentiation**

Undifferentiated cells can be pushed down different lineages *in vitro* by altering the environment (Svendsen et al., 2001). However, the same differentiation potential is often not retained *in vivo* (Cao et al., 2001; Shihabuddin et al., 2000). Neural stem cells have potential to differentiate into neurons and glia. Their differentiation appears to be influenced both by intrinsic cell properties and extrinsic environmental properties (Hitoshi et al., 2002; Shihabuddin et al., 2000). Stem cells isolated from the adult spinal cord can differentiate into neurons and glia *in vitro*, and can differentiate into neurons when transplanted into neurogenic regions (such as the subventricular zone, or granule cell layer of the hippocampus), however, when transplanted into the intact spinal cord they do not differentiate into neurons (Shihabuddin et al., 2000). When stem cells are transplanted into the injured spinal cord—in which many neurons and oligodendrocytes have been lost and may need to be replaced—the majority differentiate into astrocytes, only a few become neurons or oligodendrocytes suggesting that a lack of cells is not sufficient to induce differentiation (Cao et al., 2001; Wu et al., 2001; McDonald et al., 1999). Pushing stem cells further down a lineage *in vitro* can alter the proproportion of
differentiated cells produced. For example, ES-cell-derived oligospheres transplanted into demyelinated spinal cord lesions are able to form a large number of myelinating oligodendrocytes (Liu et al., 2000). Likewise, neural restricted precursor cells transplanted into injury sites are able to form neurons in vivo (Yang et al., 2000). However, even precursor cells further down their lineage may not differentiate into all their potential phenotypes in vivo. O-2A progenitor cells can differentiate into oligodendrocytes and type-2-astrocytes in vitro yet predominately become oligodendrocytes when transplanted in vivo (delosMonteros et al., 1993). We are only just beginning to understand what regulates cellular differentiation. Greater understanding of it will aid in the regulation of cellular differentiation following transplantation.

GRP cells appear to retain their ability to differentiate into both oligodendrocytes and astrocytes in vivo. In this study GRP cell differentiation into oligodendrocytes (PLAP-positive/CC1-positive) appeared to be preferentially located in the ventral white matter, while GRP cell differentiation into astrocytes was more prevalent within the injury site. Unfortunately, the density of PLAP staining in and around the region precluded quantification of this response.

The results of this study show that GRP cells are able to survive immediate transplantation following spinal cord injury and that they are able to differentiate into oligodendrocytes and astrocytes and thus may be able to modulate the lesion environment following spinal cord injury. Spinal cord injury results in the formation of a cystic lesion cavity surrounded by a spared rim of white matter some of which contains intact but demyelinated axons. The formation of oligodendrocytes in vivo by GRP cells may be
restricted to unmyelinated axons \textit{in vivo}, since GRP cell differentiation into oligodendrocytes was restricted to unmyelinated sites in either immature rats or dysmyelinated rat mutants (Herrera et al., 2001), and was observed primarily in the ventral white matter in this study. GRP cells appear to differentiate into astrocytes within the lesion site. Immature astrocytes can modulate scarring after CNS injury (Smith and Miller, 1991; Smith and Silver, 1988) and can support axonal growth \textit{in vitro} (Smith et al., 1990) and \textit{in vivo} (Smith et al., 1987; Smith et al., 1986). In vitro, GRP derived astrocytes support axonal growth (Dr. Mark Noble, personal communication), thus differentiation of GRP cells into astrocytes in vivo could potentially permit axonal growth after spinal cord injury.

In summary, using GRP cells isolated from a transgenic PLAP rat we have shown that 1) PLAP transgene expression remains robust and specific for up to 6 weeks after transplantation allowing for identification of cells within the injured spinal cord, 2) GRP cells survive immediate transplantation following SCI and are observed within the lesion site 8 days and 6 weeks after injury with good host-graft integration, 3) when transplanted into an injured CNS environment GRP cells retain their differentiation potential and can differentiate down both astrocytic and oligodendrocyte lineages. How the survival and differentiation of GRP cells alter the lesion environment and whether they affect recovery from spinal cord injury remains to be determined, and is addressed in the subsequent chapters. However, the ability of GRP cells to retain their differentiation potential makes these cells a useful tool for studying both remyelination and regeneration within the CNS. The technical tools and observations developed in the present, descriptive study, should be useful in designing more quantitative attempts to gauge the
potential of GRP cells and other progenitor cells as therapies for spinal cord and brain injury.
<table>
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<tr>
<th>Primary Antibody</th>
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<th>Incubation time</th>
<th>Isotype</th>
<th>Source</th>
<th>Secondary Antibody</th>
<th>Concentration</th>
<th>Incubation time</th>
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<td>1:200</td>
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<td>CC1 (APC)</td>
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<td>2-3 days</td>
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<tr>
<td>Nestin (Rat-401)</td>
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<td>DSHB</td>
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<td>2 hrs</td>
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Table 1: List of antibodies used in Chapter 3. All antibody staining was performed by washing the tissue in buffer, blocking the tissue for 1 hour in either 0.5% Triton 100X, 5% normal goat serum in buffer, or 0.1% Tween-20, 10% milk in buffer before applying the primary antibody diluted in the blocking solution. After primary antibody incubation (4°C) the tissue was again washed before secondary antibody incubation at room temperature. Following secondary antibody incubation the slides were washed, coverslipped with Vectashld (Molecular Probes, Eugene, OR) and stored at 4°C until examined. All incubations occurred in the dark. Double-labeling was performed by initially staining for PLAP, followed by staining for either CC1, GFAP or nestin. Tissue stained for CC1 or nestin underwent antigen-retrieval prior to immunohistochemistry. The Rat-401 hybridoma was developed by Susan Hockfield and obtained from the Developmental Systems Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). CC1 is also called APC and Ab-7. Abbreviations: GFAP (Gial Fibrillary Acidic Protein), PBS (Phosphate buffered saline).
Figure 3.1: Labeled nuclei within the spinal cord 8 days following transplantation of GRP cells prelabeled with Hoechst (HO). Many HO-labeled nuclei are observed in both the white and gray matter around the injury site. (A) White matter (WM) and gray matter (GM) rostral to injury. (B) Many HO-nuclei are present within the injury site 8 days after injury. (C) HO-nuclei appear in rows in the white matter, HO-nuclei are also observed surrounding a blood vessel (arrow). (D) HO-nuclei have an irregular arrangement in the gray matter and (E) are observed surrounding neurons, but do not appear to label the nuclei of neurons (arrow). (F) HO nuclei are prominent along the central canal. (A-F) Hoechst nuclei (small bright dots; blue), (E) Neurons (arrows; green).
Figure 3.2: Hoechst nuclei within the spinal cord 8 days after transplantation were plotted onto camera lucida sections of the spinal cord, A-E dorsal to ventral. (A) dorsal columns (B) CST, (C) section at the level of the central canal, (D&E) ventral half of the cord. HO-nuclei are present throughout the spinal cord around the injection site. The greatest extension occurs in the white matter, and along the central canal. Scale bar 100µm.
Figure 3.3: Comparison of labeling between PLAP and Hoechst labeling 8 days after SCI and transplantation. (A) HO-nuclei are present within the lesion and in gray and white matter surrounding the injury (small dots). (B) PLAP-labeled GRP cells are confined to the lesion site, detected with PLAP immunohistochemistry (patch within center of cord; green). (C-E) Some PLAP-labeled cells contain HO-nuclei, however, many of the HO-nuclei do not correspond with transgenic PLAP GRP cells (arrowheads in (C) point to region of HO-positive nuclei that do not correspond to PLAP labeling). (F-H) PLAP labeling was not observed at the central canal where most nuclei were HO-labeled. (C&F) PLAP cells (green), (D&G) HO-nuclei (small dots; blue) (E) Overlay of C&D (H) overlay of F&G. (C-H) Scale bar 50 μm.
Figure 3.4: Distribution of NBT/BCIP stained transgenic GRP cells 8 days after injury. (A-D) Using alkaline phosphatase enzymatic staining, GRP cells were detected within the spinal cord. (A, B) PLAP cells were mainly confined to the lesion center (A is enlargement of B). (C) Schematic of labeled cells from an individual section. (D) overlay of sections containing labeled cells.
Figure 3.5: Distribution of NBT/BCIP stained transgenic GRP cells 6 weeks after injury. Using alkaline phosphatase enzymatic staining, GRP cells were present within the spinal cord. (A) Dense labeling was present within the lesion, with extensions rostrally and caudally (rostral to left, caudal to right). (C) Within the lesion cells were present surrounding the lesion, within the trabeculae, the ventral spared tissue and in tissue extrusions dorsally, enlarged in (C). Rostrally (B) and caudally (D) individual cells could be detected (arrows).
Figure 3.6: Nestin labeling within the injured spinal cord 6 weeks after injury. Away from the lesion site nestin labeled (A) blood vessels (arrows), (B) large multipolar cells that extended radially (arrow), (C) ventral roots (arrows), and dorsal roots (not depicted), (D) some ependymal zone cells (arrow). (E-G) Within the injury site a few nestin-positive cells were found within the PLAP-positive areas. Some nestin expression was intermixed with PLAP cells within the tissue protrusion dorsally (E). Few nestin-positive cells were located within the core of the transplant, instead they were at the transplant margin (F,G). Arrows point to nestin-positive cells. Nestin (green); PLAP (red); Hoechst (blue).
Figure 3.7: GFAP labeling within the injured spinal cord 6 weeks after injury. (A, B) Some PLAP-positive cells double label with GFAP. (B) Two double-labeled astrocytes associated with HO-counterstained-nuclei can be followed through confocal slices (arrows point to HO-nuclei within the double-labeled cells. GFAP (green); PLAP (red); Hoechst (blue).
Figure 3.8: CC1 labeling within the injured spinal cord 6 weeks after injury. (A) Some PLAP-positive cells double label with CC1. Two double-labeled oligodendrocytes associated with HO-counterstained-nuclei can be followed through confocal slices (B, C). Arrows point to the location of double labeled cells. CC1 (green); PLAP (red); Hoechst (blue).
INTRODUCTION

Spinal cord injury results in the formation of a cellular and molecular scar that is thought to act as a barrier to axonal regeneration. In the immature nervous system a reparative response occurs which minimizes glial scarring and permits tissue repair including axonal regeneration (Bregman et al., 1993; Smith et al., 1986; Schreyer and Jones, 1983). However, following injury to the mature CNS, the rapid cellular activation and response to injury can potentiate the tissue damage. The activation of the immune response, particularly the damage to the blood-brain-barrier and the infiltration of neutrophils and macrophages, plays a critical role in the development of several aspects of secondary injury including gray matter destruction, axonal damage, and cavitation. Astrocytes respond to injury by proliferating and hypertrophying to wall off the injury site (Fawcett and Asher, 1999; Amat et al., 1996; Reier and Houle, 1988). At the same time, a basement membrane containing various extracellular matrix molecules including inhibitory proteoglycans molecules, is laid down to seal off the injury site (Fawcett and Asher, 1999; Reier and Houle, 1988). Both the physical barrier produced by the hypertrophy of astrocytes and the molecular barrier produced by proteoglycans, particularly
chondroitin sulphate proteoglycans (CSPGs), have been implicated in the failure of axons to regrow following spinal cord injury (Bradbury et al., 2002; Fitch et al., 1999; Fawcett and Asher, 1999; Davies et al., 1997; McKeon et al., 1991; Reier and Houle, 1988). The transplantation of immature cells following CNS injury can attenuate the scarring response (Olby and Blakemore, 1996; Wang et al., 1995; Smith and Miller, 1991; Smith and Silver, 1988; Houle and Reier, 1988; Reier and Houle, 1988; Smith et al., 1986). The purpose of the present study was to look at the effects of acutely transplanted glial restricted precursor (GRP) cells on the lesion environment (macrophages, astrocytes, and CSPGs) 8 days after a contusive spinal cord injury.

**Glial restricted precursor cells.** GRP cells are multipotent cells that can differentiate into oligodendrocytes and both type 1 and type 2 astrocytes (Herrera et al., 2001; Rao et al., 1998). They are isolated from embryonic spinal cord and show extensive self-renewal *in vitro* (Rao et al., 1998), allowing for sufficient cells to be produced for transplantation. GRP cells appear to remain restricted to the glial lineage *in vivo* (Herrera et al., 2001), and retain their ability to differentiate into oligodendrocytes and astrocytes following transplantation into both the uninjured, demyelinated and injured CNS (Herrera et al., 2001) (Chapter 4). GRP cells could potentially ameliorate the damage after spinal cord injury by forming remyelinating oligodendrocytes, and/or a growth promoting substrate via immature astrocytes (Smith and Miller, 1991; Smith et al., 1990; Smith and Silver, 1988; Smith et al., 1987; Smith et al., 1986) (Dr. Mark Noble, personal communication). As well, early transplantation may allow GRP cells to modulate the lesion environment to make it less inhibitory, similar to changes observed following transplantation of other immature cells (Olby and Blakemore, 1996; Wang et
al., 1995; Smith and Miller, 1991; Smith and Silver, 1988; Houle and Reier, 1988; Reier
and Houle, 1988; Smith et al., 1986).

In this experiment we observed a reduction in 1) astrocytic scaring, and 2) CSPG
expression eight days after injury, and 3) a trend towards a reduction in the infiltration of
macrophages early after injury. Together these data suggest that GRP cells can modulate
the environment early after injury and may potentially make it more permissive for
axonal growth.

MATERIALS AND METHODS

Subjects

Twenty-seven female rats (Long Evans, Simonsen Laboratories) 83-95 days old
were used in this study, and all received a moderate 12.5 g-cm spinal cord contusion
injury with the NYU/MASCIS impactor.

Animals were divided into 4 groups: (1). injury control (SCI) (n=6); (2). immunosuppressant control (MP/CsA) (n=6); (3). conditioned media control (CM) (n=6);
(4). experimental group (GRP cell) (n=9), and underwent a single surgery that involved
jugular catheterization, spinal cord injury and transplantation. All procedures were
carried out using aseptic technique and in compliance with NIH guidelines and were
approved by the Institutional Laboratory Animal Care and Use Committee.

Surgical procedures

Jugular catheterization. Rats were anesthetized with 40-60 mg/kg of pentobarbital i.p.
(Abbott Laboratories, Chicago, IL) and given an injection of 25 mg/kg of keflin (Lilly,
Indianapolis IN) antibiotic sc. Animals receiving methylprednisolone sodium succinate
(MP) (Pharmacia & Upjohn, Kalamazoo, MI) and cyclosporine (CsA) (Novartis Pharmaceuticals Corporation, East Hanover, NJ), MP/CsA, CM and GRP cell groups, had a catheter inserted into their jugular vein for i.v. administration of MP (30mg/kg) 5 min, 2 hrs, and 4 hrs after injury, as previously described in Chapter 3.

**Spinal Cord Contusion and Transplantation Procedures.** Following insertion of the jugular catheter, a laminectomy was performed to expose the dura of the spinal cord at T 9-10 and a 12.5 g-cm contusion injury was induced using the NYU/MASCIS device by dropping a 10g weight from 12.5 mm onto the exposed dura of the spinal cord (Constantini and Young, 1994; Gruner, 1992). Animals were allowed to stabilize after injury, and animals receiving MP received their initial dose before the transplantation procedure. All animals had a 26S gauge Hamilton syringe (Hamilton company, Reno, Nevada, USA) needle inserted 1.5-2.5 mm into the spinal cord at the impact site. Animals received either no injection, 5 µl of GRP conditioned media or 500,000 GRP cells in 5 µl of CM slowly over 5 minutes. Following the transplantation procedure, the wound was closed in anatomical layers and animals were placed singly in temperature-controlled incubators overnight. Subsequently, they were housed in pairs in standard plastic cages and given food and water ad libitum. Keflin (25mg/kg) s.c. (Lilly, Indianapolis, IN), and cyclosporin (10mg/kg) i.p. was given once a day. Bladders were expressed twice a day. Weight was monitored and supplemental feeding with Nutrical (Evsco Pharmaceuticals, Buena, NJ) was administered if animals fell below 80% of their pre-surgery weight.

**GRP cell isolation.** GRP cell were isolated, from E13.5 rat spinal cords, by our collaborators at the University of Rochester as described previously (Herrera et al., 2001; Rao et al., 1998)(Chapter 4). The initial 6 GRP cell transplants involved cells that were
transfected with GFP and frozen in batches. These cells were thawed and grown at Ohio State until there was a sufficient number for transplantation (2-3 passages). Three additional animals were transplanted with GRP cells that were derived from a transgenic rat in which the human placental alkaline phosphatase (hPLAP) gene is inserted behind the ubiquitously expressed ROSA26 promoter (Kisseberth et al., 1999). These cells were isolated by Dr. Chris Proschel (CP) and were shipped live and transplanted the following day. Prior to injection cells were labeled with the DNA intercalating dye Hoechst 33342 (10 µg/ml for 30 minutes) as a second marker for cellular detection. Cells were then washed, and spun down to 100,000 cells/µl. Five µl were then drawn into a 26S gauge Hamilton syringe and injected slowly into the impact site with the needle bevel facing caudally within 20 minutes of the spinal cord injury.

**Perfusion procedures**

Animals were perfused 8 days after SCI. All rats were terminally anesthetized with 0.8 mg/kg of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 0.5 mg/kg of xylazine (Vedco, Inc, St. Joseph, MO) and transcardially perfused with 0.5 ml of lidocaine (Elkins-Sinn, Inc, Cherry Hill, NJ) and 0.1 ml of heparin (Elkins-Sinn, Inc, St. Joseph, MO) followed by 0.9% saline, and then by 4% paraformaldehyde in PBS, pH 7.4 (Fisher Scientific). The laminectomy site was marked and the spinal cords were removed and post-fixed for 4–8 hours in 4% paraformaldehyde. A 10 mm segment of the spinal cord centered on the lesion site was removed and placed into 30% sucrose PBS for 24-48 hrs, frozen, and stored at-80°C until cut.

**Histological Procedures**
The spinal cords were cut horizontally at 16 µm with a cryostat, and four serial sets of slides were prepared for each case. Sections were mounted on gelatin coated glass slides. Two sets were used to identify GRP cells (described in Chapter 4). Two other sets were used in this study to examine the effects of GRP cells on the lesion environment by processing one set for OX-42 immunohistochemistry and another set for GFAP and CSPG, some of the sections stained with PLAP to detect GRP cells are also described here in relation to CSPG, GFAP and OX-42 expression.

*Immunohistochemistry.* Immunohistochemistry was performed to detect PLAP, macrophages (OX-42), GFAP and CSPG (See Table 2). Antibody staining was performed by washing the tissue three times for 10 minutes each in buffer, blocking the tissue for 1 hour in 0.5% Triton 100X, 5% normal serum (of the secondary antibody) in buffer. Following primary antibody incubation (4°C) the tissue was washed three times for 10 minutes prior to secondary antibody incubation at room temperature. Slides with biotinylated secondary antibodies (OX-42 staining) were processed using the peroxidase-anti-peroxidase method. ABC (Vector standard peroxidase kit PK 4000, Burlingame, CA) was applied for 1 hour, the tissue was then washed, and stable DAB (Research Genetics, Huntsville, AL) was applied until a brown precipitate was detected. Tissue stained with DAB was then counterstained with cresyl violet, dehydrated through a graded alcohol series into histoclear and coverslipped with permount. Endogenous peroxidase within the tissue was quenched by incubating with 0.3% H₂O₂ prior to primary antibody incubation.

GFAP and CSPG primary antibodies were applied together overnight. The fluorescent secondary antibodies for GFAP and CSPG were applied consecutively. The
tissue was washed, in between and after secondary antibody application, coverslipped with Vectashild (Molecular Probes, Eugene, OR) and stored at 4°C until examined. Due to concern for non-specific IgM labeling early after injury, an additional set of slides from one animal per group was stained with a modified CSPG protocol in which the tissue was blocked for 1 hour (10% non-fat milk, 0.1% Tween in PBS), CS-56 was applied at 1:10 for 2 days at 4C, and the Alexa Fluor®-594 goat-anti-mouse secondary antibody was preabsorbed with normal rat serum (NRS) for 10 minutes before being applied. Preabsorption with NRS resulted in weak staining and required the primary antibody concentration to be increased.

Data analysis

Analysis of the lesion site. Immunohistochemical markers were used to assess alterations in the lesion site 8 days after injury. OX-42 was used to label macrophages to examine the inflammatory response, GFAP was used to examine the walling off of the lesion site by astrocytes and CS-56 was used to label chondroitin sulfate proteoglycans, an extracellular matrix protein that is known to inhibit axonal growth. All slides were examined without knowledge of group inclusion.

OX-42. Macrophages and microglia were labeled with OX-42 and counterstained with cresyl violet to examine the extent of inflammation. The injury site, defined as the area of maximal OX-42 staining, was outlined using a stereology program (The Stereologer™, Systems Planning and Analysis, Inc. Alexandria, VA) and using the Cavalieri method, measurements of lesion volume, macrophage volume and percentage of lesion occupied by macrophages were determined, based on 11 sequential sections, in the stained set, centered around the central canal. To be included in the macrophage
analysis, OX-42 positive cells needed to be round without processes, and have a
discernable cell membrane. This was to ensure that phagocytic macrophages/microglia
were counted and not activated microglia. Measurements of cord volume were also
performed, by outlining every third section in the set, using the Stereologer and
standardized to a 10 mm long horizontal section of cord. The percentage of the cord
occupied by the lesion was calculated by \((\text{lesion volume/standardized cord volume}) \times 100\). Two chords with poor preservation of the lesion were removed from the data.
Stereological analysis was performed twice for each case (once each by two independent
observers blind to the treatment groups). Criteria for inclusion were made more specific
for the second analysis, and these results are reported.

**GFAP.** GFAP was used to label astrocytes. The extent of GFAP upregulation 8
days after injury was examined around the lesion site. An observer blind to treatment
condition examined the slides, and the intensity of the GFAP staining at the lesion
boundary was ranked as high, medium or low intensity.

**CSPG.** CS-56 was used to label CSPG. The intensity of the labeling both within
the lesion, and at the perimeter of the lesion, was ranked using the same scale as for the
GFAP.

**Behavioral analysis.** Animals were tested for open field locomotion using the
Basso-Beattie-Bresnahan (BBB) locomotor test (Basso et al., 1995) at days 2 and 8. This
is a 21-point rating scale where zero is complete paralysis and twenty-one is normal
coordinated gait. Animals were placed in a pool and were scored over a four-minute
rating period, by two observers blinded to treatment group.
**Statistical Analysis.** Macrophage data was analyzed using one-way ANOVAs using SPSS (SPSS Inc., Chicago IL). CSPG and GFAP semi-quantitative ranking data was analyzed using Mann-Whitney U tests. Open field locomotor scores were analyzed with SAS (SAS Institute Inc. Cary, NC) using a two-way analysis of variance (ANOVA). The factors were groups (n=4) and day post-injury (n=2) with repeated measures used for the second factor. Post hoc analysis was performed using Tukey’s honestly significant difference test. Unequal group sizes were corrected using the harmonic means of the groups.

**RESULTS**

*Alterations in the lesion environment following immunosuppression and GRP cell transplantation*

As reported in the previous chapter, GRP cells were able to survive immediate transplantation, and using an antibody to PLAP, were detected within the lesion site 8 days after injury. GRP cells were located within the macrophage filled lesion site. An astrocytic border surrounded the injury site, which contained proteoglycan and macrophage labeling. The distribution of the markers relative to one another is depicted in Fig. 4.1.

*Macrophages.* Upon histological examination, immunosuppressive treatment appeared to decrease macrophage infiltration compared to the injury alone group; a further reduction was observed in groups receiving either conditioned media or GRP cells (Fig. 4.2A). However, when the macrophage response was quantified using The Stereologer®, a significant difference between the groups was not observed for: 1) lesion
size (mm$^3$) 2) volume of lesion occupied by macrophages; or 3) percent of a 10 mm segment of spinal cord occupied by the lesion (Fig. 4.2 B,C,E). There was a trend towards differences in the percentage of lesion occupied by macrophages (ANOVA: $F=2.82$; $p=0.06$; df: 3, 21) across the four groups, with the CM and GRP cell groups being lowest, however this was not statistically significant (SCI vs. CM $p>0.1$; SCI vs. GRP cells $p>0.2$)(Fig. 4.2D).

Proteoglycans. Normally after SCI, CSPGs are upregulated at the lesion site, with CSPG expression correlating with the location of macrophages and opening of the blood brain barrier (Fitch and Silver, 1997). In the untreated SCI lesion, a thin line of CSPG expression was observed delineating the boundary between the injury site and the spared white matter (Fig. 4.3E). Another high area of CSPG expression was observed within the lesion core, here the CSPG staining was very intense and almost granular, and in some places was associated with blood vessels (Fig. 4.3C). CSPGs were highly expressed on blood vessels in and around the lesion. Throughout the remainder of the lesion site the CSPG expression was matrix-like, surrounding areas in which macrophages were located. Following transplantation of GRP cells, CSPG staining persisted within the lesion site (Fig 4.3A). When the most intense area of staining within the lesion was analyzed semi-quantitatively, a reduction was observed only in the GRP cell transplant group (Fig. 4.3B) (Mann-Whitney: $U=4$; $p<0.01$; $n_1=6$, $n_2=9$) (Fig. 3.3B). It is conceivable that the area of intense staining could correspond to the needle insertion site.

CSPGs were expressed within the lesion site in all groups 8 days after injury. Alterations in the expression of CSPGs at the lesion margins were observed between the groups (Fig. 4.3E). In the injury alone group, a clear line of CSPG expression was
apparent between the spared white matter and the injury site. Following GRP transplantation this boundary was no longer as distinct. Instead a gradation of CSPG expression from the injury site into the white matter was observed (Fig. 4.3E). Semi-quantitative analysis of the distinction between the lesion and the spared white matter based on CSPG labeling showed significant differences between the groups (Fig. 3.3D). The MP and GRP cells groups had a significant decrease in intensity compared to the SCI group (SCI vs. MP: Mann-Whitney: U=5; p=0.02; n₁=6, n₂=6; SCI vs. GRP cells: Mann-Whitney: U=3; p<0.01; n₁=6, n₂=9). The CM group did not show this reduction, instead there was an increase in CSPG expression within the lesion and an intermittent boundary at the lesion edge.

On a technical note, subsequent staining for CSPG (in an unrelated study) revealed non-specific staining with the Alexa-594 IgM goat-anti-mouse secondary, which was not initially observed in control sections with no primary antibody (unpublished data). Problems with mouse IgM antibodies cross-reacting with endogenous rat antibodies early after injury have previously been reported (Moon and Fawcett, 2001). Pre-incubation of the secondary antibody with normal rat serum for 10 minutes prior to incubation decreased the non-specific secondary binding. By increasing the concentration of the primary antibody similar, but weaker, CSPG staining was observed when one animal per group was stained and examined. Staining of blood vessels within the lesion, and co-localization of CSPG with astrocytes within the ventral white matter was observed.

_Astrocytes._ Following SCI, astrocytes hypertrophy and increase their expression of the intermediate filament protein GFAP (Baldwin et al., 1998). Eight days after a
contusive spinal cord injury, an upregulation of GFAP was observed in astrocytes as they walled off the injury site (Fig. 4.4). Following GRP cell transplantation, there was a decrease in the astrocytic scar surrounding the injury site (Fig. 4.4C). Semi-quantitative ranking of the intensity of the GFAP staining in the spared white matter by an observer blinded to group inclusion was performed (Fig 4.4B). Injury alone resulted in the greatest GFAP expression. Cases in the GRP cell transplant group had the least GFAP expression, and MP/CsA or CM control groups were intermediate (Mann-Whitney U see Figure 4.4 for significance levels). Eight days after injury GRP cells did not appear to express GFAP, because there was not an increase in GFAP expression within the lesion site associated with the transplanted cells.

Behavior

Recovery of hindlimb function was detected in all animals in the open field between 2 and 8 days. Initially after SCI, animals showed hindlimb paralysis, by 8 days after injury animals scored between 8 and 11 on the BBB scale. By 8 days animals were able to plantar place their hindlimb and some animals showed weight supported stepping. Open field locomotion did not differ between the four groups at either 2 or 8 days after injury (2 way ANOVA F=1.58 p=0.22 df=3).

DISCUSSION

Glial restricted precursor cells are multipotent cells of the glial lineage that are derived from E13.5 rat spinal cord (Rao et al., 1998). GRP cells are able to differentiate down oligodendrocyte and astrocyte lineages in vitro (Gregori et al., 2002; Rao et al., 1998) and in vivo (Herrera et al., 2001). In the previous chapter we showed that GRP
cells were able to survive immediate transplantation, were present within the lesion site 8 days and 6 weeks after injury, and that a proportion of them were able to differentiate into oligodendrocytes and astrocytes 6 weeks after injury. Many transplant studies have focused on the differentiation potential of transplanted precursor cells (Herrera et al., 2001; Wu et al., 2001; Kitada et al., 2001; Sasaki et al., 2001; Chow et al., 2000; Liu et al., 2000; McDonald et al., 1999; Whittemore, 1999; Shihabuddin et al., 1997; Giovanini et al., 1997; Houle and Reier, 1988). However, fewer have examined how the transplanted cells influence the injury environment (Olby and Blakemore, 1996; Smith and Silver, 1988; Houle and Reier, 1988; Reier and Houle, 1988). In the present study we examined the infiltration of macrophages, and the expression of GFAP reactive astrocytes and chondroitin sulfate proteoglycans 8 days after GRP cells were transplanted into a moderate contusion injury. Results of GRP cell transplantation were compared with animals that received an injury alone (SCI), immunosuppression (MP/CsA) or immunosuppression plus an injection of GRP cell conditioned media (CM).

The interplay between the inflammatory response, the astrocytic response and the expression of inhibitory molecules after spinal cord injury, is not yet clearly understood. In this study macrophages and CSPGs were observed within the injury site 8 days after injury. CSPG expression was highest within the center of the lesion, along blood vessels and along the margin between the injury site and the spared white matter. GFAP expression by astrocytes was highest at the lesion margin, between the injury site and the spared tissue. Transplantation of GRP cells immediately after injury altered the lesion environment. By transplanting the GRP cells immediately after injury we were able to decrease the astrocytic scarring and proteoglycan deposition 8 days after injury.
The acute response to spinal cord injury involves the breakdown of the blood brain barrier and the infiltration of immune cells. Neutrophils and macrophages, play a critical role in the development of several aspects of secondary injury including gray matter destruction, axonal damage, and cavitation (Popovich et al., 1999; Taoka et al., 1997; Blight, 1994), and potentially astrocytic activation and migration (Fitch et al., 1999), and expression of proteoglycans (Fitch and Silver, 1997). The damaging effects of spinal cord injury can be partially ameliorated through inhibition of the innate cellular immune response (Popovich et al., 1999; Taoka et al., 1997; Blight, 1994). Depletion of either neutrophils or macrophages affects the progression and consequences of injury, increasing tissue sparing and altering lesion development (Popovich et al., 1999; Taoka et al., 1997; Blight, 1994).

In addition to the normal progression of the inflammatory response to CNS tissue damage, transplantation of allogenic cells runs the risk of initiating an immune response that causes transplant rejection. Transplantation of GRP cells did not alter the macrophage infiltration or the lesion size 8 days after injury. In this experiment we administered MP and CsA together, CsA to prevent the rejection of GRP cells, and MP because it is the standard therapy for SCI. Although both MP and CsA are thought to be neuroprotective (Diaz-Ruiz et al., 2000; Hall, 1992; Hall and Wolf, 1984), there are contradictory reports on their effectiveness following spinal cord injury (Takami et al., 2002; Rabchevsky et al., 2002; Rabchevsky et al., 2001; Oudega et al., 1999; Diaz-Ruiz et al., 1999; Behrmann et al., 1994; Means et al., 1981). Administration of MP following experimental transection (TX) or hemi-section (HX) spinal cord injury has been reported to result in decreased infiltration of neutrophils and macrophages (Oudega et al., 1999;
Bartholdi and Schwab, 1995), increased tissue sparing (Oudega et al., 1999), decreased
dieback and the promotion of regeneration in some fiber systems (Oudega et al., 1999;
Chen et al., 1996). As well, previous reports from our lab (Behrmann et al., 1994) have
shown modest behavioral recovery in the rat following contusion injury. In the current
study, MP/CsA did not increase tissue sparing or reduce macrophage infiltration 8 days
after injury compared to injury alone. Nor did it result in behavioral alterations in the
open field. Although our results are contradictory to reports of decreased macrophage
infiltration following TX or HX (Oudega et al., 1999; Bartholdi and Schwab, 1995), and
behavioral recovery following SCI (Behrmann et al., 1994), our results are in agreement
with recent studies in which the administration of CsA or MP, using the same injury
severity and model as in this study (12.5 mm injury with the NYU device), had no effect
on lesion development or behavior (Takami et al., 2002; Rabchevsky et al., 2002;
Rabchevsky et al., 2001).

The interplay of endogenous responses early after injury affects the morphology
of the chronic lesion. Many of the endogenous processes that decrease tissue damage,
also prevent axonal regeneration. Both astrocytes and proteoglycans have critical roles in
maintenance of the intact CNS. Alterations in GFAP and CSPG expression after injury
likely limit the spread of tissue damage (Moon et al., 2002; Bush et al., 1999; Hoke and
Silver, 1996), however at the same time they form a barrier to axonal growth (Moon et
al., 2002; Bush et al., 1999; Hoke and Silver, 1996). Following SCI, astrocytes
hypertrophy and increase their expression of GFAP as they wall off the injury site
(Baldwin et al., 1998). Following contusion injury astrocytic alterations in cell/process
size are observed 2 days after injury with maximum alterations and GFAP expression
observed 7-14 days after injury and persisting at the lesion site (Baldwin et al., 1998). In this study GFAP-positive astrocytes walled off the injury site eight days after a contusive spinal cord injury, similar to previous reports. The astrocytic response to injury is associated with the formation of a physical and molecular barrier associated with the failure of axons to regrow following spinal cord injury (Bradbury et al., 2002; Fitch et al., 1999; Fawcett and Asher, 1999; Davies et al., 1997; McKeon et al., 1991; Reier and Houle, 1988). Astrocytes, and other cells (activated macrophages, fibroblasts, meningeal cells) increase proteoglycan expression following CNS injury (Jones et al., 2002; Plant et al., 2001; Fitch et al., 1999; McKeon et al., 1991); as does the pia and blood vessels (Loy et al., 2002; Plant et al., 2001; Fitch et al., 1999). Robust proteoglycan expression following penetrating lesions (Moon et al., 2002; Jones and Tuszynski, 2002; Jones et al., 2002; Plant et al., 2001; Davies et al., 1997; Fitch and Silver, 1997; Levine, 1994; Dou and Levine, 1994; McKeon et al., 1991), is associated with barrier formation (Fitch and Silver, 1997), and the failure of axonal growth (Davies et al., 1997; Dou and Levine, 1994; McKeon et al., 1991). Increased axonal growth is observed after injury when either astrocytes or CSPGs are removed or absent (Bradbury et al., 2002; Moon et al., 2001; Bush et al., 1999; Davies et al., 1997).

Despite the growing evidence that CSPG expression increases following penetrating spinal cord injuries, few studies have looked at the expression of CSPG following contusive spinal cord injury (Loy et al., 2002; Lemons et al., 1999). Here we show that following contusive SCI, CSPGs (detected by the CS-56 antibody) are observed within the lesion center, associated with the needle insertion site and blood vessels, and at the lesion margin, separating the injury site from the spared white matter.
In the present study both GFAP and CSPG expression differed between the four treatment groups. Following GRP cell transplantation GFAP and CSPG expression was reduced compared to injury alone or immunosuppression. Astrocytes continued to wall off the injury site in all groups, but the GFAP labeling was less extensive following GRP cell transplantation. The reduction in GFAP expression corresponded to a reduction of CSPG expression at the lesion margin. It is likely that part of the observed reduction is due to immunosuppression, since MP/CsA administration also reduced astrocytic scarring and CSPG expression-although to a lesser extent. The attenuated GFAP and CSPG expression following MP/CsA treatment, or GRP cell transplantation, may be a result of delayed macrophage infiltration. Activated macrophages are involved in cavity formation and scarring (Popovich et al., 1999; Fitch and Silver, 1997). Activated macrophages can induce astrocyte migration, cavity formation and increased CSPG expression in vitro, suggesting that they contribute to lesion development and scarring (Fitch and Silver, 1997). The role of macrophages in scarring is further supported by in vivo studies in which decreased macrophage infiltration is associated with decreased cavitation and increased axonal sprouting (Popovich et al., 1999). Together this suggests that by delaying macrophage infiltration after injury, lesion development can be altered. Although we did not observe any significant alterations in macrophage infiltration 8 days after injury, MP has been shown to decrease the macrophage response 2 days after injury (Rabchevsky et al., 2002). The early reduction observed by Rabchevsky, might result in the alterations in CSPG and GFAP expression observed in this study.

Although immunosuppression can decrease CSPG expression, immunosuppression does not necessarily prevent CSPG expression. Injection of CM in
conjunction with immunosuppression resulted in CSPG expression persisting at the lesion margin adjacent to the spared white matter. It is possible that factors within the CM promoted the proliferation of CSPG expressing cells within the injury site. The CM contains both exogenous growth factors required for GRP cell survival (including but not limited to bFGF, BDNF, insulin and progesterone) and endogenous growth factors produced by GRP cells. It is not yet known what growth factors GRP cells produce. bFGF is involved in cellular proliferation of spinal cord cells *in vitro* (Shihabuddin et al., 1997) and *in vivo* (Kojima and Tator, 2002; Kojima and Tator, 2000), and other growth factors have similar roles. It is therefore possible that CM administration caused proliferation of endogenous cells, resulting in the observed increase in CSPG expression.

The attenuated CSPG and GFAP expression at the lesion margin and the reduction of CSPG expression within the lesion core observed following transplantation, implicate GRP cells in the alteration of the lesion environment. Immature cells can alter the lesion environment following implantation. Transplantation of immature glial cells (Olby and Blakemore, 1996; Wang et al., 1995; Smith and Miller, 1991; Smith et al., 1990; Smith and Silver, 1988; Smith et al., 1987; Smith et al., 1986) or fetal tissue (Houle and Reier, 1988; Reier and Houle, 1988), decreases astrocytic scarring and in some cases allows for increased axonal growth. How immature cells alter the environment is not well understood. GRP cells and mature GRP-derived-astrocytes express glutamate transporters (GLAST, GLT1 and EAAC1) and when placed onto spinal cord slices can increase glutamate uptake (Maragakis et al., 2001). This suggests that transplantation of GRP cells acutely after injury may modulate the acute glutamate toxicity. GRP cell alterations of the acute injury response could explain the decrease in astrocytic scarring observed.
By altering the astrocytic response, GRP cells may reduce the number of CSPG expressing astrocytes around the lesion margin, resulting in the observed decrease in CSPG expression. Within the lesion center, GRP cells may limit the infiltration of CSPG-expressing meningeal cells or fibroblasts, which infiltrate as a result of the needle penetration, and thus decrease the CSPG expression. Alternatively, GRP cell-derived-astrocytes may reestablish the BBB following transplantation, similar to transplanted astrocytes (Bush et al., 1999), since CSPG expression correlates to the opening of the BBB (Fitch et al., 1999; Fitch and Silver, 1997), reestablishment of the BBB may correspond to a decrease in CSPG expression.

Previously, transplantation of E14 fetal cells failed to alter CSPG expression surrounding the contusion injury site, and the transplant increased its CSPG expression over time (Lemons et al., 1999). The differences in CSPG expression following GRP cell transplantation verses fetal tissue transplantation (Lemons et al., 1999) may be partially explained by differences in staining protocols and time of transplantation. The Lemons study, (Lemons et al., 1999), used the C6SPG antibody to examine changes in CSPG expression. The C6SPG antigen site requires digestion of the glycosaminoglycan (GAG) side chains for immunohistochemical detection (Lemons et al., 1999). C6SPG and CS-56 antibodies have very different expression patterns. C6SPG detects all the CSPGs within the cord, including those expressed in the normal intact cord. The Lemons (Lemons et al., 1999) study used delayed transplantation paradigm. The differences between acute transplantation of GRP cells and delayed transplantation of fetal tissue, suggests that although delayed transplantation of cells results in improved cell survival, good host graft integration, and some alterations in astrocytic scarring, it may not be able to alter the
lesion scar once it has developed. The improved cell survival and good host graft integration observed following delayed transplantation are likely due to stabilization of the lesion and the decline in the macrophage response, the same factors that early transplantation affects.

Alteration of the glial scar may not be required for behavioral recovery following injury, since stem cell transplantation can alter behavioral recovery following delayed transplantation (McDonald et al., 1999). However, removal of the lesion scar is also associated with improved function (Bradbury et al., 2002). The limited behavioral recovery observed following SCI is likely be achieved by multiple mechanisms. GRP cells offer the ability to study multiple strategies by altering the lesion environment to make it more permissive, by providing potential substrate for axonal growth and by providing oligodendrocytes, which can remyelinate axons.

In Chapter 3, we showed that GRP cells survive immediate transplantation following SCI, are observed within the lesion site 8 days and 6 weeks after injury with good host-graft integration, and that when transplanted into an injured CNS environment they retain their differentiation potential and can differentiate down both astrocytic and oligodendrocyte lineages. Here we add to that and show that transplanted GRP cells modulate the lesion environment early after injury by reducing astrocytic scarring and the expression of inhibitory CSPGs. Additionally, we suspect that GRP cells may modulate the infiltration of immune cells early after injury. Whether these alterations affect the development of the chronic lesion cavity and allow for axonal regeneration is examined in the next chapter.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Buffer</th>
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<tbody>
<tr>
<td>Name</td>
<td>Concentration</td>
<td>Incubation time</td>
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<tr>
<td>OX-42 (CD11b/c)</td>
<td>1:500</td>
<td>O/N</td>
</tr>
<tr>
<td>GFAP</td>
<td>1:2000</td>
<td>O/N</td>
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<tr>
<td>CSPG (CS-56)</td>
<td>1:200</td>
<td>O/N</td>
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<td>1:10</td>
<td>2 days</td>
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Table 2: List of antibodies used in Chapter 4. Antibody staining was performed by washing the tissue (three times for 10 minutes each) in buffer, blocking the tissue for 1 hour in 0.5% Triton 100X, 5% normal serum (of the secondary antibody) in buffer. For CSPG staining with the primary antibody at 1:10 the tissue was blocked with 0.1% Tween-20, 10% milk in buffer, but the milk was omitted in the antibody diluent. Following primary antibody incubation (4°C) the tissue was washed (three times, 10 minutes each) prior to secondary antibody incubation at room temperature. Slides with fluorescent secondary antibodies were washed and coverslipped with Vectashield (Molecular Probes, Eugene, OR) and stored at 4°C until examined. Slides with biotinylated secondary antibodies were processed using the peroxidase-anti-peroxidase method. ABC (Vector standard peroxidase kit PK 4000, Burlingame, CA) was applied for 1 hour, the tissue was then washed, and stable DAB (Research Genetics, Huntsville, AL) was applied until a brown precipitate was detected. Tissue stained with DAB was then counterstained with cresyl violet, dehydrated through a graded alcohol series into histoclear and coverslipped with permount. Endogenous peroxidase within the tissue was quenched by incubating with 0.3% H2O2 prior to primary antibody incubation. GFAP/CSPG double labeling was performed by applying primary antibodies together and secondary antibodies sequentially. Abbreviations: CSPG (Chondroitin Sulphate Proteoglycan), GFAP (Glial Fibrillary Acidic Protein), NRS (Normal Rat Serum), PBS (Phosphate buffered saline).
Figure 4.1: Relationship of GRP cells, macrophages, proteoglycans and astrocytes following transplantation. GRP cells (A) are located within the macrophage filled lesion site (B). The lesion site is delineated by an astrocytic border (D). CSPGs (C) are located within the lesion and co-localize with the region of macrophage infiltration. Rostral (small R) is to the left, caudal (small C) is to the right.
Figure 4.2: Macrophage response. A) Appearance of OX-42 staining at the lesion site at the level of the central canal across treatment groups. The injury site is filled with OX-42$^+$ macrophages in all cases. All groups receiving treated with methylprednisolone and cyclosporin (MP/CsA; CM; GRP cell groups) had a reduction in the percentage of the lesion occupied by macrophages, as observed by the decreased intensity of OX-42 staining. Groups receiving an injection of either CM or GRP cells had a trend towards a decreased lesion size. However this was not found to be significant when quantified (B). No differences were observed in (C) the volume of lesion occupied by macrophages or (E) the percentage of a 10mm segment of spinal cord occupied by lesion. (D) Animals receiving an injection of either CM or GRP cells show a trend towards a reduction in the percentage of the lesion occupied by macrophages (ANOVA: F=2.82; p=0.06; df 3,21).
Figure 4.3: CSPG labeling. Alterations in CSPG expression were observed between the four groups at the lesion core and at the lesion margin. (A) Low magnification image of the CSPG labeling at the lesion across treatment groups. CSPG expression was highest with the lesion center and around the margin of the lesion after SCI. CSPG staining at the lesion center is reduced following GRP cell transplantation (B&C). CSPG staining is very intense at the needle insertion site (arrow in A). Transplantation of GRP cells immediately following SCI significantly decreases the intensity of CSPG staining at the lesion site compared to needle insertion alone (Mann-Whitney). (D&E) The boundary of CSPG staining between the lesion and the spared white matter is reduced following GRP cell transplantation. CSPG staining clearly delineates the lesion boundary after SCI alone (E: SCI). Treatment with MP/CsA alone or transplantation of GRP cells significantly reduces the CSPG boundary (Mann Whitney)(E: MP/CsA and GRP cells). The CM appears to reduce the line of CSPG staining observed in the SCI alone, but the boundary between the lesion and the spared white matter is still clearly distinguishable (E: CM). Arrows delineate lesion boundary. Number in bottom right corner is rating score.
Figure 4.4: GFAP staining in the spared white matter is reduced following GRP cell transplantation. GFAP positive astrocytes surround the lesion cavity following contusive SCI (A,C). Treatment with MP/CsA or CM significantly reduced the amount of GFAP staining in the spared white matter compared to injury alone (B). Transplanting GRP cells resulted in a further reduction that was significantly different from all three-control groups (B). Number in bottom right corner is rating score.
CHAPTER 5

CHRONIC INFLUENCES OF GLIAL RESTRICTED PRECURSOR CELLS ON THE LESION ENVIRONMENT AND AXONAL GROWTH

INTRODUCTION

Despite the innate ability of many axons to sprout and persist chronically, most axons fail to grow across the lesion cavity following spinal cord injury. A number of factors are likely to be involved in the failure of axonal regeneration including insufficient growth promoting molecules, growth inhibitory molecules, and the lack of an appropriate substrate present within the permanent cystic cavity (McKerracher, 2001; Fawcett, 1998; Schwab and Bartholdi, 1996). Transplantation of cells from a variety of sources may increase the survival, sprouting and elongation of specific populations of axons after transplantation into spinal cord injuries by providing a substrate for axonal growth and/or by modulating the lesion environment to make it more permissive (Cao et al., 2001a; Coumans et al., 2001; Ramon-Cueto et al., 2000; McDonald et al., 1999; Liu et al., 1999a; Liu et al., 1999b; McTigue et al., 1998; Menei et al., 1998; Ramon-Cueto et al., 1998; Blesch and Tuszynski, 1997; Bregman et al., 1997; Li et al., 1997; Li and Raisman, 1997; Cheng et al., 1996; Xu et al., 1995a; Xu et al., 1995b; Paino et al., 1994; Jakeman and Reier, 1991; David and Aguayo, 1981). The following study was designed to assess the effect of transplanted GRP cells on 1) the lesion environment and 2) axonal growth 6 weeks after injury.
Recent transplantation experiments have focused on the use of stem cells, because of their ability to differentiate into multiple cell types (Cao et al., 2002a; Cao et al., 2001; Rao and Mayer-Proschel, 2000; Chow et al., 2000; McDonald et al., 1999), and reports of functional improvement following implantation after injury (McDonald et al., 1999). Neuronal stem cells can differentiate into both neurons and glia \textit{in vitro} and \textit{in vivo} (Wu et al., 2001; Chow et al., 2000; Shihabuddin et al., 2000; Doetsch et al., 1999; Chiasson et al., 1999; Temple and Alvarez-Buylla, 1999), and have been shown to improve locomotor performance in rats when transplanted into contusive spinal cord injuries (McDonald et al., 1999). It is uncertain how stem cells mediate recovery following SCI. The small proportion of neurons formed following transplantation (Cao et al., 2001; McDonald et al., 1999) suggests that the alteration may be glially mediated, either through remyelination (Liu et al., 2000) or by altering the lesion environment to make it more permissive for axonal growth (Smith and Miller, 1991; Smith and Silver, 1988). The use of glial restricted precursor cells allows us to address the effects of transplanting immature glia into CNS injury sites.

Glial restricted precursor (GRP) cells are multipotent cells that can differentiate along oligodendrocyte and astrocyte lineages both \textit{in vitro} and \textit{in vivo} (Herrera et al., 2001; Rao et al., 1998)(Chapter 3). The multipotentency combined with the specificity of GRP cells to the glial lineage makes them a good cell type for examining the effects of transplanted glia following SCI. In the preceding chapters we have shown that following SCI, transplanted GRP cells survive transplantation, maintain their differentiation potential and modulate the acute injury environment (Chapters 3&4).
By altering the lesion environment, axonal regeneration can be promoted (Bradbury et al., 2002; Takami et al., 2002b; Coumans et al., 2001; Ramon-Cueto et al., 2000; Ramon-Cueto et al., 1998; Blesch and Tuszyński, 1997; Xu et al., 1995a; Xu et al., 1995b; Paino et al., 1994; Schnell and Schwab, 1990; David and Aguayo, 1981). GRP cells may alter the endogenous repair process as well as providing an additional substrate for axonal growth. Fetal tissue transplants can ameliorate tissue damaged chronically (Anderson et al., 1995; Houle, 1992), and allow for increased axonal growth (Anderson et al., 1995; Bregman et al., 1993; Jakeman and Reier, 1991). Immature astrocytes, unlike their adult counterpart, can promote axonal regeneration in vitro and in vivo (Smith et al., 1990; Smith and Silver, 1988; Smith et al., 1987; Smith et al., 1986). GRP-cell-derived-astrocytes, like immature astrocytes and fetal tissue, permit axonal growth in vitro (Dr. Mark Noble, personal communication), whether axons are able to grow on GRP cells in vivo has not previously been determined.

This experiment was designed to assess 1) whether the acute alteration in the glial scar observed following GRP cell transplantation persists chronically and 2) whether GRP cells support axonal growth of descending axons from the cortex and brainstem. Immunohistochemistry was used to detect transgenic PLAP GRP cells, astrocytes, and raphespinal axons in and around the injury. Corticospinal axons were examined by anterograde tracing.

Six weeks after injury, immature GRP-cell-derived-astrocytes were observed within the transplant and PLAP-positive GRP cells were associated with a reduction in astrocytic scarring around the lesion. CST axons were observed within GRP cell transplants at the rostral lesion margin, and there was an increase in CST terminal sprouts
with growth cone-like morphologies, suggesting that GRP cells provide a suitable substrate for CST axonal growth. However, the alterations in the growth cones did not correspond to an obvious increase in axonal elongation of either the CST or the raphespinal tract. Together the alteration in the lesion environment and the CST axons, suggests that GRP cells are permissive for axonal growth in vivo, and might be used as a therapy. However, under the conditions of the present study, the transplants did not yield dramatic changes in recovery of function.

**MATERIALS AND METHODS**

**Subjects**

Thirty-two female rats (Long Evans, Simonsen Laboratories) 85-94 days old were used in this study and all received a moderate 12.5 g-cm injury with the MASCIS/NYU impactor, as well as injection of the anterograde tracer Fluoro-ruby into the somatosensory cortex.

The thirty-two rats were divided into 4 groups: (1) injury control group (SCI) (n=8); (2) immunosuppressant control group (MP/CsA)(n=8); (3) conditioned media control group (CM) (n=8); and (4) experimental group (GRP cells) (n=8). Animals underwent a single surgery that involved anterograde labeling of CST fibers, jugular catheterization, spinal cord injury and transplantation. All procedures were carried out using aseptic technique and in compliance with NIH guidelines and were approved by the Institutional Laboratory Animal Care and Use Committee.

**Surgical Procedures**
Anterograde labeling of CST. Rats were anesthetized with 40-60 mg/kg of pentobarbital i.p. (Abbott Laboratories, Chicago, IL) and given an injection of 25 mg/kg of keflin (Lilly, Indianapolis IN) antibiotic s.c. and 1mg/kg s.c. of atropine (Fujisawa USA, Inc., Deerfield, IL) to maintain airway patency. Rats were placed in the stereotaxic device and the scalp was incised to expose Bregma and Lambda. Burr holes over the intended injection sites were made with a number 9 dental burr and enlarged with rongeurs. One percent (w/v) fluoro-ruby (FR) (25mg/2.5ml) (dextran, tetramethylrhodamine, 10,000 MW, lysine fixable (fluoro-ruby), Molecular Probes, Eugene, OR) was pressure injected through a pulled glass pipette (Radnoti star-bore tubing: Radnoti, Monrovia, CA) with a beveled tip (20-40 µm in diameter). For labeling CST axons, 3 injections (100 nl each) of FR were made into the sensorimotor cortex (anterior-posterior (AP) -0.7 mm, -1.4 mm, -2.3/-2.5 mm, medial-lateral (ML) +/-2.3 mm, depth (D) –1.5 mm) on each side of the brain, using Bregma as a landmark for AP and ML and the dura as a landmark for depth.

Jugular catheterization. Animals receiving methylprednisolone sodium succinate (MP) (Pharmacia & Upjohn, Kalamazoo, MI) and cyclosporine (CsA) (Novartis Pharmaceuticals Corporation, East Hanover, NJ), MP/CsA, CM and GRP cell groups, had a catheter inserted into their jugular vein for i.v. administration of MP (30mg/kg) 5 min, 2 hrs, and 4 hrs after injury. Animals were removed from the stereotax and placed on a heating pad and a piece of pulled PE 50 tubing was inserted into the jugular vein and secured, as previously described in Chapter 3.

Spinal cord injury and transplantation. Spinal cord injury and transplantation was performed as previously described (Chapter 4). Briefly, animals received a 12.5 g-cm
contusion injury using the NYU/MASCIS device by dropping a 10g weight from 12.5 mm onto the exposed dura of the spinal cord. Following SCI, a Hamilton syringe was inserted 1.5-2.5 mm into the center of the impact site for 5 minutes, and animals received either no injection, 5 µl of GRP CM or 500,000 GRP cells in 5 µl of CM slowly over 5 minutes. The wound was closed in anatomical layers and rats were placed singly in temperature-controlled incubators overnight. Subsequently, they were housed in pairs in standard plastic cages and given food and water *ad libitum*. Keflin (25mg/kg) s.c. (Lilly, Indianapolis, IN) was given daily for the fist 10 days. Cyclosporin (10mg/kg) was given daily (i.p. for the first week and then added to the drinking water for the remainder of the study). Bladders were expressed twice a day until reflex urination returned. Animals had their weight monitored and supplemental feeding with Nutrical (Evsco Pharmaceuticals, Buena, NJ) was administered if animals fell below 80% of their pre-surgery weight.

**GRP cell isolation.** GRP cells were isolated from E13.5 rat embryonic spinal cords as previously described (Herrera et al., 2001; Rao et al., 1998) (Chapter 3). Using immunopanning, cells were negatively selected for N-CAM, and positively selected for A2B5. This resulted in a purified population of GRP cells (>98% purity for A2B5+ cells). In this experiment all GRP cells were isolated from a transgenic PLAP rat in which the hPLAP gene is inserted behind the ubiquitously expressed ROSA26 promotor (Kisseberth et al., 1999). The alkaline phosphatase (AP) expression can then be detected via histochemistry or immunohistochemistry (as described in Chapter 3). The cells were isolated by Dr. Chris Proschel (CP) and were shipped live and transplanted the following day. Five µl (100,000 cells/µl were then drawn into a 26S gauge Hamilton syringe and
injected slowly with the needle bevel facing caudally into the impact site within 20 minutes of the spinal cord injury.

Perfusion procedures

Animals were perfused 6 weeks after SCI. All rats were terminally anesthetized with 0.8 mg/kg of ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 0.5 mg/kg of xylazine (Vedco, Inc, St. Joseph, MO) and transcardially perfused with 0.5 ml of lidocane (Elkins-Sinn, Inc, Cherry Hill, NJ) and 0.1 ml of heparin (Elkins-Sinn, Inc, St. Joseph, MO) followed by 0.9% saline, and then by 4% paraformaldehyde (Fisher Scientific). The spinal cords were removed, blocked and post-fixed for 4–8 hours in 4% paraformaldehyde. Blocks were then placed into 30% sucrose PBS for 24–48 hrs. The spinal cords were frozen and stored at −80°C until cut.

Histological Procedures

A 15 mm segment of the spinal cord containing the lesion center was cut longitudinally at 20 μm (three serial sets of slides per animal). Sections were mounted on gelatin coated glass slides and stored at −80°C until stained. One set was used to examine the scarring response 6 weeks after injury, by staining for either GFAP and PLAP, or GFAP and CSPG. Another set was used to examine the axonal response of the CST and 5-HT to GRP cell transplantation.

Immunohistochemistry. Immunohistochemistry was performed to detect PLAP, GFAP, CSPGs and 5-HT (See Table 3). All antibody staining was performed by washing the tissue three times (10 minutes each) in buffer, blocking the tissue for 1 hour in either 0.5% Triton 100X, 5% normal serum (of the secondary antibody) in buffer, or 0.1% Tween-20, 10% powdered-milk in buffer before applying the primary antibody diluted in
the blocking solution. The milk was omitted in the CS-56 antibody diluent. After primary antibody incubation (4°C) the tissue was again washed (three times, 10 minutes each), before secondary antibody incubation at room temperature. Following secondary antibody incubation the slides were washed, coverslipped with Vectashield (Molecular Probes, Eugene, OR) and stored at 4°C until examined. All incubations occurred in the dark. Slides stained for 5-HT were dehydrated and coverslipped with entellan (Electron Microscopy Science, Fort Washington, PA), for examination of both 5-HT and FR-labeled CST fibers. Double-labeling for GFAP and CSPG was performed by incubating the primary antibodies together, and the secondary antibodies consecutively. Double labeling for PLAP and GFAP was performed by serial incubations.

Data Analysis

Slides were examined using a Zeiss Axioplan2 microscope (Carl Zeiss, Hallergmoos, Germany) that also has a CARV attachment (Atto instruments, Rockville, MD). Slides were viewed using both standard fluorescence and non-laser confocal fluorescence, using the CARV unit. Standard fluorescent digital images were taken using an Optronics Dei 470 digital CCD camera (Optronics, Goleta, CA), directly imported into a Pentium II computer (Dell, Round Rock, TX) and overlaid in Photoshop (Adobe, Systems Inc., San Jose, CA). Confocal images were obtained using the CARV attachment with a high-resolution digital CCD camera for confocal imaging (Hamamatsu Photonics, model C4742-95-12, Hamamatsu, City, Japan), using CARVer software version 1.2 (Atto instruments, Rockville, MD), as described in Chapter 3. Confocal images with the color replaced were examined for tissue with CST fibers within PLAP areas. Retraction bulb confocal images were saved as 3-dimensional stacked images.
Axon counts. CST axons were counted as previously reported (Chapter 2). Briefly five sections per cord were chosen based on the distribution of the CST: one section above top of CST; two sections through the dorsal CST; one section at the level of the central canal; and two sections below the end of the central gray in the ventral white matter. The distance between the rostral edge of the tissue section and the rostral edge of the lesion was determined on the tissue section that contained the central canal. This distance was designated the ‘cavity edge’ or 0-mm point for all the sections, allowing the sections to be put in register for measurements. This procedure addressed the problem of variability in the rostral extent of the lesion between tissue sections.

Calculations for the rostral-caudal locations of the axon counts within each case were determined, on images of the tissue sections in which the CST axons were to be counted. To do this the 0-mm point was determined on the tissue section containing the central canal as described above. This distance was then measured on each section and denoted as 0 mm; 0.5 mm intervals corresponding to the axon count locations were then calculated around this point and plotted on the images for each tissue section. Calculations were then made to scale for the monitor on which the axons were counted. Axon counts were made at 0.5 mm intervals from 2 mm rostral to 2.5 mm caudal to the designated 0 point. All axons were counted from the monitor image and double-checked for accuracy through the microscope with the 20X objective (Axioplan2, Carl Zeiss, Hallergmoos, Germany). As well, axon count locations were double-checked between the monitor and the printed images containing the calculated locations. Five cases were removed from the CST analysis due to either enlarged lesion cavities (n=2) or problems with CST labeling (n=3).
Behavioral analysis. Animals were tested for open field locomotion using the Basso-Beattie-Bresnahan (BBB) locomotor test (Basso et al., 1995) at 2 days post injury and weekly thereafter for the remainder of the study, as described in Chapter 4.

Statistical Analysis. Open field locomotor scores (BBB) and axon count data were analyzed with SAS (SAS Institute Inc. Cary, NC) using a two-way analysis of variance (ANOVA). For the BBB scores the factors were groups (n=4) and trials (n=7) with repeated measures used for the trials. For the axon count data the factors were groups (n=4) and location (n=10) with repeated measures used for location. Post hoc analysis was performed using Tukey’s honestly significant difference test. Unequal group sizes were corrected using the harmonic means of the groups.

RESULTS

GRP cell modulation of the lesion environment

Lesion Cavity. Contusive spinal cord injury results in the formation of a chronic cystic lesion cavity that is surrounded by a spared rim of white matter. Tissue strands, referred to as trabeculae, are attached to the spared rim of white matter and extend into the cavity where cellular islands are occasionally observed (Figure 5.1). Macrophages were observed within the lesion cavity of all cases 6 weeks after injury. In addition to macrophages, other cells infiltrated the lesions in all groups. Insertion of the needle opens up the dura altering the lesion. In the transplant group, GRP cells were located within the lesion cavity (Figure 5.1A). Cystic cavities persisted after transplantation, but good host-graft integration was observed. Cellular masses within the GRP cell transplants contained GRP cells as well as endogenous cells (Figure 5.1A).
**Glial Scarring.** PLAP-positive GRP cells were observed within the lesion in longitudinal section (Figure 5.1A). Comparisons of GFAP staining between the four treatment groups showed alterations in astrocytic scarring at the lesion margin (Figure 5.1 C-F). GRP cell transplants reduced the intensity of GFAP expression at the lesion border, this corresponded to a reduction in the number of large GFAP-positive cells and the appearance of thinner GFAP-positive processes in the GRP cell transplant group (Figure 5.2A-D). Six weeks after injury, GFAP expression within the lesion of GRP cell transplanted animals colocalized with PLAP staining (Figure 5.1B and Figure 5.2E&F). GRP-derived-astrocytes had many thin, delicate processes, which were heavily interdigitated, and created a fine meshwork of GFAP expression within the transplant (Figure 5.1C and Figure 5.2F,H). In non-transplanted groups (SCI, MP/CsA and CM), GFAP expression within the lesion was less extensive, consisting of a few thick GFAP-positive processes within the trabeculae and the cellular mass within the lesion (Figure 5.2G). Six weeks after SCI, GRP cells did not express CSPGs; the increase in GFAP expression within the lesions of GRP cell transplants did not correspond to CSPG expression (data not shown).

**Lesion margin.** The lesion margin differed between the four groups. In the SCI alone group, the lesion margin was smooth in 75% of the cases, while in the remaining groups the lesion margin was more variable (Figure 5.3C). Normally a thin layer of basal lamina is laid down after injury, resulting in a smooth lesion edge (Figure 5.3A). Following MP/CsA, CM or GRP cell administration, the lesion margin was not smooth. Instead the rostral lesion margin was irregular; tissue protruded into the injury site, and in some cases macrophages abutted this tissue (Figure 5.3B).
Axonal Response to GRP cell transplantation

*CST.* The appearance of FR-labeled CST fibers was similar to that previously reported in Chapter 2 (Figure 2.3A,B). CST fibers were predominantly confined to the dorsal funiculus, but occasionally a dorsolateral or ventral CST fiber was observed. CST fibers appeared as lines of fluorescence within the dCST. As they approached the injured area they appeared either thin and varicose or thick and wavy. The fibers terminated in retraction bulbs proximal to the injury site. The splaying of axons out of the confines of the dorsal funiculus as they approached the lesion varied between the treatment groups. Such splaying was observed more often in the injury control group than following GRP cell transplantation (Figure 5.3D). MP/CsA and CM controls had a few cases in which the axons splayed but were intermediate between the injury alone and the GRP cell group. The alteration in axonal splaying around the lesion site is unlikely a result of decreased sprouting since all groups showed a similar number of cases with labeled CST fibers in the gray matter just rostral to the lesion (SCI 50%; MP/CsA 62.5%; CM 62.5%; GRP cells 62.5%). Labeled CST axons were not observed within the center of the lesion. However, a number of cases contained CST axons in trabeculae attached to the rostral lesion margin. More cases that received GRP cells or CM than injury alone or MP/CsA had labeled CST fibers in the trabeculae at the rostral lesion margin (Figure 5.3E).

Differences in CST terminal endings were observed between the four treatment groups. Previously we described the appearance of CST retraction bulbs rostral to the lesion site (Chapter 2). In the current experiment the majority of CST fibers terminated in round retraction bulbs (Figure 5.4 A,B), similar to our previous report. However, two additional ending phenotypes were observed on CST axons in this study. Some retraction
bulbs terminated in a point (fusiform rb) (Figure 5.4C,D), while others terminated in a retraction bulbs with many fine processes extending off of it (filopodial rb) (Figure 4E,F). The presence of these two endings may represent sprouting CST fibers. All four groups exhibited CST fibers with fusiform or filopodial retraction bulbs. The number of fusiform profiles in each case differed between the groups, while the number of cases containing fusiform profiles did not differ. A similar number of animals in each group contained fusiform retraction bulbs (Figure 5.4H). Fewer axons terminated in fusiform retraction bulbs in animals in the injury alone group than the other three groups (Figure 5.4I). Three of the GRP cell group animals contained sections through the CST with 5-20 fusiform retraction bulbs in a section. MP/CsA and CM groups also had more fusiform profiles than injury alone. The proportion of animals with filopodial retraction bulbs was greatest following GRP cell transplantation and lowest in the SCI along group (Figure 5.4J). CST fibers were observed interspersed with PLAP labeled GRP cells (Figure 5.5), suggesting that CST axons are able to grow on GRP cells.

No significant differences in sprouting or the elongation distance of CST fibers were observed between the four treatment groups, when the number of CST axons that approached the lesion was sampled at 0.5 mm intervals from the designated cavity edge in longitudinal sections. The majority of CST fibers terminated between the lesion edge and 1 mm rostral to it. Only a few fibers were counted past the rostral lesion edge (Figure 5.6). The intensity of CST labeling and the lesion length were similar between all cases, and thus did not influence the axon counts.

5-HT. We examined the extent of 5-HT staining 6 weeks following GRP cell transplantation (Figure 5.7). 5-HT fibers were observed surrounding neuronal cell bodies
in the gray matter rostral and caudal to the injury. Extensive 5-HT labeling was observed in the lateral white matter in all cases (Figure 5.7C,F). Intensely stained 5-HT profiles were thin and varicose in both the gray and white matter. 5-HT fibers formed patches containing multiple thin varicose fibers, in and around the lesion site. All cases contained patches of 5-HT fibers entering the trabeculae within the lesion site (Figure 5.7B,E). Rarely were 5-HT fibers found within the center of the lesion, instead they tended to enter trabeculae, following along blood vessels. Semi-quantitative analysis of the extent of 5-HT labeling, by blind-ranking of the amount of staining, did not detect any significant differences in 5-HT labeled axons between the groups (data not shown).

Open field locomotion

Animals in all groups showed an initial paralysis followed by hindlimb improvement over the first two weeks. Animals plateaued at a BBB score of 11; they were able to consistently plantar step but did not develop forelimb-hindlimb coordination. No long-term behavioral effect was observed 6 weeks following GRP cell transplantation. The BBB recovery curve following GRP cell transplantation was not significantly different from the three control groups.

DISCUSSION

The ability of GRP cells to modulate the lesion environment early (Chapter 4) and to persist and differentiate into immature astrocytes within the chronic lesion site (Chapter 3), may make the lesion environment more permissive and allow for axonal growth following injury. Previously, we examined the axonal response to injury, and showed that descending fibers from the cortex and brainstem were able to initiate a
sprouting response after injury, but their response was limited and delayed (Chapter 2). In this experiment we examined the glial scar and the extent of axonal growth from the corticospinal and raphespinal tracts, to determine if the altered scarring observed early after injury persisted in the longer-term, and whether these changes toward a more permissive environment, and the presence of a GRP cell-derived-astrocyte substrate, could increase sprouting of descending axons 6 weeks after injury.

Progression of cavitation. Spinal cord injury results in the development of a chronic contusion injury lesion over time (Loy et al., 2002; Casella et al., 2002; Grossman et al., 2001; Hill et al., 2001; Rosenberg and Wrathall, 1997; Basso et al., 1996; Martin et al., 1996; Guizar-Sahagun et al., 1994; Bresnahan et al., 1991; Noble and Wrathall, 1989; Iizuka et al., 1987; Noyes, 1987; Noble and Wrathall, 1985; Bresnahan, 1978; Balentine, 1978a; Balentine, 1978b). A rim of spared fibers and partially demyelinated axons surrounds a central fluid filled cavity separated into chambers by cellular tissue bridges, called trabeculae (Bunge et al., 1994; Noble and Wrathall, 1989; Noble and Wrathall, 1985; Bresnahan, 1978; Balentine, 1978a; Balentine, 1978b). The walls of the chronic lesion appear smooth as the spared white matter is walled off from the fluid filled cavity by the glia limitans (Shearer and Fawcett, 2001; Brook et al., 1998; Stichel and Muller, 1998; Martin et al., 1996; Bunge et al., 1994; Guizar-Sahagun et al., 1994).

Contusive SCI, like transection, disrupts the blood-brain-barrier, allowing the influx of macrophages and fibroblasts, producing an anisomorphic (open) injury (Bovolenta et al., 1992). Endogenous cells (astrocytes, microglia/macrophages, ependymal cells), infiltrating mesodermals cells (fibroblasts, endothelial, meningeal and
hematogenous cells) and the production of a basement membrane (collagen, laminin, fibronectin, proteoglycans) form the glia limitans lining the injury (Shearer and Fawcett, 2001; Brook et al., 1998; Stichel and Muller, 1998; Martin et al., 1996; Bunge et al., 1994; Guizar-Sahagun et al., 1994).

Usually the dura remains intact after contusion injury, limiting the influx of cells into the lesion. In this study the dura was opened, in all animals, to insert the needle for transplant. Dural opening appeared to increase the amount of tissue present within the chronic injury site, likely due to the infiltration of mesodermal cells (fibroblasts, Schwann cells and meningeal cells) (Beattie et al., 1997), similar to that observed after transection injuries (Krikorian et al., 1981).

The formation of the glia limitans can be altered by glucocorticoid administration (Li and David, 1996) and fetal tissue transplants (Kruger et al., 1986), reducing the number of meningeal cells and the compacted nature of astrocytic processes. In the present study administration of methylprednisolone and cyclosporine or the transplantation of GRP cells, increased the amount of tissue within the lesion and appeared to reduce the formation of the glia limitans, as indicated by the altered lesion margins and the lack of smooth walls surrounding the injury (Fig. 5.3).

Similar to SCI controls, the lesions of animals receiving immunosuppression or GRP cells contained trabeculae and variable amounts of tissue and macrophages (Figure 5.1). Administration of methylprednisolone and cyclosporin (MP/CsA, CM and GRP cell groups) qualitatively increase the amount of tissue within the lesion. It is likely that the cells that contribute to the tissue within the lesion differ between the MP/CsA, CM and GRP cell groups. MP increases tissue sparing within the lesion, likely via neuroprotection
preserving some of the endogenous cells, which results in decreased cavitation (Takami et al., 2002a). The majority of cells within the lesion following transplantation were GRP cell derived, as detected by PLAP labeling. The tissue sparing, observed following MP administration is likely supplemented by the proliferation of endogenous cells following administration of GRP cells or of GRP cell conditioned media. GRP cell conditioned media contains exogenous growth factors (including but not limited to bFGF, BDNF, insulin and progesterone), as well as factors produced by GRP cells that have not yet been determined. How these factors influence lesion development is not yet known, however, bFGF is associated with increased neuronal survival (Teng et al., 1999), tissue sparing (Rabchevsky et al., 2000; Lee et al., 1999; Rabchevsky et al., 1999) and cellular proliferation (Kojima and Tator, 2002; Kojima and Tator, 2000) in vivo, suggesting that even a single injection (Teng et al., 1999) of exogenous and endogenous factors can influence chronic lesion morphology.

SCI alone resulted in a lesion cavity with a relatively smooth wall that surrounded the lesion (Fig. 5.3A). Administration of MP or the injection of GRP cells, resulted in a lesion edge that was jagged and uneven with multiple small tissue protrusions (5.3B); injections of CM were intermediate. Similar group differences were observed, 8 days after injury, when CSPG expression was examined, suggesting that the altered CSPG expression may reflect early changes in the formation of the glia limitans. Others have shown that matrix molecules are laid down at the interface between astrocytes and meningeal cells (Shearer and Fawcett, 2001; Stichel and Muller, 1998), two components of the glia limitans.
Glial scarring. The formation of the glia limitans after injury is essential for limiting tissue damage (Bush et al., 1999), however, it also results in the failure of axonal regeneration (Bush et al., 1999). Axons are able to grow along the basal lamina but do not grow through it (Shearer and Fawcett, 2001; Stichel and Muller, 1998). In transection lesions, it forms an impenetrable barrier (Stichel and Muller, 1998), while in the contusion injury, axons splay out of the CST and deviated around the injury site into the spared white matter laterally (Chapter 2). Astrocytes play essential roles in basal lamina production. They both limit tissue damage, and inhibit axonal regeneration after injury. Transplantation of either fetal tissue or immature astrocytes can modulate the astrocytic response to injury by limiting tissue damage and facilitating repair (Olby and Blakemore, 1996; Wang et al., 1995; Smith and Silver, 1988; Smith et al., 1987; Smith et al., 1986). Immature astrocytes are able to decrease glial scar formation, inhibit bleeding and secondary necrosis, and promote axonal regeneration (Firkins et al., 1993; Smith et al., 1987; Smith et al., 1986; Noble et al., 1984; Barrett et al., 1984).

The ability of the immature nervous system to respond better to injury than the mature CNS is often attributed in part to the ability of immature astrocytes to alter the lesion environment, to make it more permissive, and to provide a substrate for axonal growth (Bernstein-Goral et al., 1997; Olby and Blakemore, 1996; Wang et al., 1995; Smith and Miller, 1991; Smith et al., 1990; Smith and Silver, 1988; Smith et al., 1986). In this experiment we transplanted GRP cells into contusion injuries to see if they continued to alter the glial scar six weeks after injury, and if they could provide a substrate suitable for axonal growth. Six weeks after transplantation, the reduced astrocytic scarring initially observed 8 days after injury persisted. GRP cell transplants reduced the number
of large GFAP-positive cells surrounding the lesion, particularly in areas adjacent to the transplant. GRP-derived-astrocytes were present within the transplant. These astrocytes formed a fine mesh within the lesion indicative of an immature phenotype. CSPGs did not colocalize with these immature astrocytes within the lesion, suggesting that GRP cells, unlike the fetal tissue (Lemons et al., 1999), do not increase CSPG expression within the lesion. Instead it appears that GRP cells are able to modulate the lesion to make it more permissive for axonal growth, by decreasing both the astrocytic scar and the formation of the glia limitans, and by forming GRP-derived-immature-astrocytes within the lesion. In vitro, GRP-derived and immature astrocytes are able to support axonal growth (Dr. Mark Noble, personal communication)(Smith et al., 1990; Noble et al., 1984). To test whether GRP cells were able to support axonal growth in vivo, we examined the response of the corticospinal tract and the raphespinal tracts 6 weeks after injury.

Relationship of axons with the lesion cavity

*Relationship of descending CST axons with the lesion cavity.* Following spinal cord injury the proximal portion of damaged axons retract away from the injury site, form retraction bulbs, and mount a sprouting response (Chapter 2). In this study we examined the CST response to GRP cell transplantation. We chose the CST because we previously characterized the formation of retraction bulbs and the initiation of CST sprouting (Chapter 2), it is a confined tract that is easy to label, and unlike many of the descending spinal cord tracts, CST fibers do not survive a moderate contusion injury (Chapter 2)(Kim et al., 2002). The CST has been shown to sprout following transplantation, usually in conjunction with growth factors or neutralization of inhibition (Ramon-Cueto
et al., 2000; Bregman et al., 1997; Grill et al., 1997; Li et al., 1997; Cheng et al., 1996; Bregman et al., 1995; Schnell and Schwab, 1993). However, the regenerative response of the CST in generally limited following therapeutic manipulations (Oudega et al., 1999; Weidner et al., 1999; Menei et al., 1998; von Meyenburg et al., 1998; Blesch and Tusznynski, 1997; Bregman et al., 1997; Grill et al., 1997; Guest et al., 1997b; Ye and Houle, 1997; Chen et al., 1996; Martin et al., 1996; Xu et al., 1995a; Xu et al., 1995b; Paino et al., 1994; Schnell et al., 1994; Schnell and Schwab, 1993; Schnell and Schwab, 1990; Pallini et al., 1988; David and Aguayo, 1981).

In the present study, the morphology and location of CST fibers was similar to previously reported (Chapter 2). After injury, fibers terminated in retraction bulbs proximal to the lesion site. Following contusion injury, CST fibers descended along the base of the dorsal funiculus and splayed as they reached the rostral lesion edge, deviating around the injury site and extend for a short distance in the spared white matter laterally. After GRP cell transplantation, the basal lamina was altered affecting the CST response. The CST fibers descended along the base of the dorsal funiculus like usual, but when they reached the lesion edge, they failed to grow around the rostral edge. Instead the CST fibers abutted the rostral lesion margin and in some cases penetrated into the rostral trabeculae. Due to the irregular, altered lesion edge it was not possible to determine if these fibers grew into the rostral trabeculae, or were spared due to treatment. The presence of CST fiber intermixed with GRP cells at the rostral lesion margin (Fig. 5.5), and the alterations in retraction bulb morphology (Fig. 5.4), discussed below, suggest that GRP cells are able to support axons growth.
In the current study the majority of CST fibers terminated in round retraction bulbs, similar to previously reported (Chapter 2). However, fusiform and filopodial retraction bulbs were also observed. Fusiform retraction bulbs terminated in a point (Fig. 5.4 C,D). Filopodial retraction bulbs had fine processes extending from an enlarged ending (Fig. 5.4 E,F). The morphology of these two endings are similar to those described during CST development (Joosten and Bar, 1999) and may represent sprouting CST fibers (Joosten and Bar, 1999; Mason and Wang, 1997; Tosney and Landmesser, 1985). All four groups exhibited CST fibers with fusiform or filopodial retraction bulbs. A similar proportion of animals contained fusiform retraction bulbs across the groups. However, GRP cell transplants had more axons/case with fusiform retraction bulbs than the other three groups, and a greater proportion of animals with filopodial retraction bulbs. MP/CsA and CM groups were intermediate between injury alone and GRP cell transplantation. Although MP/CsA treatment may influence the formation of retraction bulbs by the CST in response to injury, transplantation of GRP cells resulted in the greatest alteration in CST retraction bulbs suggesting that they are able to alter the sprouting of CST fibers 6 weeks after injury.

Although GRP cells were able to support CST axonal growth and increase axonal sprouting this did not correspond to an increase in long distance axonal growth. Similar results have been observed following fetal tissue transplantation (Bregman et al., 1997), where fibers are able to penetrate short distances into the transplant but do not grow through. All four groups had a similar number of axons, when the number of axons were sampled around the lesion. It is possible that 6 weeks is too early to look at long distance regeneration of the CST. Previously we observed delayed CST sprouting. Initial CST
sprouting was observed 21 days after injury with an increase in sprouting between 21 days and 14 weeks and an increase in elongation of the CST into the center of the lesion between 14 weeks and 6 months following contusion injury (Chapter 2), suggesting that the lack of elongation may arise from insufficient growth time. However, others have observed CST elongation from 2 weeks to 2 months and beyond after SCI (Bregman et al., 1997; Cadelli et al., 1992) weeks. Reports of CST elongation following transplantation have predominantly involved transplantation in conjunction with additional manipulations (Coumans et al., 2001; Bregman et al., 1995), suggesting that transplants alone may not be sufficient to induce long-distance regeneration. Although GRP cells may provide a substrate for axonal growth, the lack of long-distance regeneration suggests that either a longer time is required for long-distance regeneration or that the transplant needs to be supplemented with either growth promoting factors or neutralization of inhibitory factors. It is also possible that GRP cells inhibit axonal growth by expressing inhibitory molecules. Both oligodendrocyte myelin and NG2 expression (Gregori et al., 2002), (a CSPG not detected by the CS-56 antibody), can preclude long-distance regeneration. However, the presence of CST fibers in association with PLAP-positive GRP cells (Fig. 5.5) and the presence of sprouting axons make this seem unlikely.

**Relationship of 5-HT axons with the lesion cavity.** Brainstem-spinal axons (vestibulospinal, reticulospinal, rubrospinal, raphespinal) have an increased propensity to regrow after axotomy compared to CST axons, especially when provided with a favorable growth environment. Brainstem-spinal axons, but not corticospinal axons, have been shown to grow into peripheral nerve grafts (Cheng et al., 1996; David and
Aguayo, 1981) and Schwann cell grafts with exogenous factors added. The addition of either methylprednisolone (MP), or specific neurotrophins (brain derived neurotrophic factor and neurotrophin-3) to Schwann cell grafts results in brainstem-spinal axons penetrating into the graft (Chen et al., 1996; Xu et al., 1995a). The presence of a small number of reticulospinal fibers (Chapter 2) and 5-HT fibers within the trabeculae (present study) after contusive SCI, suggests that trabeculae offer an environment conducive to axonal growth, but not an environment sufficiently enriched for substantial growth. In this experiment the response of serotonergic fibers to GRP cell transplants was examined. Serotonergic fibers originate in the raphe in the brainstem and descend all the way to the lumbrosacral spinal cord (Saruhashi et al., 1996). These fibers are partially spared following injury (Kim et al., 2002) and may regenerate after injury (Liu et al., 1999b; Bregman et al., 1997; Saruhashi et al., 1996; Anderson et al., 1995; Houle and Reier, 1988). Following SCI 5-HT fibers are lost caudal to the injury (Holmes et al, 2000; Saruhashi et al., 1996). Replacement or sparing of 5-HT innervation is associated with recovery of locomotion, respiration, bowel, bladder, and sexual functions (Pikov and Wrathall 2000; Slawinska et al., 2000). Transplantation of 5-HT cells injected into or rostral to the lumbar enlargement improves function more than injection into the lesion site (Ribotta et al., 2000). Together this suggests that sparing or sprouting of 5-HT fibers has important functional consequences after SCI and warrants examination.

Six weeks after contusion injury, 5-HT fibers innervated neurons in the gray matter rostral and caudal to the injury. 5-HT fibers were present within the dorsal and ventral horns and along the IMLCC as previously described (Saruhashi et al., 1996; Marlier et al., 1991; Newton and Hamill, 1988). 5-HT fibers were able to descend along
the spared rim of white matter and penetrate into the trabeculae within the lesion, they did not however, reach the center of the lesion in any of the groups. The extent of 5-HT axonal labeling was similar between the four treatment groups. Serotonergic fibers endogenously respond to SCI (Saruhashi et al., 1996) and are able to infiltrate the trabeculae. The relatively strong endogenous response made detection of differences between the groups difficult. Brainstem-spinal axons are able to respond better to injury than CST axons, however in this study semi-quantitative ranking of the amount/intensity of 5-HT did not detect differences in 5-HT staining between the treatment groups. The relatively strong endogenous response made detection of differences via ranking difficult. Better quantification methods may be able to detect subtle differences between the groups.

Open field locomotion

Transplantation of ES cells results in improved locomotor recovery when transplanted into a 25 mm injury 10 day after injury (McDonald et al., 1999). ES cells are tripotential and can generate neurons, as well as glia (both oligodendrocytes and astrocytes). In the McDonnal study, the majority of cells produced from ES cells transplanted into contusion injuries developed into oligodendrocytes and astrocytes; only a small percentage became neurons. Therefore, it is unlikely that ES cells improve locomotor recovery through the development of new neuronal connections, but instead locomotor improvement may be glially mediated. Transplantation of GRP cells allowed us to test the effects of transplanted glia on recovery of function 6 weeks following injury. In this study, no long-term behavioral effect was observed 6 weeks following transplantation of GRP cells. Animals plateaued at a score of 11; consistent plantar
stepping, no forelimb-hindlimb co-ordination. Animals frequently plateau here following moderate contusion injury. This may be due to damage of propriospinal fibers, which are located medially and co-ordinate interlimb movements. It is possible that alterations in behavior may have been observable if a more severe injury had been used; this would fit well with the ES cells transplantation data from McDonald in which ES cells were transplanted into 25 mm injuries with the NYU device (McDonald et al., 1999). Alternatively, it is possible that the behavioral effect observed in the ES cell transplantation experiment is due to increased remyelination by oligodendrocytes as a result of delayed transplantation. Remyelination may require myelin clearance (Li et al., 1999). Immediate transplantation may preclude significant GRP cell derived remyelination. If either of these hypotheses is correct, then acute transplantation into a severe injury or delayed transplantation should result in improved open field locomotion. It should also be noted that in this experiment, animals that received MP/CsA did not show improvement in BBB score. This corresponds with 2 recent studies in which administration of MP failed to result in behavioral improvement 6 weeks (Rabchevsky et al., 2002) or 10 weeks after injury (Takami et al., 2002a), using the same injury model and a similar dosing regimen.

The present study was designed to assess the effect of acute GRP cell transplantation on the chronic lesion environment. Specifically, we wanted to determine whether the alterations observed in the acute lesion environment permitted increased axonal growth at chronic time points, and whether alterations in the lesion environment persisted chronically. Anterograde labeling of the CST was used to examine these fibers as they approached the lesion site, and immunohistochemistry was used to examine 5-HT
fibers, and GFAP expression in and around the lesion. GRP cells were able to differentiate into immature astrocytes within the lesion, and were able to reduce astrocytic scarring around the lesion, 6 weeks after injury. Transplanted GRP cells were able to support axonal growth, and induce CST growth cone formation, but did not produce long-distance regeneration of the corticospinal or raphespinal tracts. The alterations in growth cones observed following GRP cell transplantation, and the presence of CST fibers intermixed with GRP cells suggests that GRP cells are able to support axonal growth in vivo. Further studies are required to determine if a longer time course is required for enhanced CST elongation, or if delayed transplantation can result in remyelination and behavioral improvement.
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
<th>Buffer</th>
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<tr>
<td>Name</td>
<td>Concentration</td>
<td>Incubation time</td>
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<tr>
<td>human placental alkaline phosphatase (hPLAP)</td>
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<td>O/N</td>
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<td></td>
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<tr>
<td>GFAP</td>
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<tr>
<td>CSPG (CS-56)</td>
<td>1:10</td>
<td>2 days</td>
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<tr>
<td>5-HT</td>
<td>1:500</td>
<td>O/N</td>
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Table 3: List of antibodies used in Chapter 5. All antibody staining was performed by washing the tissue (three times for 10 minutes each) in buffer, blocking the tissue for 1 hour in 0.1% Tween-20, 10% milk in buffer before applying the primary antibody diluted in the blocking solution. The milk was omitted in the CS-56 antibody diluent. After primary antibody incubation (4°C) the tissue was again washed three time for 10 minutes each before secondary antibody incubation at room temperature. Following secondary antibody incubation the slides were washed, coverslipped with Vectashield (Molecular Probes, Eugene, OR) and stored at 4°C until examined. All incubations occurred in the dark. Double-labeling for PLAP, was performed by initially staining for PLAP, followed by staining for either GFAP. GFAP/CSPG double labeling was performed by applying primary antibodies together and secondary antibodies sequentially. Abbreviations: CSPG (Chondroitin Sulphate Proteoglycan), GFAP (Glial Fibrillary Acidic Protein), NRS (Normal Rat Serum), PBS (Phosphate buffered saline), 5-HT (Serotonin).
Figure 5.1: Comparison of the lesion sites and astrocytic response between treatment groups 6 weeks after injury. (A) PLAP-labeled GRP cells are present within the lesion and (B) some colocalize with GFAP within the lesion (● GRP cells colocalized with astrocytes; ★ tissue within the lesion without GRP cells). (B, C) GFAP expression around the lesion is reduced in area adjacent to GRP cells (*). GFAP is expressed along the lesion margin in all groups: (C) GRP cell transplant group, (D) SCI alone group, (E) MP/CsA group, (F) CM group. (C-F) Cystic cavities and trabeculae (large arrow) attached to a central tissue mass (arrowheads) are present within the lesion site of all groups. Macrophages are present within the lesions (small arrows). Scale 1 mm.
Figure 5.2: Comparison of GFAP expression at the lesion margin and within the lesion between treatment groups. (A-D) Astrocytes hypertrophy and increase GFAP expression. Thick processes wall off the lesion. Astrocytes within the GRP cell group (D) are smaller and have thinner processes than the other treatment groups (A-C). (E) PLAP expression is present within the lesion, and corresponds with an increase in thin, mesh-like astrocytic processes (F, H). A few enlarged GFAP processes are present within the injury alone group (G). MP/CsA and CM groups were similar to the injury alone group (not depicted). Scale bars 100 µm. Cell bodies (arrows), Processes (arrowheads).
Figure 5.3: Appearance of the lesion margin at the CST after GRP cell transplantation. CST axons descend along the base of the dorsal funiculus (A, B). (A) After SCI alone, CST axons splay around the smooth rostral lesion margin. (B) Following GRP cell transplantation the lesion margin is irregular, with an uneven, serrated edge with multiple tissue protrusions (large arrowheads), some of which are attached to trabeculae. Macrophages are observed adjacent to the notched lesion edge (small arrows). CST fibers extend into the rostral trabeculae and the tissue protrusions (small arrowheads). Semi-quantitative rating of the lesion was performed for the presence of a smooth lesion margin (C), splaying axons (D) or CST axons within the rostral trabeculae. Scale bars 100 μm.
Figure 5.4: Alterations in CST retraction bulb morphology. Retraction bulbs were either (A, B) round, (C, D) fusiform, or (E, F) filopodial. Filopodial retraction bulbs had thin filamentous extension off the retraction bulb (small arrows). Cases were examined for the presence of the three types of retraction bulbs and the relative number of endings per case. All cases contained round retraction bulbs (G). The majority of animals contained fusiform retraction bulbs (H). GRP cell transplants increase the relative number of fusiform retraction bulbs (I), and the number of cases containing filopodial retraction bulbs (J) compared to SCI alone.
Figure 5.5: CST axons within the GRP cell transplants. (A-D) Fluoro-ruby labeled CST fibers (arrows)(red) were found within areas of PLAP labeled GRP cells (green). (A) Low magnification, (B-D) high magnification.
Figure 5.6: Number of labeled axons sampled around the lesion cavity 6 weeks after injury, compared between treatment groups. Imaginary lines through five tissue slices were used to count the number of labeled axons around the cavity. Displayed is the mean of the total number of sampled axons counter per animal (see chapter 2, Fig. 2.1, for sample sites). No significant differences were observed between treatment groups. CST axons in all groups reduced in number as they approached the lesion edge, with a few fibers extending past the rostral lesion edge.
Figure 5.7: Raphespinal fibers in and around the cavity 6 weeks after injury. All groups contained 5-HT labeled axons rostral and caudal to the lesion. An increase in 5-HT was observed in the spared white matter laterally and within the trabeculae in all groups; injury alone (A-C) and GRP cell transplant (D-F) are depicted. Patches of 5-HT fibers containing multiple thin varicose fibers enter into the trabeculae (B, E). Intensely stained 5-HT thin varicose fibers were present within the spared white matter laterally. Scale bar 500 µm.
Figure 5.8: Open field locomotor score 6 weeks after injury, comparison between treatment groups. Injury resulted in initial paralysis in all animals. Over the first two weeks animals gained improved use of their hindlimbs. By 15 days post injury animals obtained their maximum BBB score of 11; they were able to consistently step, but did not exhibit forelimb-hindlimb coordination. No differences were observed in locomotor recovery between the four treatment groups. Data is expressed as the mean score of both hindlimbs.
CHAPTER 6

SUMMARY AND CONCLUSIONS

The current studies were designed to examine the endogenous responses of descending systems after contusive spinal cord injury and whether these responses could be altered by the transplantation of glial restricted precursor cells. The formation of a cystic lesion cavity after SCI limits the endogenous response of axons. After injury descending fibers are able to initiate a sprouting response but this response is limited and delayed. Early after injury, descending corticospinal axons splay out of the dorsal funiculus into the gray matter. Over time they are able to elongate to a modest extent along the spared white matter surrounding the cystic cavity and, to a more limited extent, into the tissue bridges that form within the lesion.

The axonal response to injury can be altered by manipulating the lesion environment. Cellular transplantation can increase the regeneration of a number of fiber systems. In particular, brainstem-spinal axons (vestibulospinal, raphespinal, reticulospinal) and propriospinal axons respond well to transplantation. Their responses can be further augmented by the administration of neurotrophins (Coumans et al., 2001; Menei et al., 1998; Xu et al., 1995a). Conversely, CST axons do not respond as robustly to transplantation or other manipulations (Oudega et al., 1999; Weidner et al., 1999; Menei et al., 1998; von Meyenburg et al., 1998; Blesch and Tuszynski, 1997; Bregman et
al., 1997; Grill et al., 1997; Guest et al., 1997; Ye and Houle, 1997; Chen et al., 1996; Martin et al., 1996; Xu et al., 1995a; Xu et al., 1995b; Paino et al., 1994; Schnell et al., 1994; Schnell and Schwab, 1993; Schnell and Schwab, 1990; Pallini et al., 1988; David and Aguayo, 1981). However, they are able to initiate a limited sprouting response. In this study we show that GRP cells provide a substrate suitable for CST axonal growth. Similar to fetal tissue transplants, CST axons only penetrate a short distance into the transplant (Bregman et al., 1997). However, given the number of treatments that fail to elicit a CST response, the increase in growth-cone-like morphologies and the penetration of CST fibers into the margins of the rostral trabeculae is very promising.

Additional studies are required to determine if axonal regeneration can be further increased by a longer survival time. This is particularly important given the delayed endogenous response of the corticospinal tract (Hill et al., 2001). As well, recent promising findings of increased regeneration following either fetal tissue transplantation and exogenous neurotrophin administration, or transplantation of neurotrophin-expressing fibroblasts, suggest that administration of neurotrophins in conjunction with GRP cell transplantation may further increase axonal regeneration (Coumans et al., 2001; Liu et al., 1999; Blesch and Tuszynski, 1997; Grill et al., 1997).

Examination of the response of other labeled descending systems will also help to elucidate the effects of GRP cells. Contusion injury results in the partial sparing of many fiber systems. Unlike the CST, which is completely severed following moderate contusion injuries, damage to other descending systems is proportional to the injury severity. Two recent studies evaluate the extent of fiber sparing following contusion
injury via axonal labeling (Kim et al., 2002; Basso et al. 2002). These studies provide important baselines for examining the response of descending systems following therapeutic manipulations, an area that thus far has been largely ignored in the contusion injury model.

In addition to the effect on CST axonal growth, GRP cell transplants altered glial scarring. The reduction in glial scarring observed eight days after injury, persisted at six weeks and corresponded to alterations in the morphology of the lesion. Thus, transplantation of GRP cells altered the endogenous response to injury.

In addition to examining the axonal response to injury, and the alterations in the lesion, we characterized some of the basic biological properties of GRP cells. By isolating GRP cells from transgenic rats were able to clearly identify the transplanted cells, solving a problem that plagues the transplantation literature, and our initial studies. We demonstrated that GRP cells are able to survive transplantation into the hostile injury environment, and can differentiate into oligodendrocytes and astrocytes at the lesion site by six weeks after injury.

GRP-derived-astrocytes were present, preferentially, within the lesion site. Unlike endogenous reactive astrocytes—which have large cell bodies and thick GFAP-positive processes that wall off the injury site and sparsely penetrate into the trabeculae and central tissue mass—GRP-derived-astrocytes had many fine GFAP-positive processes that were intertwined and formed a fine mesh-work of cells within the lesion. The presence of CST axons on these immature astrocytes suggests that they were able to
support axonal growth similar to previous experiments that transplanted immature astrocytes.

The presence of GRP-derived-oligodendrocytes in the ventral white matter suggests that they are able to remyelinate axons. We observed CC1-positive-oligodendrocyte cell bodies surrounded by many PLAP-positive processes, suggesting that they are surrounding axons; specific documentation of GRP cells myelinating axons after injury remains to be shown.

Spinal cord injury results in the formation of a spared rim of demyelinated axons around the lesion site over time. Given that myelinating cells need naked axons to wrap, delayed transplantation of GRP cells after SCI may be more appropriate for examination of the extent of remyelination by GRP cells. The recent behavioral recovery observed following delayed transplantation of ES cells into contusion injuries, and the subsequent demonstration that ES cell-derived-oligospheres can remyelinate axons in vitro, supports this line of study (Liu et al., 2000; McDonald et al., 1999).

The recent isolation and characterization of GRP cells means that many questions remain to be answered about this precursor cell population. The results of this initial qualitative study indicate that transplantation of GRP cells into the injured spinal cord may be useful in repairing the spinal cord after injury. The methodology developed in the current study will aid in the development of future quantification studies.
BIBLIOGRAPHY


Plant GW, Bates ML, Bunge MB (2001) Inhibitory proteoglycan immunoreactivity is higher at the caudal than the rostral Schwann cell graft-transected spinal cord interface. Molecular and Cellular Neuroscience 17: 471-487.


