ASTROCYTES IN DEVELOPMENT AND IN THE INJURED CNS

by

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Abstract

After a traumatic injury to the brain or spinal cord, the adult mammalian central nervous system (CNS) has a limited ability to repair itself. The reasons why CNS neurons are unable to grow and make functional connections after injury are beginning to be elucidated. There is evidence to suggest that both the intrinsic response of the neuron to injury and the environment the surrounding injured neuron contribute to the absence of anatomical and functional recovery after axotomy. In the developing chick embryo, the onset of myelination by oligodendrocytes is associated with a transition from a growth permissive to a growth restrictive extraneuronal environment. This thesis examined the development of astrocytes, another glial cell, in chick development. Astrocytes begin to show a differentiated phenotype at embryonic day 12, which is just prior to the onset of myelination. In the adult, reactive astrocytes are a prominent feature of the glial scar that forms after injury. Although it has never been proven, the astroglial scar is less permissive to axonal growth than the surrounding CNS environment. It was the aim of my research to investigate different methods of reducing or removing the astroglial scar after a spinal cord injury in the adult rat. These approaches included 1) targeted antibody and complement-mediated lysis of astrocytes, 2) the reduction of transforming growth factor beta and 3) the ablation of astrocytes using l-alpha aminoadipate (L-AAA). Of all these treatments, L-AAA was found to be the most successful at removing astrocytes from the site of injury over a one-week period. In the future, L-AAA may be useful in the investigation of the role astrocyte have in CNS development and regeneration after injury.

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Abbreviations

-/-	null mutation
BBB	blood brain barrier
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CSPG	chondroitin sulfate proteoglycan
CST	corticospinal tract
DREZ	dorsal root entry zone
E	embryonic day
ELISA	enzyme-linked immunosorbant assay
FGF	fibroblast growth factor
GFAP	glial fibrillary acidic protein
GAP-43	growth associated protein
GCV	ganciclovir
IHC	immunohistochemistry
IL	Interleukin
IR	immunoreactivity
L-AAA	L-alpha aminoadipate
μm	microns
02-A	oligodendrocyte type-2 astrocytes
P	post hatchling
PBS	phosphate buffer saline

PFA	paraformaldehyde
PN	peripheral nerve
PNS	peripheral nervous system
RAG	regeneration associated gene
RST	rubrospinal tract
SD	Sprague Dawley
T	thoracic
TK	thymidine kinase
TGF-β	transforming growth factor - beta
VIM	vimentin

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General Introduction

It has long been noted that the mammalian central nervous system (CNS) lacks the capacity to repair itself after injury (Ramon y Cajal, 1928). Severing a CNS axon typically results in a permanent loss of functional connections whereas functional recovery and anatomical regeneration is usually observed after a peripheral nervous system (PNS) axotomy. The reasons underlying the disparity in healing between these two systems are now beginning to be elucidated. Regeneration research has focused on the intrinsic ability of neurons to initiate a regeneration response as well as the extrinsic environment axotomized neurons must navigate in order to re-innervate their targets.

This thesis will briefly review an injured CNS neuron's intrinsic capacity for growth and will then focus on the environment in which growth must occur, specifically examining the glial scar and the molecules expressed at the scar. Finally, a number of strategies for reducing the glial scar after injury will be discussed.

CNS Response to Injury

During neuronal development, neurons must initiate axonal growth in order to reach an appropriate target. Initiation and perpetuation of growth are associated with a change in gene expression and with the subsequent expression of several proteins. For example, the structural protein α tubulin, required for microtubule formation, is expressed at high levels during CNS growth. After the growing neuron reaches its target, α tubulin is down-regulated (Bamburg et

al., 1973; Miller et al., 1987). Other proteins, such as growth associated protein (GAP) – 43, are also expressed during periods of axonal growth. GAP - 43 is first detected after a neuron has undergone its final cell division and before neuronal extension begins (Dani et al., 1991). High levels of GAP-43 are strongly corelated with the growth of developing axons (Benowitz and Routtenberg, 1997). For example, GAP-43 is co-localized to the growing phrenic and brachial axons at the brachial plexus (Allan and Greer, 1998). The expression of particular genes is associated with the growth of axons in the developing CNS.

In the adult, an axotomized peripheral axon is capable of regrowing toward its target and reestablishing functional connections, where a central axon is capable of neither. A factor contributing to this discrepancy may be the intrinsic ability of the PNS neuron to initiate a growth program after an injury. For example, following a facial nerve axotomy and subsequent axonal regrowth, there is an increase in the synthesis and expression of GAP-43, tubulin and actin mRNA in the facial nucleus. The expression of these genes is a recapitulation of genes associated with growth during neuronal development (Tetzlaff et al., 1991; Tetzlaff et al., 1988). These growth-associated genes, such as GAP-43 and tubulin, have been referred to as regeneration-associated genes or RAGs. In the CNS, RAG expression in the red nucleus is not induced after a thoracic axotomy of the rubrospinal tract, but is induced in a cervical axotomy (Tetzlaff et al., 1991). No growth of the RST is observed after either of these injuries. Only when a more permissive pre-degenerated sciatic nerve graft is implanted into the injury site will the cervically axotomized rubrospinal axons grow into the graft. Under the same conditions, thoracic axons are not able to extend axons into the graft (Fernandes et al., in press; Richardson et al., 1984). Growth into the graft is correlated with the expression of RAGs at the red nucleus

(Kobayashi et al., 1997). Thus the ability of a neuron to initiate growth after an axotomy is associated with the capacity of the neuron to elicit a RAG response. Peripheral nerve (PN) transplant studies indicate that if they are to regenerate, CNS neurons require both a permissive environment and RAG expression.

Other avenues of research have revealed that extrinsic factors may contribute to the absence of CNS regeneration. For example, when a CNS optic nerve is grafted into an axotomized tibial nerve, the PNS axons do not grow into the graft - yet they will readily grow into another PNS graft (Weinberg and Raine, 1980). This illustrates that the CNS environment may be less permissive to axonal growth in the PNS even when RAG expression is present.

While transplantation studies indicate that the mature CNS is a less permissive environment for RST regeneration than the PNS (Ramon y Cajal, 1928; Richardson et al., 1984), during development, CNS neurons must grow through the CNS in order to make appropriate connections. As the CNS develops, the environment becomes more prohibitive to axonal growth. This shift towards a less permissive growth environment in the CNS has been examined in different model systems and species. The expression of myelin by oligodendrocytes has been closely examined as an inhibitor of axonal regeneration. In the chick, the switch from a permissive to less growth-permissive environment occurs at embryonic (E) stage 12 (Hasan et al., 1993). This change correlates to the onset of myelination by oligodendrocytes (Keirstead et al., 1992). If a spinal cord axotomy is performed before the onset of myelination at E12, anatomical and functional repair of the spinal cord can be observed (Hasan et al., 1993). By delaying myelination immunologically, the permissive period for axonal reconnections at E13

can be extended a maximum of three days (Keirstead et al., 1992). Research has shown that in the adult chick or rodent, making the CNS more permissive by immunologically blocking, disrupting or removing myelin results in axonal regeneration or sprouting (Dyer et al., 1998; Keirstead et al., 1995; Schnell and Schwab, 1990; Schnell and Schwab, 1993). These studies indicate that myelin is one of the factors preventing CNS regeneration. Other glial cells are also associated with the lack of CNS regeneration after injury. In particular, microglial cells (Lazarov-Spiegler et al., 1998; Rapalino et al., 1998; Zeev-Brann et al., 1998) and astrocytes (Davies et al., 1997; Davies et al., 1999) have been shown to be associated with lack of growth of CNS neurons after injury.

Astrocyte function

The word glia is derived from the Latin word for glue. Many years ago, the perceived function of glial cells was to passively hold axons together while providing a homeostatic support system. However, research has shown that astrocytes perform a variety of roles within the CNS. Recent evidence indicates that astrocytes provide ionic homeostatis at internodes of axons (Nagaraja and Brookes, 1998; Sontheimer et al., 1994; Thangnipon et al., 1983), act as antigen presenting cells (Gold et al., 1996; Mossner et al., 1990; Takiguchi and Frelinger, 1986), aid in boundary formation (Gonzalez et al., 1993; Gonzalez and Silver, 1994), assist in neurite extension and patterning (Goodman et al., 1993), complement deactivation (Gasque et al., 1995; Gasque et al., 1995), provide neuronal trophic support (reviewed in Eddleston and Mucke, 1993; Ridet et al., 1997), phagocytose debris (Aldskogius and Kozlova, 1998; Roldan et al., 1997), provide glycogen stores for neurons (Dringen et al., 1993; Lomako et al., 1993; Magistretti et al., 1994; Pfeiffer et al., 1992; Swanson and Choi, 1993), and aid in the formation of the blood brain

barrier (BBB) during development and after a penetrating injury (Fitch and Silver, 1997; Golding and Cohen, 1997; Janzer and Raff, 1987; Tout et al., 1993).

A penetrating injury to the CNS elicits edema and rapid local inflammation. A plethora of cytokines, chemokines and inflammatory molecules which have pleurotrophic effects on many of the surrounding glial cells are also released as a result of the injury (Eddleston and Mucke, 1993; Fawcett, 1994; Logan and Berry, 1993; Norenberg, 1994; Ridet et al., 1997). Concurrently, astrocytes begin to form an astroglial scar as invading macrophages and leptomeningeal cells enter the CNS via the disruption of the blood brain barrier (Bell et al., 1994; Ness and David, 1997; Zhang et al., 1997). One of the main characteristics of a glial CNS injury response in mammals, owing to the subsequent rapid proliferation and hypertrophy of astrocytes, is the resulting astrogliotic scar around the injury site (Dusart and Schwab, 1994). Also known as reactive astrogliosis, it is denoted by an increase in a distinct intermediate filament protein found in astrocytes, namely glial fibrillary acidic protein (GFAP) (Bignami, 1975; Bignami and Dahl, 1975; Eng, 1985; Eng and Ghirnikar, 1994; Oblilnger et al., 1993; Tardy et al., 1993; Vijayan et al., 1990). In the rodent, increases in GFAP mRNA and protein occur within the first hour after injury and reach a maximal level within the first seven days (Mucke et al., 1991; Vijayan et al., 1990). This rapid increase in GFAP expression is considered the key feature of astrogliosis (Aldskogius and Kozlova, 1998; Eddleston and Mucke, 1993; Eng, 1985; Eng and Ghirnikar, 1994; Eng et al., 1987; Hatten et al., 1991; Norenberg, 1994; Ridet et al., 1997; Stichel and Muller, 1998; Vijayan et al., 1990). The astroglial scar, consisting mainly of reactive astrocytes, forms a physical (Liuzzi and Lasek, 1987) and/or chemical barrier to axonal regeneration

(Carlstedt et al., 1988; Carlstedt et al., 1987; Davies et al., 1997; Fawcett, 1994; Guenard et al., 1994; McKeon et al., 1991; Ridet et al., 1997).

Astrogliosis in Humans

The difficulty of obtaining spinal cord tissue at early and consistent time points after injury makes the acute astroglial response after a spinal cord injury (SCI) harder to examine in humans. Postmortem autopsies of gunshot or stab wound victims offer an insight into the human astroglial response after a penetrating injury. Between eight days to five weeks after a gunshot wound at the second cervical vertebrae, there is no apparent change in GFAP-immunoreactivity (IR) near the degenerating neurons of the dorsal columns (Puckett et al., 1997). An increase in astrocyte expression of GFAP-IR occurs only four months after injury, after which a decrease of GFAP-IR is seen after one year. The processes of the reactive astrocytes appear longer and have more GFAP-IR than uninjured tissue (Puckett et al., 1997). The discrepancy in GFAP expression between humans and murines remains unexplained, and other molecules expressed by murines at the astroglial scar such as CSPG, tenascin-C, collagen IV or laminin have yet to be examined. Since an increase in GFAP-IR is only one of the indicators of astrogliosis, the latent GFAP-IR response to injury cannot be compared to other model systems when the changes in expression of other factors have not been examined.

Models of astrogliosis

In vitro models of astrogliosis

It is impossible to study non-reactive astrocytes *in vitro,* since astrocytes are thought to enter a reactive state when harvested for tissue culture. Scratching the astrocyte monolayer creates a "pseudo astroglial" response (Ghirnikar et al., 1994; Hou et al., 1995; Yu et al., 1993). However, this technique may only amplify the already reactive response of the astrocytes rather than mimicking the changes that occur when an astrocyte becomes reactive *in vivo*. Since this protocol requires monolayers of astrocytes, the response of other glial cells to an *in vitro* injury cannot be examined. As astrocytes are passaged with increasing frequency, their *in vitro* ECM expression changes. By selecting for specific changes, astrocytes were grown that expressed higher amounts of chondroitin sulfate proteoglycan (CSPG) or tenascin (Meiners et al., 1995). Although astrocyte cell lines may not be representative of their expression of extracellular matrix moleclules (ECMs) before or after injury, they do provide some insight into the properties of the ECMs expressed by reactive astrocytes. Different neuronal responses can be observed when mixed glial cells are cultured together as opposed to pure monolayers. For example, a dorsal root ganglia's (DRG) growth cones collapse when grown on oliogodrocyte cultures (Kobayashi et al., 1995). However, neurite growth is observed when DRGs are grown on a mixed population of astrocytes and oligodrocytes (Fawcett et al., 1992).

The spatial representation of the astrocytes *in vitro* differs from that found *in vivo*. *In vitro*, astrocytes form a monolayer of cells on the culture dish which growth cones must navigate. The monolayer culture system is an inaccurate representation of the orientation of both neurons and astrocytes *in vivo*. To alleviate this potential problem, three-dimensional culture systems have

been used to better represent the cyto-architecture of the CNS. These experiments show that adult DRGs are only able to navigate astrocytes cultures which are less than 10 days old, whereas DRGs grow through Schwann cell cultures at any age (Fawcett et al., 1989). These *in vitro* results are more representative of the behavior of DRGs *in vivo* than two-dimensional *in vitro* systems.

Explant and transplant models of astrogliosis

In order to create a better *in vitro* scar model, McKeon and colleagues (1991) attempted astrocyte explant experiments. These experiments involved implanting nitrocellulose paper into the adult murine cortex. Astrocytes then invaded the pores of the paper forming a glial scar (Rudge et al., 1989). The nitrocellulose paper was then excised and used as an *in vitro* substrate for RGC and DRG cultures. Adult explant scar tissue was less permissive to growth than embryonic scar tissue or unlesioned tissue (Rudge and Silver, 1990). The excised nitrocellulose tissue was immunohistochemically positive for Laminin, collagen IV, tenascin, and CSPG, all of which have been shown to be expressed *in vivo* at a CNS injury site (McKeon et al., 1991).

One weakness of the explant model is that it consists mainly of a two-dimensional *in vitro* system where neurons attempt to grow on top of the gliotic tissue. To create a more realistic three-dimensional substrate, gliotic tissue has been transplanted into the growing CNS olfactory system (Anders and Hurlock, 1996). The optic nerve of the rat was axotomized to elicit astrogliosis. The resulting scar was then transplanted into the olfactory bulb, an environment containing neurons continuously growing towards their targets. In contrast to an uninjured optic nerve transplant, the astroglial transplant resulted in a complete cessation of growth. These

findings indicate that when compared to uninjured tissue, the glial scar creates an environment less permissive to growth (Anders and Hurlock, 1996).

Dorsal Route Entry Zone (DREZ) as a model for reactive astrocytes

In the reactive state, astrocytes produce numerous cytokines, trophic factors, extracellular matrix molecules, proteases and adhesion molecules (reviewed in Eddleston and Mucke, 1993). The varied and dramatic changes in expression of these factors are thought to have both beneficial and detrimental effects on the regenerative response in the CNS (Ridet et al., 1997). The expression of these molecules is viewed as an attempt to recapitulate a developmental program in order to facilitate repair and prevent further damage. During development, astrocytes assist in the formation of the blood brain barrier (Cassella et al., 1996; Goldstein, 1988; Hayashi et al., 1997). The putative function of this barrier is to control the entry or exit of cells after neurogenesis is complete.

The peripheral and central nervous systems share a transition point in the dorsal horns of the spinal cord, also known as the dorsal root entry zone (DREZ). Adult mammalian neurons are unable to grow through the PNS-CNS transition zone in the spinal cord (Carlstedt, 1997). In the adult rodent, DREZ astrocytes are phenotypically similar and express many of the same ECMs as reactive astrocytes found at the glial scar (Fraher, 1992; Golding et al., 1997; Kozlova et al., 1995; Pindzola et al., 1993). Consequently, many studies have used the DREZ as a model to examine the gliotic scar. During development, many of the growth-inhibitory ECMs are not expressed by astrocytes, thus allowing sensory axons to enter. NT-3 dependant sensory fibres are able to enter the murine spinal cord at E14 to reach their motor neuron targets. From E14 to

P7, axotomized sensory fibres are able to innervate their targets by regrowing through the DREZ and into the spinal cord (Carlstedt, 1988; Carlstedt et al., 1987). After P6, sensory afferents fail to grow past the DREZ and the growth cones stop at astrocytic processes on the edge of the DREZ (Liuzzi and Lasek, 1987). At P7, the change from an environment permissive to the growth of sensory fibres to one that is restrictive to that growth is correlated to the appearance of CSPG. Astrocytes become immunoreactive (IR) for CSPG at P7 when a group of uncharacterized cells disappear. These cells normally separate the astrocytes from Schwann cells (Golding and Cohen, 1997). The loss of these uncharacterized cells at the DREZ leads to initial contact between astrocytes and the PNS. This correlates to the astrocytes becoming reactive and expressing CSPG.

To further demonstrate that the adult DREZ is inhibitory to growing axons, embryonic DRGs were transplanted into the ganglionic capsules of adult rat hosts. Embryonic DRGs have a good growth ability, but they circumvented the DREZ by entering the spinal cord through the pia matter surrounding blood vessels (Kozlova et al., 1995; Rosario et al., 1993). When embryonic astrocytes were transplanted into the adult DREZ, sensory fibres were able to regrow past the reactive astrocytes (Kliot et al., 1990). Removal by X-irradiation is another effective method for avoiding reactive astrocytes at the DREZ. When the dorsal spinal cord is X-irradiated after birth, causing the depletion of astrocytes, the depleted areas allow inward migration of Schwann cells from the PNS (Gilmore and Sims, 1997). Sixty days after a dorsal root axotomy and X-irradiation, regenerating sensory fibres were seen entering the DREZ (Sims and Gilmore, 1994). This growth may have resulted from the changes occurring in the glial environment. Following X-irradiation, there were fewer reactive astrocytes present, and growth permissive Schwann cells

were then able to invade the CNS. Thus the ability of sensory fibres to grow into the DREZ may be partly dependent on the removal of reactive astrocytes.

These studies indicate that adult reactive astrocytes contribute to the growth-inhibitory environment of the CNS. After a penetrating injury to the adult CNS, a PNS-CNS interface similar to that seen at the DREZ forms around the lesion. This new barrier of reactive astrocytes, which is a putative attempt to re-establish the division between the central and peripheral nervous systems, may inadvertently create an environment which is less permissive to axonal growth than the uninjured CNS.

Astrogliosis in vivo

There are a variety of models to study astrogliosis *in vivo*. Spinal cord injury models in the rat range from a weight drop compression to stab wound models (Taoka and Okajima, 1998). The contusion method is well-characterized in various mammalian species (Basso et al., 1996; Finkelstein et al., 1990; Hackney et al., 1994; Kuhn and Wrathall, 1998; Noble and Wrathall, 1985; Rosenberg and Wrathall, 1997). The main advantage of the stab wound model, in comparison to the contusion model, is the consistency of fibres axotomized.

Recently, one transplantation technique has illustrated that the glial scar in the spinal cord and cortex is inhibitory to sensory fibres. When dissociated DRGs were injected into a rat cortex, robust growth was only observed when a gliotic reaction was not initiated (Davies et al., 1997). This was accomplished by making a minute lesion using micro-injection techniques. These

techniques minimized the impact on the cyto-architecture of the surrounding cells. When injections elicited an astrocytic response and CSPG was produced, the injected DRGs, recognized for their vigorous capacity for growth *in vivo* and *in vitro*, were unable to grow past the gliotic scar. Furthermore, when dissociated DRGs were transplanted many segments rostral to the spinal cord lesion, the DRGs extended processes as far as the edges of the gliotic scar (Davies et al., 1999). This data suggests that the gliotic environment inhibit DRG growth in the adult CNS.

Thesis Objectives

Astrocytes are considered important in neurodevelopment and regeneration. In chapter I, the expression of astrocyte markers in the developing chick spinal cord will be examined. It is the objective of this chapter to determine when GFAP and vimentin are expressed in the developing chick spinal cord. It is my hypothesis that astrocytes will begin their final differentiation stage before the final stage of oligodendrocyte development and after neuronal connections has been made. This characterization of glial maturation will lead to a greater knowledge of chick glial maturation.

In Chapter II, differing approaches of reducing the astroglial scar will be examined. The astroglial scar has been shown to contribute to the lack of CNS regeneration observed after a CNS axotomy. One potential problem when studying the astroglial scar is the limited number of techniques available to reduce and/or remove reactive astrocytes from the injury site. Chapter II investigates three potential methods for reducing and/or removing reactive astrocytes after

injury, with the objective of finding a method that can remove astrocytes from the injured CNS with minimal damage.

Chapter I:

GFAP and Vimentin Expression in the Embryonic Chick

Introduction

The embryonic chick spinal cord has been used to investigate changes from a growth permissive to a growth restrictive environment (Hasan et al., 1993). Previous work has shown that this transition period occurs at E13 and coincides with the onset of myelination by oligodendrocytes (Hasan et al., 1993; Keirstead et al., 1992). Furthermore, myelin suppression experiments suggest that myelin is a major cause of this transition (Keirstead et al., 1995; Keirstead et al., 1992). The maturation astrocytes at E13 have not been examined. In this chapter the expression of astroglial cytoskeletal proteins will be examined because reactive astrocytes are believe to contribute to the regenerative failure seen in the adult mammalian CNS (Davies et al., 1997; Davies et al., 1999; Liuzzi and Lasek, 1987).

Astrocytes in the CNS have unique cytoskeletal proteins, which have allowed researchers to easily identify and study astrocytes *in vivo*. Antibodies that recognize epitopes on these cytoskeletal proteins are used to identify astrocytes. The major cytoskeletal framework in astrocytes is comprised of intermediate filament proteins (IF). The regulation of these proteins is central to determining aspects of astrocyte function. There are five types of intermediate filament proteins in mammalian tissues. Types I and II IFs include a large group of acidic and basic keratins, type III IFs include VIM, GFAP and periphern, type IV IFs are neurofilament

proteins and type V are the nuclear lamins (Steinert and Liem, 1990). Astrocytes express GFAP and VIM, the only CNS type II IFs. Developing astroglial cells within the CNS may express VIM initially and as astroglial cells mature, they begin to express GFAP. The unique expression of VIM and GFAP within astrocytes has facilitated research on glial development.

Vimentin, a 57 kDa protein, is expressed in immature astroglial cells of the meningial and ependymal varieties (Bignami et al., 1982; Dahl et al., 1981; Dahl et al., 1982). This structural protein is believed to stabilize the glial cell before it is replaced by GFAP. Previous *in vivo* studies have shown that VIM expression is high during development, but quickly decreases as GFAP expression increases (Dahl, 1981; Elmquist et al., 1994) whereas in culture, the expression of both GFAP and VIM has been shown to increase with time (Sancho-Tello et al., 1995). In development and in adults, the role of VIM alone is not entirely clear since VIM -/- mice appear normal but demonstrate some reduction in renal function (Terzi et al., 1997). This implies that there may be overlap or compensation of function of another structural protein within glial cells.

The type II IF protein GFAP is expressed in what is believed to be the final stage of differentiation of astrocytes. The expression of this 50 kDa IF increases as the expression of VIM decreases within the developing CNS (Dupouey et al., 1985). The reason for the change in expression from VIM to GFAP remains unclear. A possible explanation is that VIM IFs are more efficient reorganization that may be beneficial during mitosis (Steinert and Liem, 1990). *In vitro*, astrocytes have demonstrated a reduced ability to extend processes if antisense GFAP is transfected into an astrocytoma cell line upon neural induction (Weinstein et al., 1991). *Gfap* -/-

mice developed normally, but as adults had a defective blood brain barrier, abnormal myelination, and increased CNS extracellular space (Gomi et al., 1995; Liedtke et al., 1996). Due to putative compensatory mechanisms such as an increase in VIM expression, the role GFAP on the development of the nervous system cannot be accurately investigated using *Gfap* -/- mice.

The optic nerve is one of the best-characterized systems of astrocyte and oligodendrocyte development. Early *in vitro* work led to a classification of glia precursor cells as type-1 astrocyte progenitors and oligodendrocyte type-2 astrocyte progenitors (O-2A, Raff, 1989). *In vitro* studies indicate that O-2A progenitor cells were immunoreactive for the antigen A2B5 whereas type-1 astrocytes did not express A2B5 (Raff et al., 1983). An O-2A cell can develop into an oligodendrocyte or into a "type-2" astrocyte. Type-2 astrocytes express GFAP and A2B5 (Raff et al., 1983). When O2-A progenitors are cultured in serum free medium, they differentiate into oligodendrocytes and therefore this is considered the default pathway. With the addition of serum into the medium, O2A progenitors differentiate into type-2 astrocytes. *In vitro* studies indicate that platelet-derived growth factor (PDGF) is one of the mitogens of $A2B5^+$ O-2A cells. PDGF is produced by some neurons and type-1 astrocytes (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988). The PDGF-alpha receptor is localized to oligodendrocyte precursors or O2A progenitor cells, further implicating PDGF in the role of glial cell differentiation (Pringle et al., 1992). It seems likely that the differentiation of oligodendrocyte precursors may be partly controlled by astrocytes through PDGF. Although the above research has primarily been conducted in the optic nerve, similar results have been observed when cells were cultured from

the cerebellum (Levi et al., 1986; Trotter and Schachner, 1989), brain stem (Dutly and Schwab, 1991), cortex (Trotter and Schachner, 1989) and the spinal cord (Fok-Seang and Miller, 1994).

The development of astrocytes from an O-2A progenitor has been suggested to be a phenomenon unique to *in vitro* studies. There was no evidence of astrocyte differentiation *in vitro* when O-2A progenitor cells were dissociated from freshly isolated rat optic nerves or from O-2A immortalized cell lines (Espinosa de los Monteros et al., 1993; Groves et al., 1993). When 0-2A cells modified to express β-galactosidase were injected into demyelinating lesions of the rat spinal cord, only oligodendrocytes had β-galactosidase expression (Groves et al., 1993). These results suggest that astrocyte differentiation *in vitro* may differ from that *in vivo.*

Of the proliferating glial precursor cells in the ventral ventricular zone of the developing spinal cord, 60% become glatactocerebroside $(GC)^+$ oligodendrocytes *in vitro* (Noll and Miller, 1993). *In situ* hybridization experiments have indicated that precursor cells within the ventral ventricular zone express PDGF-α receptors (Pringle and Richardson, 1993). Thus both the *in vitro* and *in vivo* data indicate that some of the glial precursors in the spinal cord are in the ventral ventricular zone. From this zone, precursors have been shown to migrate radially and dorsally (Noll and Miller, 1993; Warf et al., 1991). Cell cultures from the spinal cord have indicated that the proliferating cells from the ventral zone are able to differentiate into oligodendrocytes and astrocytes whereas cells from the dorsal zone generally become astrocytes (Warf et al., 1991).

Other factors have been implicated in the differentiation of progenitor glial cells into astrocytes. Ciliary neurotrophic factor (CNTF) causes stem cells to differentiate into astrocytes (Rajan and McKay, 1998). Because the signaling moieties of the CNTF receptor are identical to the leukemia inhibitory factor (LIF) β receptor, the effects of the two factors are very similar (Grotzinger et al., 1997; Ip et al., 1993; Kallen et al., 1999). Adult mice lacking the LIF β receptor when compared to wild type mice have been shown to have fewer astrocytes (Ware et al., 1995). Furthermore, LIF and CNTF have both been shown to cause glial progenitor cells to differentiate into astrocytes (Nakagaito et al., 1995; Richards et al., 1996). Hence, CNTF and LIF may play a role in astrocyte differentiation.

Not all of the glial precursors differentiate into mature glial cells. Barres (1994) and colleagues demonstrated that only those oligodendrocytes which myelinate electrically active axons survive. The non-myelinating population of oligodendrocytes in the rat optic nerve appear to undergo programmed cell death (Barres and Raff, 1994), functionally controlling oligodendrocyte numbers. A different mechanism exists to control astrocyte numbers. In the optic nerve, the number of astrocytes was shown to be controlled by neuronal active transport (Burne and Raff, 1997). This indicates that a potential signal from neurons might cause astrocyte maturation.

In the chick, brainstem-spinal projections required for locomotion have made their final connections by E12 (Okado and Oppenheim, 1985) and have initiated electrical activity and the transport of target derived factors. Oligodendrocytes have been shown to initiate myelination of these electrically active axons by E13 (Barres and Raff, 1994). By secreting PDGF, astrocytes may aid in the maturation of glial precursors to oligodendrocytes (Noble et al., 1988; Raff et al.,

1988; Richardson et al., 1988). Therefore it is likely that astrocytes mature before oligodendrocytes. Furthermore, astrocyte proliferation may depend on an axonally transported factor from the neuron. It is my hypothesis that the expression of GFAP, a sign of late stage astrocyte differentiation, will occur before the expression of late stage oligodendrocyte markers and after E12. This hypothesis will be tested by using astrocyte specific antibodies on chick spinal cords at various time periods throughout development.

Materials and Methods

Animals

Fertilized White Leghorn chicks, *Gallus gallus domesticus*, (Coastline Chicks, Abottsford Canada) were incubated at 37°C in an automatic rotating incubator (Peacock Equipment Limited, Aldergrove B.C.). Animals were staged to the appropriate developmental time period (Hamburger and Hamilton, 1951). Table 1 indicates the number of animals examined for immunohistochemical analysis.

Embryonic Age	Animal Numbers
E9	6
E10	6
E11	6
E12	6
E13	6
E14	6
E15	6
E16	5
E17	6
E18	4
P1	8

Table 1

The number of animals used to analyze the developmental expression of GFAP-IR and VIM-IR at various time points in the developing chick spinal cord.

Perfusion / cryosectioning

Embryos examined before E11 were decapitated and immediately fixed in 4% PFA. Embryos examined after E11 were perfused intracardially with 37°C 0.1 M PBS, pH 7.4 followed by 4% w/v Paraformaldehyde (BDH #294474L; PFA) in 0.1 M PBS. Since the pain pathways which result in the consciences perception of pain are formed after E14, 0.5 mls of the anesthetic Sodium Pentobarbital (M.T.C. Pharmaceuticals, #141690) was dripped onto the chorioallantoic membrane of embryos older than E14 or was injected into the tibial muscle of the postnatal chick before perfusion. After perfusion of animals older than E11, the spinal cord was removed and the tissue post-fixed for 24 hours in 4% PFA at 4°C. Tissue was cryoprotected with sequential 8

hour exposures of 12, 16 and 22% w/v sucrose solutions in PBS. After cyroprotection, tissue was mounted on filter paper with Tissue-Tek (J.B.EM. Services Inc. #JBF172) and rapidly frozen at –50 °C in supercooled 2-methylbutane (BDH, #B29452-74). Serial sections were cut at10 μm and every other section was placed on an adjacent slide for immunohistochemistry. This tissue was cut at -22°C using a Zeiss cryostat and the sections were then placed on warmed superfrost plus slides (Fisher # 12-550-15) and stored at -70°C.

Histology

All antigens were localized using indirect immunofluorescence techniques. Standard immunohistochemical (IHC) techniques and controls for indirect-immunofluorescence were used in order to visualize specific antigens on cryosectioned tissue. This included the omission of the primary antibody and processing all the tissue of the same ages together (Figure 3). Slides were brought to room temperature and separated into two groups. One group was used for vimentin visualizaion and the other slide was used for GFAP visualization, allowing for comparison of the serial sections or the slides had both the monoclonal vimentin antibody and the polyclonal GFAP antibody. Concentrations of the antibodies used are listed in table 2. The slides were washed in 0.1M PBS for 20 minutes and 200 μl primary antibodies and buffer were pipetted onto the slides. Both antibodies were used in a solution of 0.1% Triton-X100 (BDH, #R 06433) and 0.1 M PBS. Plastic coverslips were placed over the slides and incubated overnight at 4°C. After incubating, the slides were washed for 30 minutes in PBS, after which a blocking solution of 10% normal goat serum (Jackson Immunoresearch Laboratories Inc., #005-000-121) and 0.1% Triton-X100 in PBS was added for 20 minutes at room temperature. Excess blocking solution was removed from the slides. The secondary antibodies, donkey anti-mouse Cy3 and goat anti-rabbit Rodal

Green conjugates, were added for one hour at room temperature. After the secondary antibodies had incubated, the slides were washed for 30 minutes in PBS with the final wash of PBS containing 0.001 mg/ml of Hoechst 33258. Coverslips where placed on the slides with a 3:1 solution of glycerol: PBS. A fluorescent microscope (Zeiss, Axioskop) was used to visualize fluorescently labeled tissue. Images were captured with a Spot CCD digital camera (Diagnostic Instruments Inc. model 1.1.0) and Photoshop (Adobe, Version 5.1) was used to create photomontages and make minor adjustments for brightness and contrast of the whole image. For the control images in figure 3, the brightness and image intensity was increased more than the images in figures 1 and 2. This was done to further illustrate the non-specific binding of the secondary antibodies used.

ANTIBODIES / STAINS	CLONE	COMPANY/CATALOGUE NUMBER	CONCENTRATION
rabbit anti-glial fibrillary acidic protein (GFAP)		Dakopatts Corp. /Z334	1/600
monoclonal anti-glial fibrillary acidic protein (GFAP)	G-A-5	Sigma / G-3893	1/700 (0.0093 mg/ml IgG)
monoclonal anti-vimentin	VIM-13.2	Sigma / V-5255	1/200
goat anti-rabbit Rodal Green conjugate		Molecular Probes R-6470	1/200 (0.01 mg/ml of protein)
donkey anti-mouse Cy3 conjugate (H+L)		Jackson Research Laboratories Inc. 715-165-150	1/200 (0.0025 mg/ml of protein)
Goat anti-rabbit Alkaline Phosphatase conjugated		Sigma A-5153	1/7000
Hoechst 33258		Sigma / B2883	1/1000 (0.001 mg/ml)

Table 2

A list of all the antibodies and stains used.

Results

GFAP-IR and Vimentin-IR expression in the developing chick spinal cord.

Embryonic and postnatal chicks were examined for the expression of GFAP and vimentin at various developmental stages. Cervical cross-sections (figure 1) and horizontal sections (figure 2) of the spinal cord were taken at various stages throughout the chick development period. Omission of the primary antibodies did not reveal any immunoreactivity (Figure 3).

Cells begin to express vimentin-immunoreactivity (VIM-IR) in the central canal of the spinal cord at E9. Also at this time, there is weak VIM-IR at the ventral horns in the spinal cord. Hoechst 33258 nuclear staining indicates only a few cells in this area, while around the central canal a considerable number of cells have VIM-IR. By E11, VIM-IR cells have their filamentous processes aligned in a dorsal-ventral orientation in relation to the central canal. Some VIM-IR is also observed around the edge of the spinal cord.

GFAP-IR first appears around the central canal at E11 and then at the ventral horns of the spinal cord at E13 (figure 1). GFAP-IR can be seen outside the central canal, and also in the grey matter (figure 2). The GFAP-IR appears brighter in the grey matter at E13 in figure 2 when compared to figure 1. By E17, GFAP-IR can be seen ventrally with the lateral sides of the spinal cord also showing limited GFAP-IR. The dorsal columns express lower levels of GFAP-IR than the ventral portion of the spinal cord (figure 1). GFAP-IR is also seen radiating from the central

canal at E17 (figure 2) with some VIM-IR cells located in the central canal. By P1, GFAP-IR is fairly uniform across the spinal cord (figures 1 and 2) with some VIM-IR radiating from the central canal in a dorsal-ventral orientation (figure 1).

Figure 1

Photomicrographs of cervical cross-sections of the chick spinal cord demonstrating the developmental pattern of glial fibrillary acidic protein (GFAP) immunoreactivity (IR), vimentin –IR and the staining of the nuclei of cells with the nuclear stain Hoechst 33258. At embryonic day (E) 9, vimentin-IR occurs in the cells that surround the central canal. By E11, the initial GFAP-IR is present with labeled fibres radiating from the central canal. Vimentin-IR is seen in a similar pattern to that of GFAP but there is also vimentin-IR at the meninges of the developing spinal cord. From E13 to post-hatching day (P) 1, there is an increased expression of GFAP-IR which initially occurs ventrally and then becomes more dorsal at each successive interval examined. Vimentin-IR is consistently observed in the meninges of the spinal cord and in the cells surrounding the spinal canal. Hoechst 33258 nuclear stain indicates the nuclei of cells at each particular time point. In all photomicrographs, the dorsal aspect of the spinal cord is on the top of the frame and the ventral portion of the cord appears at the bottom of the frame. Scale bar = 1 mm

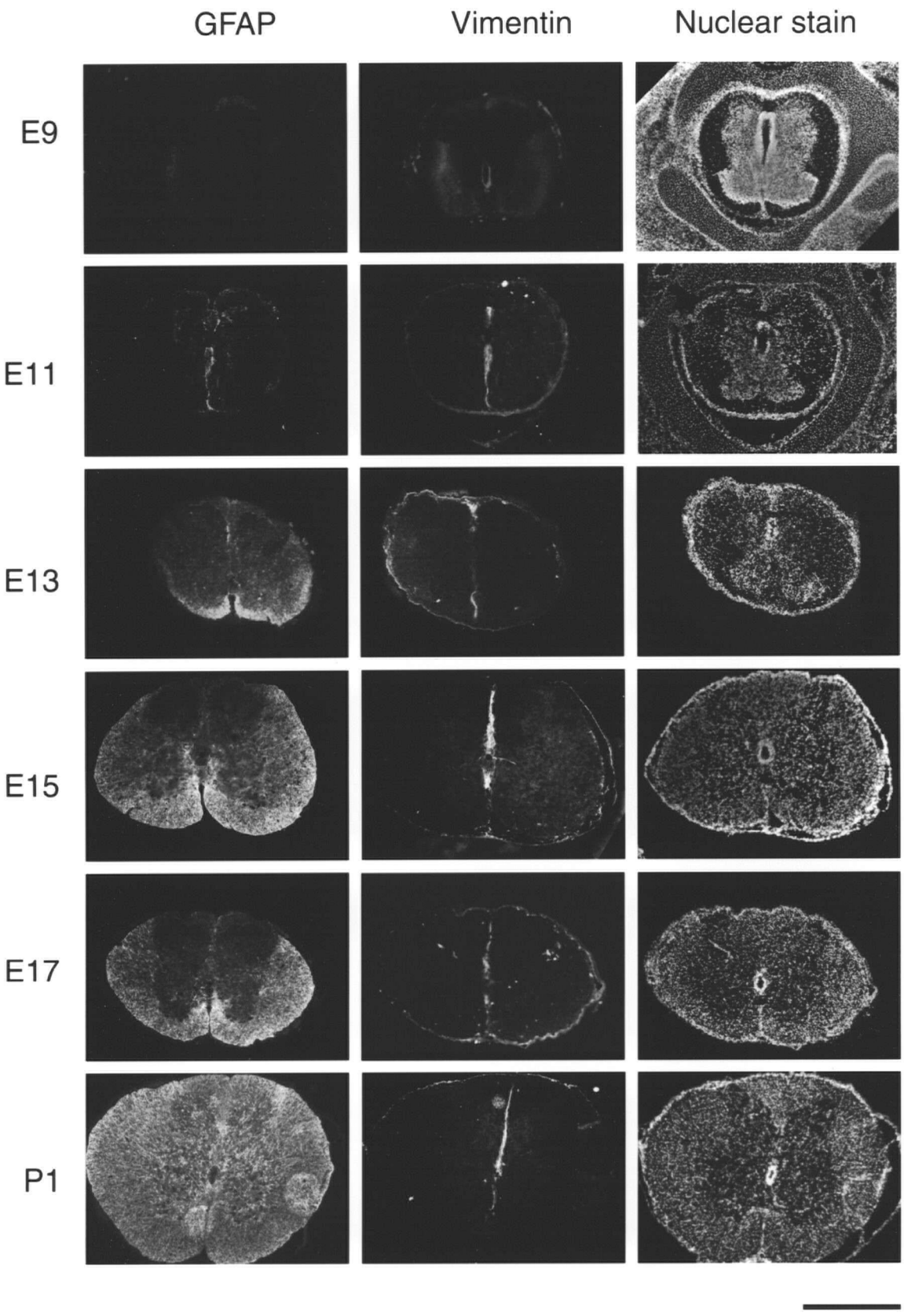

GFAP
Vimentin
Nuclear stain
E9
E11
E13
E15
E17
P1

Figure 2

Photomicrographs in horizontal section of the chick spinal cord demonstrating the developmental pattern of glial fibrillary acidic protein (GFAP) immunoreactivity (IR), vimentin-IR and the staining of the nuclei of cells with the nuclear stain Hoechst 33258. At embryonic day (E) 9, vimentin-IR can be seen throughout the cord. The E9 vimentin photomicrograph appears to show uneven immunoreactivity of vimentin. This was caused by the improper alignment of the fluorescent bulb when the image was captured, leading to uneven illumination of the fluorochrome labeled vimentin. At successive stages, vimentin-IR appears near the exterior of the central canal and at the edge of the spinal cord. By E11, GFAP-IR is seen in cells surrounding the central canal. At each time point after E13, GFAP–IR can be observed with increasing intensity in the grey matter of the spinal cord. Hoechst 33258 nuclear stain clearly defines the cellular nuclei. Cells surrounding the central canal can be clearly be seen. In all photomicrographs, the rostral aspect of the spinal cord is at the top of the frame, the caudal end of the spinal cord appears at the of the frame, and the sides show the most lateral section of the spinal cord. Scale bar = 500 μm

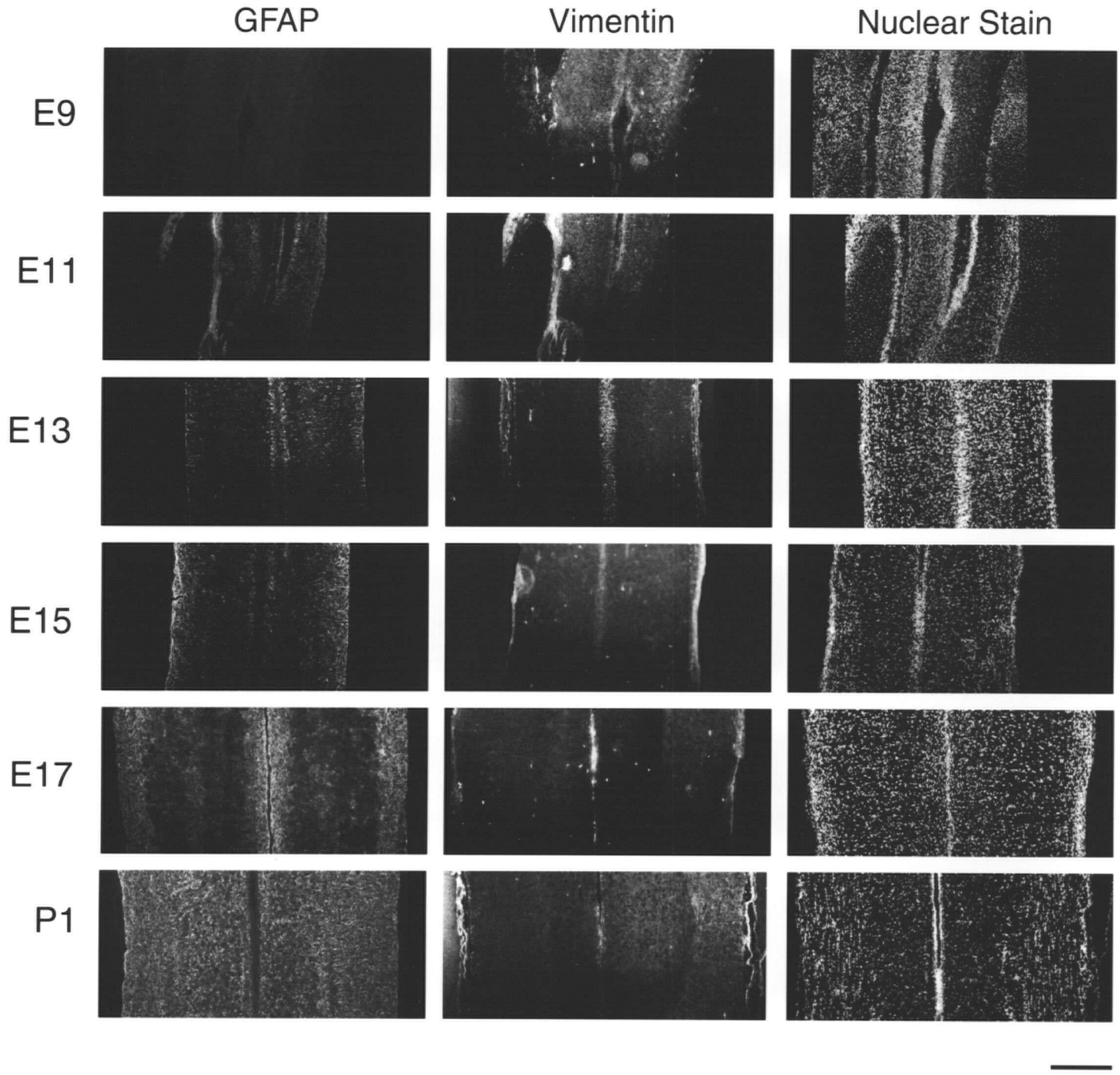
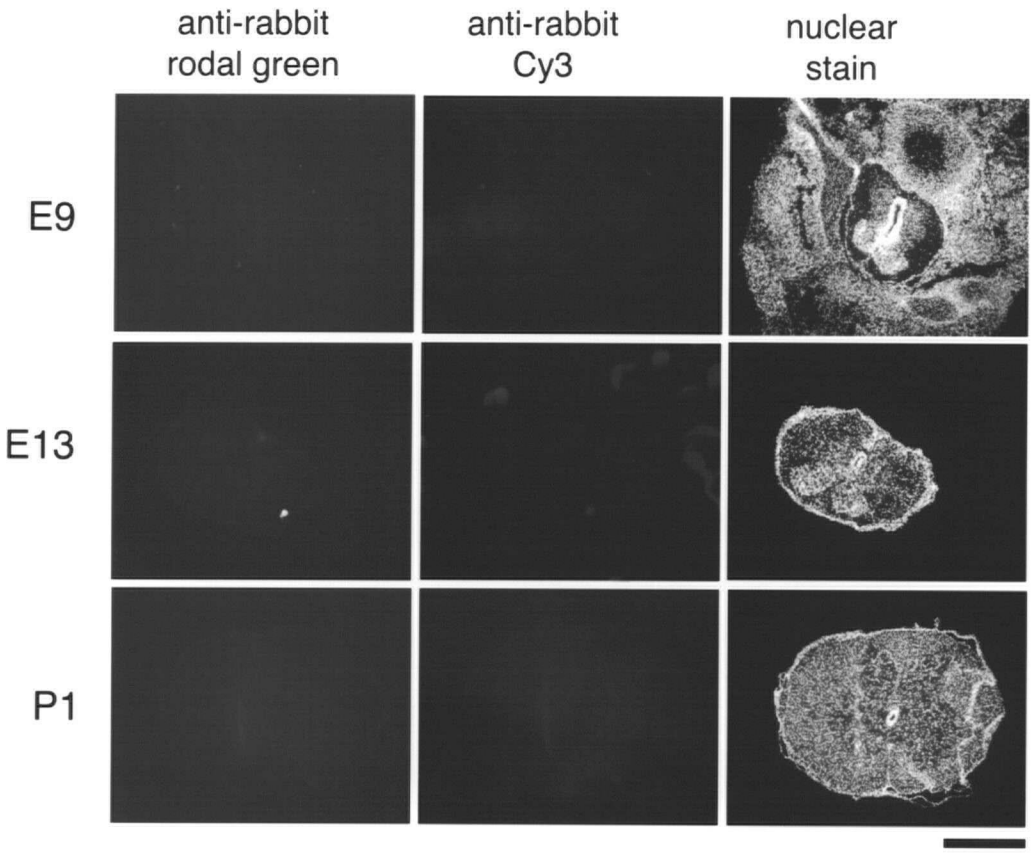

GFAP
Vimentin
Nuclear Stain
E9
E11
E13
E15
E17
P1

Figure 3

Photomicrographs of cervical cross-sections of the chick spinal cord demonstrating the non-specific binding of the secondary antibodies used in the figure 1 and 2. The brightness has been increased twice as much as Figures 1 and 2 to better visualize any non-specific binding the secondary antibodies my have to the tissue. The nuclear stain Hoechst 33258 illustrates the area the spinal cord cross section is on the photomicrograph. Scale bar = 0.5 mm


anti-rabbit
rodal green
anti-rabbit
Cy3
nuclear
stain
E9
E13
P1

Discussion

Astroglial development in the chick spinal cord was investigated for the expression of both GFAP and VIM using immunohistochemical techniques. Early morphological studies in the chick spinal cord suggested that glial precursor cells first appear within the ventricular zone (VZ) and migrate towards the developing white matter (Fujita, 1965). In the spinal cord, precursors of astrocytes and oligodendrocytes originate from the ventral portion of the VZ (Hirano and Goldman, 1988). From the ventral VZ, the precursors migrate dorsally and then radially (Miller, 1996). At E9, VIM+ cells were observed surrounding the central canal and ventral portion of the spinal cord (Figure 1Figure 2). The VIM-IR in Figure 2 shows uneven immunoreactivity. This was caused by the improper alignment of the fluorescent bulb when the image was captured, leading to a gradient from light to dark of illumination of the fluorochrome labeled vimentin. By E11 there are fibres radiating from the central canal which show GFAP- and VIM-IR in the same area. These fibres are immunoreative for VIM and are arranged dorsal to ventral (figure 1), a pattern which appears consistently in similar studies of the rat and opossum (Elmquist et al., 1994; Oudega and Marani, 1991). VIM+ cells were seen in each developmental stage and continue into the post-natal chick. This agrees with to reports of glial precursor cells in the spinal cord of adult rats (Johansson et al., 1999). Since some differentiating neurons and their processes in the spinal cord contain a small amount of vimentin, it is possible that the VIM-IR seen is not exclusively of glial origin (Bignami et al., 1982). This is unlikely since all of the neurons have differentiated at the developmental stages examined.

By E12, oligodendrocytes begin to express GC in the developing chick spinal cord (Keirstead et al., 1992). GC is one of the indicators of a fully differentiated oligodendrocyte. Astrocytes produce PDGF and oliogodedrocytes express the PDGF – α receptor. These factors are believed to allow oligodendrocyte precursors to enter their final stage of differentiation (Noble et al., 1988; Pringle et al., 1992; Raff et al., 1988; Richardson et al., 1988). Other immunohistochemical studies in the rat and mouse have shown that GFAP expression appears to precede the development of oligodendrocytes (Choi, 1988; Schnitzer and Schachner, 1982). This occurs either late in the fetal stage or early in the postnatal stage of neuronal development (Choi, 1988; Malloch et al., 1987; Schnitzer et al., 1981). As expected, this study showed that astrocytes began to express GFAP before E13, indicating they are mature and possibly involved in oligodendrocyte maturation.

From E13 to E17, expression of GFAP in the ventral half of the chick spinal cord increased. There is a variation in GFAP expression between the dorsal and ventral halves of the spinal cord. In rats, GFAP+ astrocytes in the dorsal half of the spinal cord arise from the dorsal neuroepithelium, while the cells in the ventral ventricular zone differentiate into GFAP+ cells in the ventral portion of the spinal cord (Pringle et al., 1998). The final brainstem-spinal connections are completed by E12 (Okado and Oppenheim, 1985), the same stage at which astrocytes begin to express GFAP. This is not unexpected since neuronal brainstem spinal connections are completed at this time. In another neuronal system, the developing rat optic nerve, the proliferation of astrocytes has been shown to be dependant on a signal from the retinal ganglion cell (RGC) axons (Burne and Raff, 1997). It appears that astrocytes may become GFAP+ after neuronal connections are successfully completed.

From E13 to E17, a higher concentration of GFAP+ astrocytes appears around the ventral half of the spinal cord than that which is present in the dorsal half. Oligodendrocytes are known to differentiate and migrate in a ventral-dorsal direction (Nishiyama et al., 1996; Pringle et al., 1992; Warf et al., 1991). Since astrocytes are implicated in the differentiation of oligodendrocytes, it was expected that astrocytes would express GFAP earlier in the ventral half of the spinal cord than in the dorsal half.

It is also of note that the onset of GFAP expression occurs just before E13. The loss of regenerative capacity of the embryonic chick spinal cord coincides with the onset of myelination at E13 (Hasan et al., 1993; Keirstead et al., 1992). Thus the maturation of both astrocytes and oligodendrocytes coincides with the loss of a regenerative response in the developing chick spinal cord. In the adult, astrocytes around a lesion may inhibit the regenerative response of axotomized neurons. Therefore strategies to reduce the number of astrocytes at the lesion seem warranted.

There are many potential difficulties when investigating astrocyte ablation strategies in the chick. Many of the reagents and antibodies are mammalian species specific, limiting the number experiments that can accomplished. Therefore, chapter II will focus on potential scar reducing strategies in the adult rat.

Chapter II:

Methods of Reducing of the Astroglial Scar

Introduction

The lack of spontaneous regeneration within the injured CNS may be attributed to a variety of factors that are intrinsic to injured neurons and are present in the environment in which the neuron must navigate. The astroglial scar is a feature particular to the injury site, and may have a considerable effect on the regrowing axons. More specifically, molecules and proteins associated with the astrogliotic scar are known to have both growth-permissive and inhibitory cues.

Reactive astrocytes express a wide variety of ECMs and growth factors that are not permissive to the growth of axons. In particular, proteoglycans, a family of highly sulfated ECMs, are implicated in creating a non-permissive environment for neuronal growth (Davies et al., 1997; Davies et al., 1999; McKeon et al., 1991; Pindzola et al., 1993). CSPG, a specific class of proteoglycan, is the most inhibitory glycosaminoglycan (GAG) assayed. When used as a substrate *in vitro*, CSPG retards DRG and retinal ganglion cell (RGC) neurite extension (Smith-Thomas et al., 1994; Snow et al., 1990). Cleaving off specific glycosaminoglycan (GAG) side chains from CSPG by using Chondroitinase A and C enzyme pretreatment reduces the inhibition of CSPG for axonal growth *in vitro,* as does blocking the epitopes with functional blocking antibodies (Bovolenta et al., 1993; Meiners et al., 1995). The inhibitory nature of CSPG is only

slightly reduced by chondroitinase pretreatment, suggesting that the core glycoprotein also inhibits axonal outgrowth. When astroglial scar explants are used as substrates for DRGs *in vitro*, their inhibitory properties positively correlate with the increased expression of CSPG (McKeon et al., 1991). Furthermore, *in vivo,* when dissociated DRGs are injected into the corpus callosum, $CGRP^+$ neurites are only able to make long projections along the white matter tracts when the astrocytes are not $CSPG^+$ (Davies et al., 1997). At the glial scar, there are other ECMs and soluble factors that may inhibit the regeneration of axons.

There are many other inhibitory molecules associated with gliosis. For example, collagen IV is expressed at the injury site (McKeon et al., 1991; Stichel et al., 1999). When the post-commissural fornix is axotomized, growth across a lesioned area occurs when either the collagen synthesis inhibitor alpha'-dipyridyl or collagen IV functional blocking antibodies are injected into the injury site (Stichel et al., 1999). Another ECM expressed at the glial scar is tenascin (McKeon et al., 1991; Pindzola et al., 1993; Zhang et al., 1997). *In vitro*, astrocyte cell lines which express high amounts of tenascin or certain splice variants which are used as substrates have been shown to inhibit the growth of cultured neurons (Fok-Seang et al., 1995; Meiners and Geller, 1997; Meiners et al., 1995). It is unlikely that all of the molecules associated with the astroglial scar are known. The expression of Sema III, a guidance molecule, was only recently found in glial scars at the olfactory bulb (Pasterkamp et al., 1999). It is not known if sema III is expressed at the glial scar in the spinal cord. Approaches that reduce the expression of one specific growth-inhibitory molecule are limited since many other key factors may be expressed.

Approaches in which astrocytes are reduced or removed after injury may provide some benefit by preventing the expression of the inhibitory factors associated with astrogliosis. It is my

overall hypothesis that killing astrocytes at the injury site is a viable method of preventing the formation of the glial scar. Specifically, I will use three different approaches with which to reduce astrogliosis after a spinal cord injury. The first method will attempt to functionally block transforming growth factor beta, which is one of the initiators of astrogliosis. The second method involves the infusion of the glial toxin L-alpha amino adipate. In the third approach I will attempt to kill astrocytes through the use of specific antibodies and complement. I will then compare the size of the astrocytes-free zone around the infusion cannula to vehicle treated animals.

Methods of reducing the astroglial scar

There are many triggers for an astroglial response. These range from a change in pH (Oh et al., 1995) to increases in cytokine levels (Fawcett, 1994; Logan and Berry, 1993; Norenberg, 1994; Ridet et al., 1997; Wu and Schwartz, 1998). Researchers have attempted to inhibit or reduce gliosis by reducing the amount of functional cytokines available at the injury site. Injections of IL-10, which is believed to inhibit microglial cytokine production, reduced the gliotic appearance of the scar tissue (Balasingam and Yong, 1996). TGF-β1 and TGF-β2 functional blocking antibodies have been successfully used to reduce the scar in the cortex (Logan et al., 1994; Logan et al., 1999). Thus, reducing the levels of active TGF-β1 and TGF-β2 is a viable method of preventing the glial scar from forming.

Fetuin to reduce the glial scar

Transforming growth factor beta (TGF-β) is a multifunctional polypeptide growth factor (Assoian and Sporn, 1986; Heine et al., 1987; Sporn et al., 1986). The TGF-β family has five distinct isoforms termed TGF-β1-5. Only TGF-β1-3 are found in mammals, and TGF-β1 is seen only in the CNS. TGF-β1 has many effects within the CNS. At the earliest stages of response, TGF-β1 acts as a mediator for inflammation, angiogenic responses and also ECM deposition (reviewed in Logan and Berry, 1993). Previous work has demonstrated that recombinant TGF-β1 injected into the cortex results in an increased astroglial scar response, and TGF-β1 may act as a chemoattractant for blood-derived monocytes (Logan et al., 1994). TGF-β2 is found throughout the blood stream, and fetuin binds to it to prevent its unwanted effects. Binding is made possible by fetuin's high degree of homology to the TGF-β receptor type II (Dziegielewska et al., 1997). TGF-β1 and 2 are shown to both initiate gliosis (Logan et al., 1994; Logan et al., 1999). *In vitro*, fetuin inhibits the effect of both growth factors by blocking the receptor-binding site on the TGF molecule (Demetriou et al., 1996). It is my hypothesis that when compared to vehicle alone, fetuin infused into a spinal cord lesion will reduce the formation of an astrogliotic scar. This reduction of astrogliosis may occur since there would be less TGF-β 1 and 2 available to initiate a gliotic reaction. The potential problem associated with targeting a select gliosis-initiating molecule is that other injury response cues may elicit a gliotic reaction. For example, fibroblast growth factor (FGF) - 2 causes astrocytes to become reactive near the injection site when it is injected into the cerebral cortex (Eclancher et al., 1996; Eclancher et al., 1990; Gomez-Pinilla et al., 1995; Menon and Landerholm, 1994). Other *in vivo* studies have shown that ciliary neurotrophic factor (Hudgins and Levison, 1998; Kahn et al., 1995; Lee et al., 1997; Levison et al., 1996; Rajan and McKay, 1998), interleukin – 1 (Balasingam et al., 1994; Chiang

et al., 1994; Giulian et al., 1988), and tumor necrosis factor – α (Balasingam et al., 1994) are released after a penetrating CNS injury. All have been shown to elicit an astroglial response.

These studies indicate that there are many factors capable of invoking an astroglial response. To circumvent this potential problem, some experimental approaches have focused on removing or reducing the number of astrocytes. One strategy has involved removing the intermediate filament proteins associated with the astrocytes, but these attempts yielded mixed results. *Gfap* -/- mice have normal development (Pekny et al., 1995) and failed to show either any difference in ECM expression or any regeneration after a CST axotomy when compared to wild type mice (Wang et al., 1997). When compared to wild type mice, *Vim* -/- mice showed normal glial responses to injury (Pekny et al., 1999). Double-knock out mice which have both vimentin and GFAP null mutations showed a decrease in endothelial and ependymal cell expression of nestin, no change in cell proliferation and reduced scar formation as revealed by HE staining after a stab wound injury (Pekny et al., 1999). Axonal regeneration, however, was not examined in these studies.

To specifically kill astrocytes, a transgenic mouse was created using the herpes thymidine kinase (TK) gene. This gene was inserted downstream of the *Gfap* promoter region (Bush et al., 1998; Delaney et al., 1996). The TK gene is only lethal to astrocytes when the anti-herpes drug ganciclovir (GCV) is administered to the animal (Delaney et al., 1996). Astrocytes were killed cytotoxically after an injury when the *Gfap* promoter was activated and ganciclovir was administered. Stab wound injuries to the thalamus with GCV administration showed an increase in neurofilament-M staining in the lesioned area when compared to the wild type or the non-

GCV administered GFAP-TK mouse. This indicates that the environment at the lesion site is more hospitable to neurons after the astroglial scar is reduced by the administration of GCV. It is not known whether the absence of the glial scar increases the propensity of the neurons to grow into the lesioned area or if the neurons are dying at a slower rate as compared to the wild type mice. Currently, transgenic approaches provide insights to the function of the glial scar and its role in CNS regeneration. While they are instructive, these experiments are not clinically feasible as therapies at the present time, nor are they easily tested in other animal species.

After a penetrating spinal cord lesion, astrocytes and other glial cells undergo cell division, filling the newly created cavity and reforming the BBB. X-irradiation has been employed to specifically kill these dividing cells (Kalderon et al., 1990). Although this approach kills any proliferating microglial cells along with adult progenitor cells, it is reported to improve functional recovery of spinal cord injured rats if administered only after the third week of the injury (Kalderon and Fuks, 1996). Anatomical studies were not carried out on the x-irradiated animals to examine the injury site. Since only about 2-4% of the astrocytes divide before becoming reactive, it is conceivable that a gliotic scar may still be present (Hatten et al., 1991; Latov et al., 1979; Mangel et al., 1985).

Complement Mediated Antibody Lysis to specifically remove astrocytes

Complement is the main component of the mammalian immune system. During an invasion of foreign cells into the body, an immune response is generated in which antibodies are targeted to specific foreign antigens. Once one IgM or two IgG antibodies bind to the same antigen, a

complement cascade is initiated, leading to lysis of the invading cell (reviewed in Law and Reid, 1989). This biological mechanism for cell destruction can be utilized to specifically target and destroy particular cell types. Antibodies that recognize a surface antigen must be used in this technique since antibodies only have access to the surface of a cell. For example, oligodendrocytes can be specifically destroyed *in vivo* and *in vitro* when antibodies recognizing the surface glycolipid galactocerebroside are used with complement (Dyer and Benjamins, 1990; Keirstead et al., 1995; Mastaglia et al., 1989; Rostami et al., 1984). Therefore antibodies raised against GFAP or vimentin would not work in this technique since they are structural proteins only found intercellularly. Until recently, antibodies recognizing surface antigens on astrocytes were unavailable due to the non-immunogenic nature of astrocyte antigens. Creating antibodies to glial antigens in general is problematic, since glial proteins are highly conserved across species. Antibodies are generated in the host animal when antigens are injected. Since glial cell structures are highly conserved across species, an injection of a foreign glial antigen from a different species many not be recognized as foreign, and thus, antibodies would not be created. This is one reason why there are a limited number of antibodies available capable of recognizing glial antigens. Despite these difficulties, an IgG antibody has been developed which recognizes a glycosylphosphatidylinositol-anchored ceruloplasmin on astrocytes (Patel and David, 1997). Ceruloplasmin's putative role is to oxidize ferrous iron to remove free radicals. This antibody, generated by Dr. S. David and termed 1A1, has been shown to be specific to astrocytes in the CNS (Patel and David, 1997). It is believed that the combination of the 1A1 antibody and serum complement administered at the site of injury in the spinal cord will destroy the astrocytes which create the glial scar.

L-Aminoadipate (L-AAA) infusion into the injured CNS

L-AAA's chemical structure, $HOOC-(CH_2)_3-CH-(NH_2)-COOH$, differs from the structure of L-glutamate or $HOOC-(CH_2)_2-CH-(NH_2)-COOH$, due to the presence of an additional CH_2 group. The similarities in structure allow L-AAA to enter astrocytes by glutamate channels. The enzyme glutamine synthetase, which normally covert glutamate to glutamine (Pow and Robinson, 1994), is unable to break convert L-AAA to a less harmful molecule. The concentration of L-AAA increases to toxic levels in the astrocyte, which leads to necrotic death of the cell (Huck et al., 1984). This glutamate homologue has also been reported to be a glutamate uptake inhibitor (Fletcher and Johnston, 1991; Robinson et al., 1991), postsynaptic excitatory amino acid receptor agonist (Hall et al., 1977), excitotoxin as well as a glial and neural toxin within the CNS (Garthwaite and Regan, 1980; Khurgel et al., 1996; Olney et al., 1980). After a brain injury resulting in neuronal damage, secondary damage occurs when glutamate is released from dying neurons. The glutamate release is a result of cells necrotically dying or lysing and releasing their glutamate stores. In ischemic conditions, the glutamate transporter can reverse, pumping glutamate into the extracellular space, eventually resulting in the presence of toxic levels of glutamate around the neurons. The high glutamate concentrations lead to increased intracellular calcium, and initiate reciprocal interactions with reactive oxygen species, the combination of which results in neurotoxic conditions (Trotti et al., 1998). In low concentrations, L-AAA has been shown to reduce the amount of glutamate released during ischemic conditions *in vitro* slice cultures, and thus reduce the overall amount of damage to the neurons (Haugstad and Langmoen, 1997).

Neuronal and astroglial death within the CNS is elicited by the interperitoneal injection of L-AAA at high concentration of 100 mg/ml, (Olney et al., 1980). The resulting neuronal damage may be caused by the massive loss of the astroglial support network. Injections of L-AAA at concentrations of 20 mg/ml do not damage CNS neurons, but are toxic to astrocytes. Since L-AAA is a glutamate homologue, and is transported by astrocytes in a Na+ dependent manner, it is believed that astrocytes transport L-AAA by an undetermined Na^+ dependent uptake transporter (Huck et al., 1984). This transporter is believed to be either GLAST or GLT-1 since these are the only known Na^+ dependent astrocytic glutamate transporters in the CNS (Gegelashvili and Schousboe, 1997).

Over the past few years, the effectiveness of L-AAA has been controversial due to conflicting results (cf. Khurgel et al., 1996; Saffran and Crutcher, 1987). There are many reasons for these inconsistencies. Early studies involving L-AAA used both the L and the D formulations (Bergey et al., 1980; Bruni and Vriend, 1984; Charles and Chang, 1983; Fex and Martin, 1980; Martin and Adams, 1979; McBean, 1990). It was later shown that the D formulation was ineffective for killing astrocytes (Kato et al., 1993). Furthermore, the method of delivery varied between studies from systemic to small focal injections (Khurgel et al., 1996; Saffran and Crutcher, 1987; Takada et al., 1990). Each group of experiments used differing approaches to deliver L-AAA to the CNS, and this may account for the difference in efficacy of L-AAA as a glial toxin. The most recent study, however, found that focal injections into the amygdala created an area free of astrocytes when compared to control injections (Khurgel et al., 1996). It is my hypothesis that L-AAA will specifically kill astrocytes at the site of infusion in the spinal cord. Infusing L-AAA into the spinal cord and quantifying the volume that is free of astrocytes will test this.

Materials and Methods

Surgery

Adult Sprague Dawley (SD) rats (weight 200-250 g) were used in this study. All surgery was performed in accordance with the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee. Sprague Dawley (SD) rats were anesthetized using Ketalean 60 mg/kg (MTC Pharmaceuticals, # 8139A) and Xylazine 7.5 mg/kg (Bayer, #186894). Once the anesthetized animal did not initiate a pain with-drawl reflex after a noxious stimuli such as a toe pinch, the hair of the rat was shaved off from thoracic (T) 7 to T12. To sterilize the rat's skin, 70% ethanol and distilled water (v/v) was sprayed on the shaved area of the back. A dorsal incision was made from T7 to T12 in the SD rat. To reduce post-operative pain and lessen blood flow to the muscle, 0.4 cubic centimeters (CC) of 2% Lidocaine: H_20 (v/v; Vétoquinol, # 652525) was then injected into the exposed superficial musculature around the spinal column. A longitudinal incision was made from T-8 to T11 on either side of the spinal column to expose the spinal column. A partial laminectomy was performed on the 10th thoracic vertebrae. Immediately adjacent to the dorsal blood vessel, a hole in the dura was made with a 28-gauge needle. Stainless steel screws were inserted into the pedicle of the 9^{th} and 11^{th} thoracic vertebrae. A 7 day mini-osmotic pump (Alzet, model 2001), with an attached brain infusion cannula (Alzet brain infusion kit, model 4760-0), was placed subcutaneously along the dorsal side and the infusion cannula and inserted into the hole created by the 28-gauge needle. The T10 vertebrae and cannula head were fixed together by placing dental cement around the screws and cannula. These pumps contained 100 units/ml of Penicillin and 100 units/ml Streptomycin

(Gibco BRL # 15140-031) with either vehicle or treatment. Table 3 lists the chemicals tested. The pumps infuse a total of 100µl at a constant rate of 1.0 µl/hr for seven days. Once the dental cement was dry, the superficial muscle layers were re-apposed with 6-0 silk and the skin closed with skin staples. Animals were then placed in a warmed recovery area and monitored until fully awake. They were kept in a special animal facility for one week after which the animals were sacrificed and the spinal cord tissue was collected (see below).

TREATMENT	CONCENTRATIONS	NUMBER OF ANIMALS FOR:		SUPPLIER
		IHC	ELISA	
Control		3	3	
Vehicle	0.1 M PBS	4	4	
L-Alpha aminoadipate (L-AAA)	20 µg/ml	4	4	Sigma (A-7275)
Fetuin	1 mg/ml	4	4	Sigma (F-3004)
Anti Cerenoplasm monoclonal antibody (1A1)	0.6 mg/ml	4	4	a gift from Dr. S. David
Guinea Pig Complement	33%	4	4	GIBCO BRL #19195-015
1 A 1 + 30% Guinea Pig Complement 0.1 M PBS	0.6 mg/ml 33%	4	4	

Table 3

A table showing the treatment groups for each astroglial scar reducing strategy.

Perfusion/cryosectioning

SD rats were injected with 1 mg/100g of a 6% Ketalean (v/v) and 3.8% Xylazine (v/v) solution in dH_20 and monitored until breathing was arrested. The animals were then perfused intracardially with 37°C 0.1 M PBS, pH 7.4 followed by 4% w/v paraformaldehyde (PFA) in 0.1 M PBS. The infusion cannula was visually inspected for a separation between the tubing and the cannula head. If a separation was found, animals were removed from the study because a consistent delivery of the treatment can not be assured. After the infusion apparatus was examined, spinal cord tissue was removed and the tissue post-fixed for 24 hours in 4% PFA at 4°C. Tissue was cryoprotected with sequential 8 hour exposures of 12, 16 and 22% w/v sucrose solutions in PBS. After cyroprotection, tissue was mounted on filter paper with Tissue-Tek (J.B.EM. Services Inc. #JBF172) and rapidly frozen in supercooled 2-methylbutane (BDH, #B29452-74). Rat tissue was sectioned at 18 μm. This tissue was cut at -22°C using a Zeiss cryostat and the sections were then placed on warmed Superfrost Plus slides (Fisher # 12-550-15) and stored at -70°C.

Histology

All antigens were localized using indirect immunofluorescence techniques. Standard immunohistochemical (IHC) techniques and controls for indirect-immunofluorescence were used in order to visualize specific antigens on cryosectioned tissue. Briefly, the slides were washed in 0.1M PBS for 20 minutes and the primary antibodies were added to the slides. All antibodies were used in a solution of 0.1% Triton-X100 (BDH, #R 06433) and PBS. Plastic coverslips were placed over the slides and incubated overnight at 4°C. After incubating the tissue

overnight, the slides were then washed for 30 minutes in PBS and a blocking solution of 10% goat serum and 0.1% Triton-X100 in PBS was added for 20 minutes at room temperature. Excess blocking solution was then removed from the slides and the secondary antibodies were added for one hour at room temperature. After the secondary antibodies had incubated, the slides were washed for 30 minutes in PBS with the final wash of PBS containing 0.001 mg/ml of Hoechst 33258 and a coverslip was placed on the slide with a 3:1 solution of glycerol: PBS (v/v). All antibodies used are listed on table 3 with concentrations used. A fluorescent microscope (Zeiss, Axioskop) was used to visualize chromophore labeled tissue. Images were captured with a Spot CCD digital camera (Diagnostic Instruments Inc. model 1.1.0) and Photoshop (Adobe, Version 5.1) was used to create photomontages and make minor adjustments for brightness and contrast of the whole image.

Tissue Quantification

Volume of Reactive-astrocyte-free zone measurements

One hundred and forty sections that were 18 μm thick were collected during cryo-sectioning of the spinal cord. From each animal, seven sections were selected at 350 μm intervals though the spinal cord. Using immunohistochemistry, these sections were fluorescently labeled for GFAP and the nuclei were stained with Hoechst 33258. These images were captured using a CCD camera and imported into Photoshop (Adobe, Version 5.1; Figure 5A). An outline was drawn around the reactive-astrocyte-free zone as indicated by GFAP-IR (Figure 5B) and then imported into the image analysis program Northern Eclipse (Empix Imaging Inc., release 2.0). On each of the seven images which had GFAP-IR, the area of the zone free of GFAP-IR zone was

determined using this program (Figure 5C). This area was termed the reactive-astrocyte-free zone. The volume of the reactive-astrocyte-free zone was calculated by averaging the area of all seven measured sections and then multiplying the averaged area by the height of the spinal cord examined (Cameron et al., 1990; Moriarty et al., 1998).

The previous calculation included the volume of the cyst cavity that may directly affect the size of the reactive-astrocyte-free zone. To account for this, the volume of the cyst cavity was subtracted from the volume of the cyst cavity in the spinal cord. For determining the volume of the reactive-astrocyte-free zone, a similar protocol was followed as above (Figure 5). The nuclei were labeled with Hoechst 33258. On each of the seven images captured per spinal cord, the area of the cyst cavity was determined as the area devoid of nuclear staining (Figure 7B). The volume of the cyst was calculated by averaging the area of the seven measured sections and then multiplying this averaged area by the height of the spinal cord. Once the volume of the cyst cavity was obtained, it was subtracted from the volume of the reactive-astrocyte-free zone. This determined the volume of viable tissue that was free of astrocytes (Figure 7C).

ELISA methods

All tissue for an enzyme-linked immunosorbant assay (ELISA) was not fixed. After the perfusion of PBS through the animal, a 0.5 cm section was removed from the spinal cord, which contained the injured area, placed in a 1.5ml centrifuge tube and rapidly frozen on dry ice. Samples were then stored at –70°C until required. Standard ELISA techniques were used to assess the amount of GFAP present (Fix et al., 1995). The tissue was thawed, 0.2% Tween-20 (PBS-Tw; BDH, #R06435) PBS (v/v) with protease inhibitor cocktail (Boehringer Mannheim

#1836153 Complete Mini™) was added to the centrifuge tube and the tissue was homogenized. The protein concentration was determined using a spectrophotometer. This was accomplished by adding 1 ml of 0.2% Tween PBS (v/v) with protease inhibitor cocktail solution and a reading at $\lambda = 280$ was obtained which was used for a zero level absorbency reading. A Pharmacia ultrospec LKB plus spectrophotometer was used for this. A 1 ml sample was placed in a cuvet and absorbency was measured at 350, 280 and 260 λ. The protein concentration adjusted for light scatter was the determined using the following calculations:

$E_{280\ corrected} = E_{280}-E_{350}$

$E_{260\ corrected} = E_{260}-E_{350}$

$E_{280\ corrected}/E_{260\ corrected}$

From this the Factor was determined and was then multiplied by $E_{280\ corrected}$. All samples were then standardized to 10 µg/ml. For GFAP protein quantification, the double antibody sandwich technique was employed (Fix et al., 1995). The monoclonal antibody to GFAP was coated on 96 well flat microtiter plates (Falcon, MicroTest III) at a concentration of 1:32,000 in coating buffer pH 9.6 for 2 hours at 37°C. Each well had 200 µl of solution the tests. After the incubation, the microtiter plates were washed 3 times in distilled water. A solution of 1% bovine albumin (Sigma, A8551) in 0.1% PBS -Tween (v/w) was added to the plate for 30 minutes at 37°C. The plates where then tapped dry and 10µg/ml of sample protein in 0.1 M PBS was added to each well for 2 hours at 37°C. The plates were washed 3 times in distilled water and a polyclonal GFAP (table 2) antibody was added at a concentration of 1/8000 and incubated for 2 hours at 37°C. The plates were washed 3 times in distilled water and a goat anti-rabbit alkaline

phosphatase conjugated secondary antibody (table 2) was added at a concentration of 1/7000 and incubated for 2 hours at 37°C. Plates were washed 3 times in distilled water and phosphatase (Sigma, P4105) was added at concentration of 1 mg/ml in 0.1 % diethanolamine in distilled water, pH 9.8. After two hours, a Biorad Model 3550 Microplate Reader was used to measure the optical absorbency at 405 nm against blank wells (a well that only contained the phosphatase and not any of the antibodies or protein). For negative controls, for differing wells an antibody or protein was omitted during the procedure. For positive controls, purified GFAP (ICN, #77-106) was used instead of the tissue sample.

Statistics

To determine the statistical significance of the reactive-astrocyte-free volume measurements an analysis of variance (ANOVA) was used to account for a difference in the means. When a difference was found, a Dunnett's multiple comparisons test was used to compare the treatment means to the control means.

Results

GFAP-IR after treatments

To infuse the desired treatment, a cannula was inserted into the spinal cord at thoracic level (T) 10. The implantation of the cannula into the thoracic spinal cord created a defined and reproducible lesion that invoked an astroglial response. Leaving the infusion cannula in the animal for the full seven day duration of the treatment created a cavity in the spinal cord. This cavity, known as a cyst, is usually seen at the epicentre of a penetrating brain injury (Beattie et al., 1997; Koshinaga et al., 1993; Raghupathi et al., 1998).

In Figure 4, GFAP-IR on the horizontal sections indicates that after all treatments there was a reactive-astrocyte-free zone around the cannula. Within each of the reactive-astrocyte-free zones, nuclear staining indicated that a cyst cavity had formed. The occurrence of the cavity overlapped with the position of the cannula. The reactive-astrocyte-free zone appeared smallest in both the vehicle (Figure 4B) and 1A1 antibody alone treatments (Figure 4E). There is also an increase in GFAP-IR around the infusion site (Figure 4B and E).

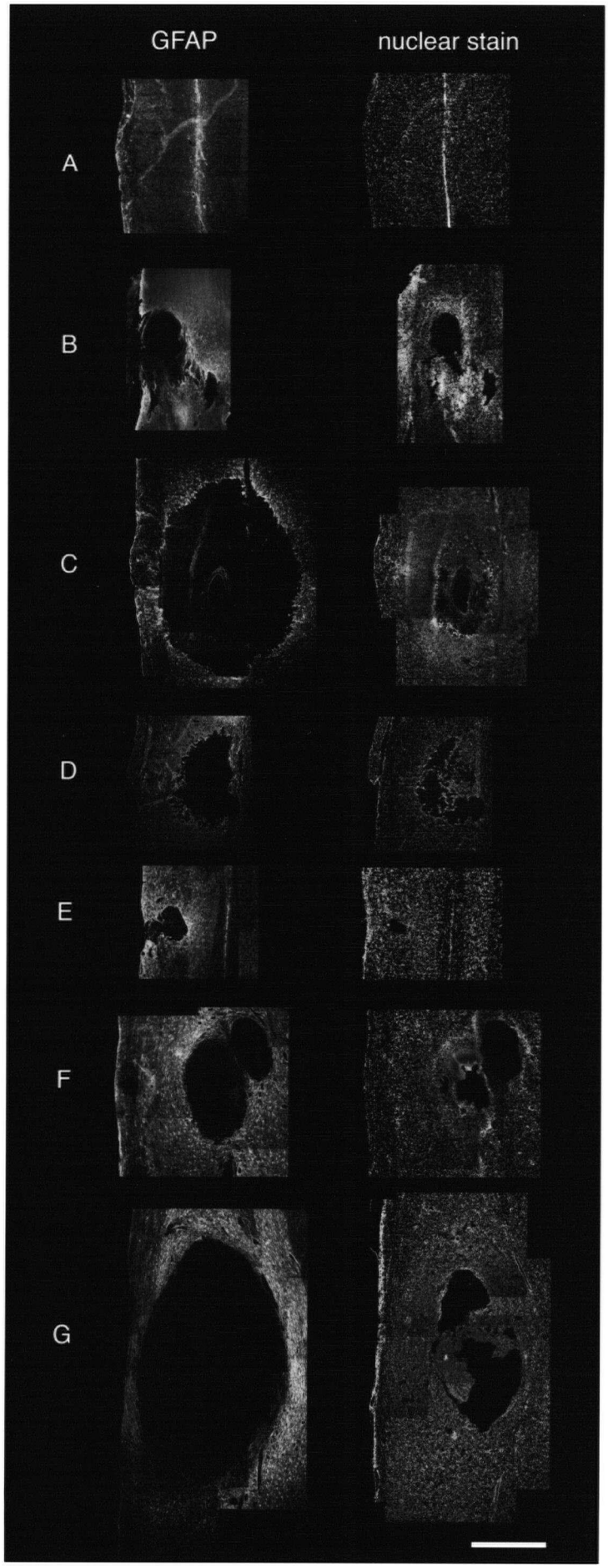
As compared to vehicle treatment, infusions of the glial toxin L-AAA resulted in a considerably larger reactive-astrocyte-free area (Figure 4C). This area was filled with many cells with small nuclei. Their number and the size of their nuclei, revealed by nuclear staining, indicates that these cells are likely to be microglial or macrophage cells originating from the CNS or from the periphery respectively. In the L-AAA treated animals, since nuclear blebbing

consistent with an active form of cell death is not apparent, astrocytes presumably die necrotically (Kerr et al., 1972).

Fetuin did not appear to reduce the amount of gliosis around the cannula when compared to vehicle treatment alone (Figure 4D). Although the fetuin-treated reactive-astrocyte-free zone around the cannula appears larger than that in the vehicle control, this was not statistically significant (Figure 6). When the astrocytes were removed through complement-mediated lysis (1A1 + complement), a large reactive-astrocyte-free zone resulted (Figure 4G). This zone had a strong astrogliotic reaction around the border, as indicated by GFAP-IR. Within this zone, nuclear stain revealed a large cyst cavity created by the treatment. Removing the astrocytes using this method appeared more detrimental to the spinal cord than the antibody or vehicle treatment. When complement alone was infused into the spinal cord, a strong astroglial reaction was observed around the infusion area (figure 4F). This was expected since high amounts of complement usually signal a strong immunological response (Baxter, 1994).

Figure 4

Representative pictures of the spinal cord at the 10th thoracic (T) level one week following the infusion of various treatments using a 7 day mini-osmotic pump. **A) Unoperated control rat.** Glial fibrillary acidic protein (GFAP) immunofluorescence (IR) and Hoechst 33258 nuclear staining of the unoperated control rat. **B) Vehicle only treatment.** GFAP-IR shows that there is a small area around the spinal cord that lacks astrocytes. This was where the osmotic pump was inserted. **C) L-AAA treatment**. The hole created by the pump cannula can clearly be seen near the center of the spinal cord. There is a large area surrounding the infusion site that lacks astrocytes, as indicated by the absence of GFAP-IR. Nuclear stain indicates that this area is not entirely devoid of cells. **D) Fetuin treatment.** GFAP-IR is absent from the area surrounding the pump. This corresponds to a lack of nuclear stain. **E) 1A1 antibody alone.** There appears to be marked astrogliosis around the cannula infusion site as seen by GFAP-IR. **F) Complement alone.** An area that does not contain GFAP-IR surrounds the pump with marked gliosis seen around the border of the infusion site. **G) 1A1 and complement**. A large area surrounding the pump implantation site area that is devoid of GFAP-IR. Around the border this area devoid of astrocytes, strong astrogliosis can be observed. The area devoid of nuclear stain indicates a large cyst cavity. In all photomicrographs, the top of the picture is rostral and the bottom is caudal. The left side of each picture is the edge of the spinal cord. Scale bar = 1 mm


GFAP
nuclear stain
A
B
C
D
E
F
G

Size of the Reactive-astrocyte-free zone

To determine whether the effects of the treatments shown in Figure 4 were significant, the volume of each animal's reactive astrocyte-free zone was measured. Figure 5 shows the method of tracing the area around the area devoid of GFAP-IR, and then quantifying that area using Northern Eclipse image analysis software (see methods). A series of these quantifications was done and the volume of the reactive-astrocyte-free zone was determined (Figure 6). Average volumes of the reactive-astrocyte-free zones resulting from the various treatments were as follows (means ± SEM): Vehicle: 1.87 ± 0.88 mm^3, L-AAA: 4.91 ± 0.92 mm^3, fetuin: 2.15 ± 0.36 mm^3, 1A1 antibody alone: 1.68 ± 0.71 mm^3, complement alone: 2.65 ± 0.52 mm^3, and complement and 1A1 antibody combined: 3.78 ± 0.51 mm^3. A comparison of the size of the reactive-astrocyte-free zone in the treatment versus vehicle animals revealed that the L-AAA ($p<0.01$) and antibody + complement ($p<0.05$) treated animals had a significantly larger reactive-astrocyte-free zone than the other experimental groups (Figure 6).

The volume of the cyst cavity and the astrocyte-free zone were measured in order to determine if the size of the reactive-astrocyte-free zone was merely a function of the size of the cyst cavity. Figure 7 demonstrates the method used to determine area of the reactive-astrocyte-free zone without the cyst cavity. The volume of the GFAP-IR-free zone and the cyst cavity were determined using the same method described earlier (see methods and Figure 5). Once the volumes of the two zones were determined, one was subtracted from the other (Figure 7). The result was the size of the reactive-astrocyte-free zone, accounting for the presence of the cyst

cavity. The average volume of the viable tissue that contained the reactive-astrocyte-free zones resulting from the various treatments were as follows (mean ± SEM): Vehicle: 1.32 ± 0.55 mm^3, L-AAA: 4.56 ± 0.98 mm^3, fetuin: 1.29 ± 0.52 mm^3, 1A1 antibody alone: 1.88 ± 0.63 mm^3, complement alone: 1.76 ± .0.45 mm^3, and complement and 1A1 antibody combined: 2.57 ± 0.71 mm^3. A comparison of the viable tissue in the reactive-astrocyte-free zone in Figure 8 revealed that L-AAA ($p<0.05$) was significantly larger than the reactive-astrocyte-free zone of other experimental groups when compared to vehicle alone. The antibody + complement treatment created a large reactive-astrocyte-free zone (Figure 6), which decreased when the formation of the cyst cavity was accounted for (Figure 8).

Figure 5

A representative series of pictures demonstrating the calculation of the reactive-astrocyte-free zone volume as determined by glial fibrillary acidic protein (GFAP) immunofluorescence (IR). **A)** A horizontal section was cut from the spinal cord every 108 μm and the tissue was processed using immunohistochemistry in order to visualize GFAP. A digital image was captured of the fluorochrome used to visualize the antibodies to GFAP. This is the same image seen in Figure 4C. **B and C)** The digital images were imported into Photoshop, and a circle was drawn around the astrocyte free zone. **D)** The area of the circle was determined using Northern Eclipse. Once the area of each section of the spinal cord was determined, their average was then calculated. The average area was then multiplied by the height of the spinal cord to determine the volume of the reactive-astrocyte-free zone (Cameron et al., 1990).

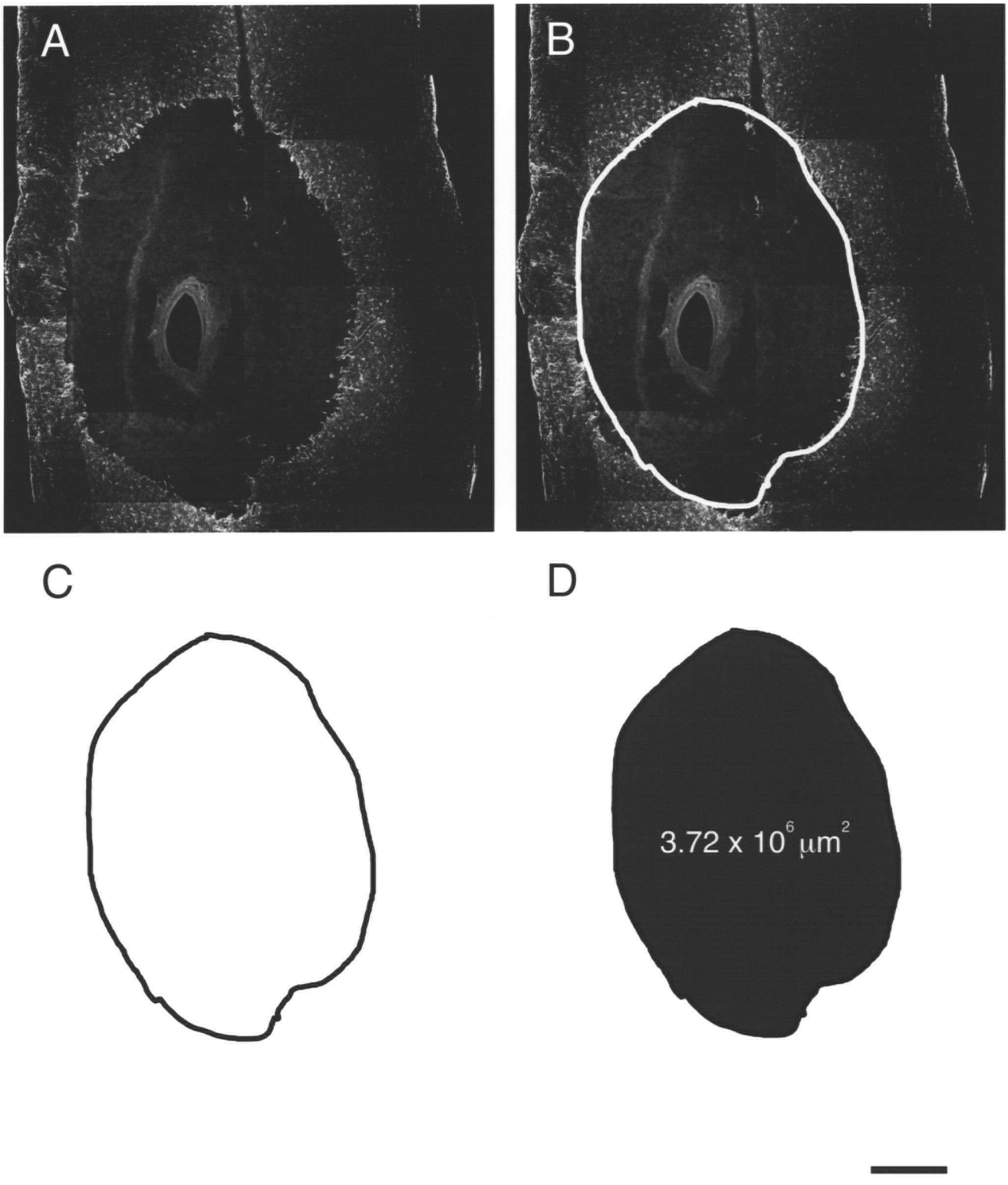

A
B
C
D
3.72 x 10^6 µm^2

Figure 6

A chart comparing the volumes of the reactive astrocyte-free zones of different treatment groups 7 days after infusion using an osmotic pump. Both the infusion of L-AAA and antibody + complement had a significantly larger reactive-astrocyte-free zone at the site of infusion than vehicle alone. Values are means ± SEM and ** indicates $P < 0.01$ and * $P < 0.05$ when compared to vehicle treatment alone. Volume is expressed in mm^3.

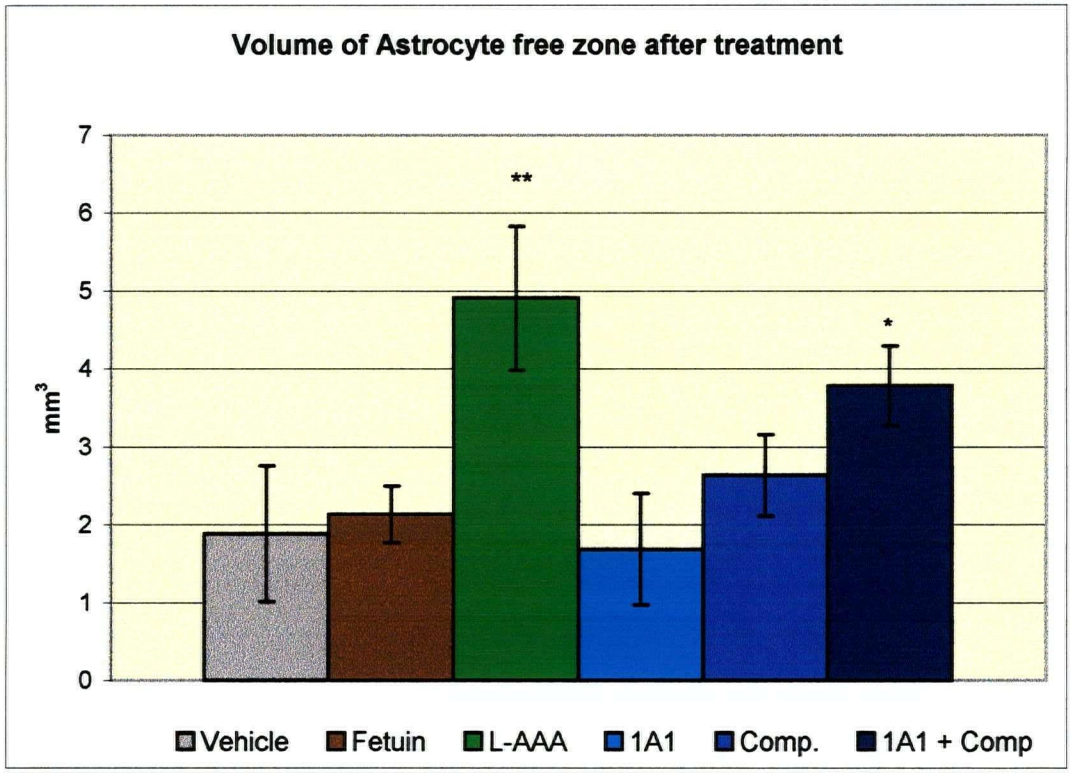
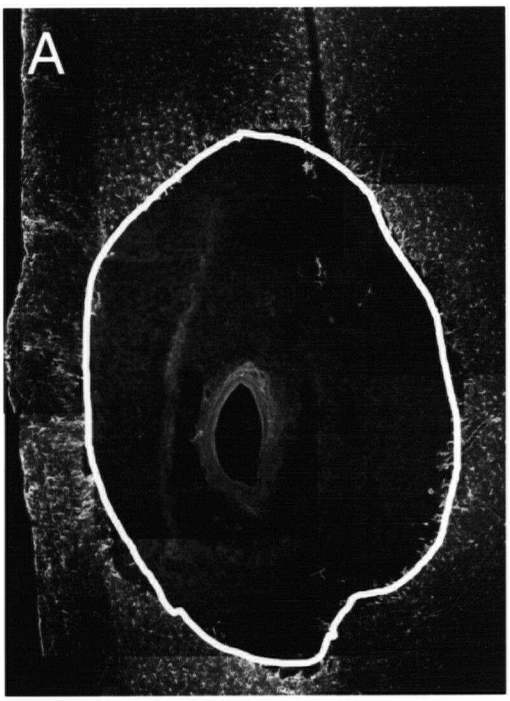
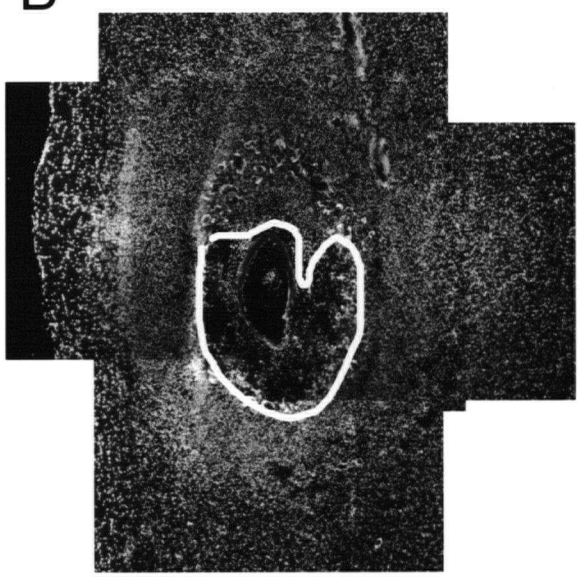

Volume of Astrocyte free zone after treatment
mm3
0
1
2
3
4
5
6
7
**
*
Vehicle
Fetuin
L-AAA
1A1
Comp.
1A1 + Comp

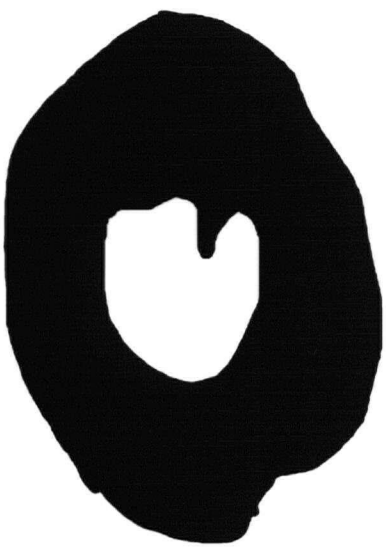
Figure 7

A different method of calculating the reactive-astrocyte-free area was used in order to investigate whether a change in the astrocyte free zone was a result of a change in size of the cyst cavity. **A)** As seen in Figure 5, seven sections from a spinal cord were labeled with Hoechst nuclear stain and GFAP-IR. A circle was made around each astrocyte free zone. **B)** Another circle was drawn around the area devoid of nuclear staining. **C)** These two areas were determined, and for each of the seven sections, the astrocyte free zone and cyst cavities were each averaged. These two average areas were then multiplied by the height of the spinal cord in order to determine the volume of the reactive-astrocyte-free area and the cyst cavity. The resultant volume of the reactive-astrocyte-free zone and the cyst cavities were subtracted from each other to obtain volume of viable tissue that was free of astrocytes.





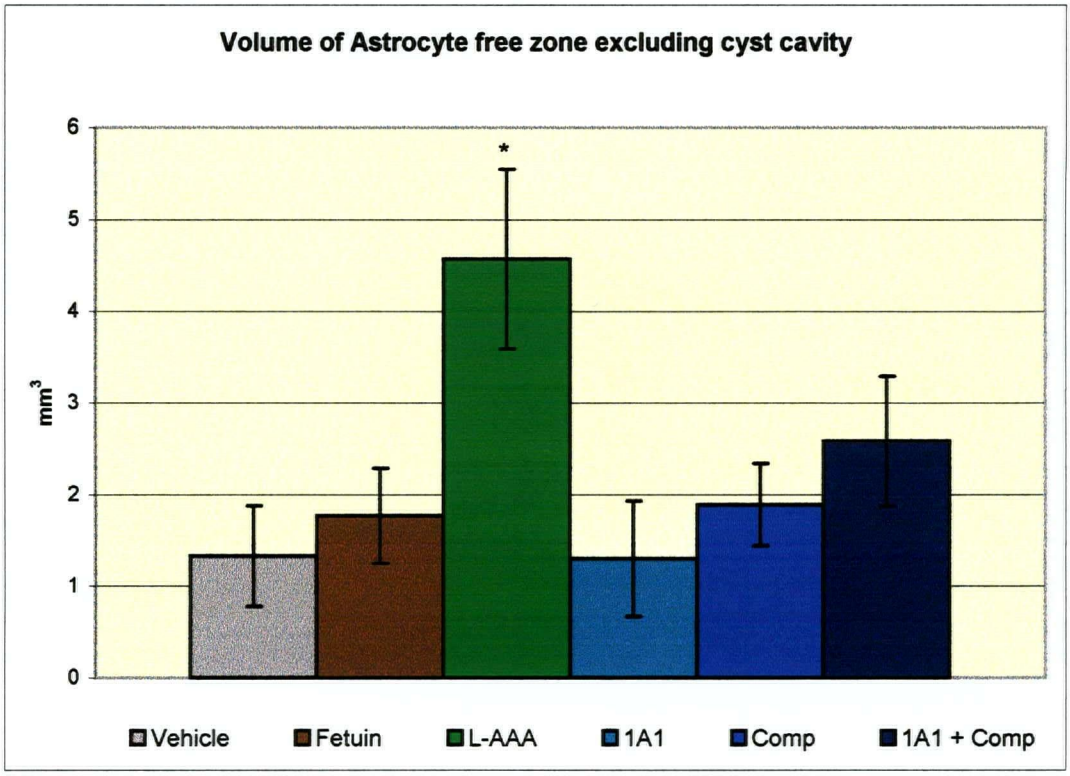
C



volume of reactive astrocyte free zone
= (Volume of A) - (Volume of B)

Figure 8

A chart comparing the volumes of the viable tissue in the reactive astrocyte-free zone of different treatment groups 7 days after infusion using an osmotic pump. The infusion of L-AAA was significantly larger reactive-astrocyte-free zone at the site of infusion than vehicle alone. Values are means ± SEM and * indicates $P < 0.05$ when compared to vehicle treatment alone. Volume is expressed in mm^3.

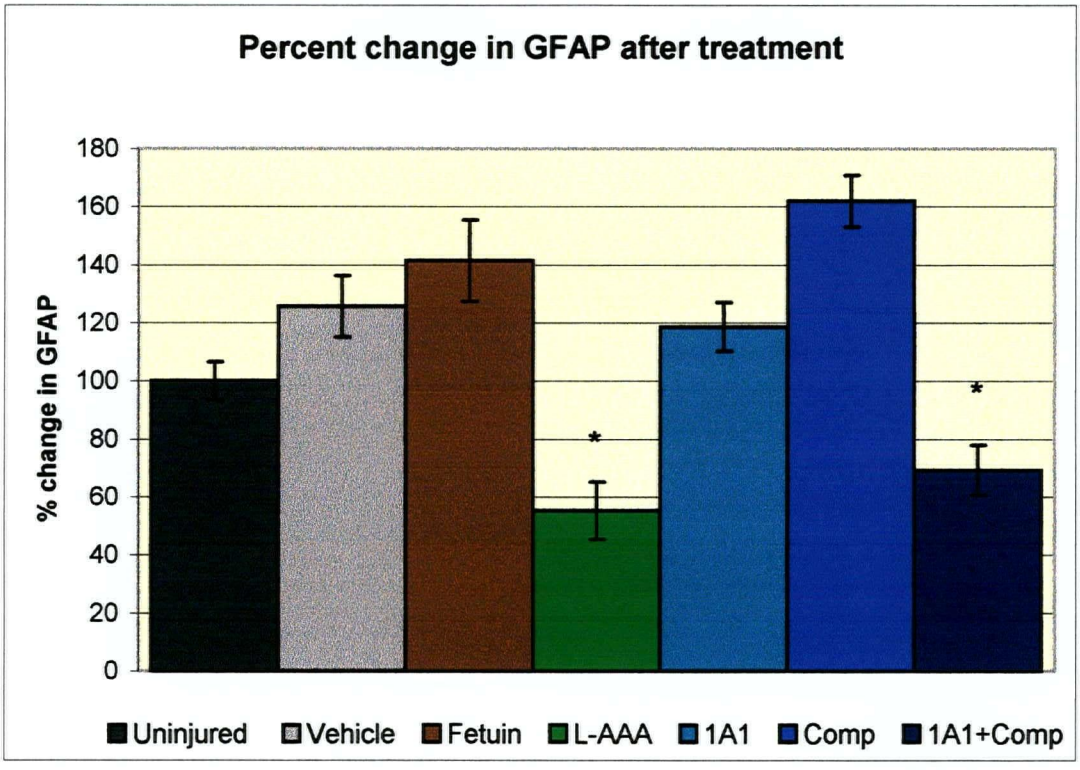

Volume of Astrocyte free zone excluding cyst cavity
6
5
4
3
2
1
0
mm3
*
Vehicle
Fetuin
L-AAA
1A1
Comp
1A1 + Comp

Change in the amount of GFAP after treatments

An ELISA was performed in order to detect changes in the amount GFAP present in the treatment area. This test was used to quantify the changes in GFAP levels present in the spinal cord after various treatments. For comparison, GFAP absorbency was normalized to uninjured spinal cord tissue (Figure 9). When compared to vehicle treatment alone, both L-AAA and 1A1+ complement had reduced levels of GFAP ($p<0.05$) as seen in Figure 9. This corresponded with the volume of the reactive-astrocyte-free space represented in Figure 4. There was more GFAP in the complement treated animals when compared to antibody or vehicle alone treatments ($p< 0.05$). An increase in GFAP expression is well-characterized as a astrogliotic post-injury reaction (Vijayan et al., 1990). It is uncertain whether the increase in GFAP present is a result of a greater number of astrocytes and/or astrocytes increasing their GFAP expression.

Figure 9

A graph of an ELISA comparing levels of GFAP expression one week after the infusion of the treatment into the thoracic spinal cord of the rat. One segment of the spinal cord around the infusion site was removed. The tissue was processed using an enzyme-linked immunosorbant assay (ELISA) which was used to determine the amount of glial fibrillary acidic protein (GFAP) present at the infusion site. All results have been normalized to the uninjured rat spinal cord. After both L-AAA and antibody + complement treatments, there was a significant reduction in GFAP at the infusion site when compared to vehicle infusions alone. All other treatments failed to show a reduction in GFAP when compared to vehicle alone. Values are means ± SEM and * indicates $P < 0.05$ when compared to vehicle treatment alone.


Percent change in GFAP after treatment
% change in GFAP
180
160
140
120
100
80
60
40
20
0
*
*
Uninjured
Vehicle
Fetuin
L-AAA
1A1
Comp
1A1+Comp

Discussion

This thesis has investigated various approaches to preventing the astroglial scar from forming. The treatments used in this series of experiments employed different approaches in an attempt to allow for a better comparison among treatments. The quantification techniques used to measure the reduction of astrocytes all have limitations. Using indirect-immunofluorescence to examine GFAP-IR is informative, but not quantitative. This technique allows one to observe the position and reactivity of the astrocytes. An increase in expression of GFAP can be observed, but the technique cannot be used to determine if the increase is statistically significant. As well, tissue sections in which the different treatment groups are shown in Figure 4 were processed on different slides. These conditions increase the potential for differences in the intensity of the fluorochrome observed.

To overcome the limitations inherent in immunohistochemistry, ELISAs were performed on tissue samples from the spinal cord (Figure 9). In Figure 4G, the antibody and complement treatment showed intense GFAP-IR around the border of the reactive-astrocyte-free zone. The ELISA data (Figure 9) indicates that there is less GFAP present in the treated spinal cord than in the uninjured spinal cord. The spinal cord samples obtained for ELISA could have consisted mainly of the reactive-astrocyte-free zone and cyst cavity. The reduction of GFAP observed after antibody and complement treatment is consistent with this possibility (figure 9).

Quantifying the volume of the reactive-astrocyte-free zone has potential problems. The formation of the cyst cavity is not accounted for in these calculations. This is problematic because if the size of the cavity changes with the differing treatments, results could show that the reactive-astrocyte-free zone was larger without implicating the shift in cavity size (Bunge et al., 1994). To overcome this limitation, the size of the cyst cavity was subtracted from the astrocyte free zone volume. Another limitation of examining the cyst cavity is that the tissue may not maintain its integrity under each set of treatment conditions. In fact, some tissue may be lost during processing. This possibility was reduced since the results were consistent within treatments. The treatments may also cause changes in the inflammatory response of the CNS after the injury and/or proliferation or migration of cells into the treatment area (Fish and Blakemore, 1983). These factors may lead to a misrepresentation of the volume of the reactive-astrocyte-free zone created by the treatment (Olby and Blakemore, 1996). While swelling of the cord may also distort results, this was not investigated since the objective of this study was to determine if any of the treatments reduced or removed astrocytes from the lesion.

Fetuin

The multi-functional polypeptide growth factors TGF-β1 and 2 are significant to inflammatory wound response, astrocyte proliferation, and gliosis. Antibodies which function to block TGF-β1 and TGF-β2 reduce astrogliosis after injury, and TGF-β2 functional blocking antibodies specifically reduce GFAP levels (Logan et al., 1994; Logan et al., 1999). It was hypothesized that using the serum product fetuin, a protein which functionally inhibits the effect of TGF-β1 and 2 *in vitro* (Demetriou et al., 1996), would have a similar effect on the astroglial response

when using TGF-β1 or 2 functional blocking antibodies. However, this was not observed. Infusions of fetuin did not significantly affect the expression of GFAP when compared to vehicle alone (Figure 4 and Figure 9), nor was the size of the reactive-astrocyte-free area significantly different to that observed in the vehicle control animals (Figure 6 and Figure 8). Even though the concentration of fetuin was similar to the activity of the functional blocking antibodies, further concentration changes will be required to reach a more certain explanation (Demetriou et al., 1996; Logan et al., 1994; Logan et al., 1999). Fetuin's biological activity was not assayed in this study, and therefore it cannot be claimed that infusions of fetuin do not inhibit the formation of the astroglial scar.

Astrocytes have been shown to become reactive as a result of increased levels of growth factors and cytokines. It is likely that these factors further instigate a gliotic reaction, and mask any effect a reduction of TGF-β1 and 2 might invoke. Logan and colleagues (1994; 1999) used a stab wound to the cerebral rat cortex, and after antibody treatment, a reduced astrogliotic reaction was observed. The stab wound was created by the insertion of a needle that was removed from the cortex immediately thereafter, as opposed to the wound in this study that was caused by a fixed cannula in the spinal cord. A fixed cannula causes sustained damage that may result in the presence of a higher number of factors associated with injury, whereas a single lesion elicits a reduced number of injury-associated molecules.

Complement mediated lysis

Targeted complement mediated lysis is a technique which has been employed in the Steeves laboratory to remove developing and mature oligodendrocytes (Dyer et al., 1998; Keirstead et al., 1995; Keirstead et al., 1992; Keirstead et al., 1997). A similar technique was used to kill astrocytes around the lesion. In view of the possible combination of an antibody-complement demyelination protocol with astrocyte ablation in future regeneration studies, a serum complement concentration equivalent to that of the demyelination protocol for SD rats was used (Dyer et al., 1998). When compared to vehicle, antibody or complement only treated animals, antibody and complement treatment created a zone free of GFAP-IR cells around the infusion cannula (Figure 4 and 6). When the cyst cavity was accounted for, these results were not significant (Figure 8). These results suggest that the treatment may cause some tissue damage at the infusion site. The higher level of tissue damage may be attributable to the high serum complement concentration and/or one of the roles astrocytes have in the CNS, namely complement deactivation. Astrocytes are known to express complement receptors on their surface (Gasque et al., 1996; Gordon et al., 1992; Gordon et al., 1993; Ischenko et al., 1998; Lacy et al., 1995). The putative role of these receptors is to bind and inactivate excess complement. Serum complement, which is not normally found in the CNS, has the potential to damage nervous tissue if not properly inactivated. It is thought that astrocytes can reduce any deleterious effect complement may have by binding and inactivating it. However, by removing this complement buffer by destroying astrocytes, further damage done by complement could be seen.

The 1A1 antibody has a low binding affinity that necessitated the use of high complement concentrations (Patel and David, 1997). To further develop this technique, the concentrations of complement should be examined along with antibodies that are either IgM or have a higher specificity to the ceruloplasmin antigen. In preliminary studies, it was determined that a single injection was not sufficient to reduce astrogliosis after an injury. A better delivery technique could be developed in order to balance the amount of chemical that can be infused to create a reactive-astrocyte-free zone and limit the size of the cyst cavity. A sub-dural catheter may serve as a potential delivery method.

L-Alpha Amino Adipate (L-AAA)

The ablation of astrocytes by the glial toxin L-AAA in the spinal cord shown in Figure 4 is consistent with similar studies in the amygdala (Khurgel et al., 1996), the substantia nigra (Takada et al., 1990), and the corpus striatum (Takada and Hattori, 1986). Similar results occur when L-AAA is placed in the culture medium of astrocytes *in vitro* (Bridges et al., 1992; Brown and Kretzschmar, 1998; Chang et al., 1997; Huck et al., 1984; Karlsen et al., 1982). After a single injection of L-AAA, it was observed that astrocytes began to repopulate the injection area within four days (Chang et al., 1993). Repopulation was prevented through repeated injections (Chang et al., 1993).

Using L-AAA for further *in vivo* studies has some limitations. Preliminary studies suggested that, in order achieve the desired effect, L-AAA would have to be continually infused into the

spinal cord. Delivery of the chemical would require the use of an osmotic pump. Presently, the nature of this delivery method limits the value of L-AAA for use in stab wound injury models. In addition, the mechanism by which L-AAA is taken up by the astrocytes is still unknown, although there is some evidence to suggest it is accomplished by one of the astrocyte glutamate transporters (Tsai et al., 1996). Despite the above limitations, L-AAA remains a useful tool for examining the role of the astroglial scar in spinal cord injury and regeneration.

Conclusions and Future Studies

Of all the approaches examined to reduce the glial scar, the glial toxin L-AAA and the 1A1 + complement treatments appeared to remove reactive astrocytes from the infusion site. L-AAA is a chemical that is able to exert it glial toxic effect on astrocytes within the CNS. However, there are many questions that are still unanswered about this chemical. The mechanism by which L-AAA enters the astrocyte is still unresolved. It is known that L-AAA enters via a Na^+ mechanism but it has not been determined which glutamate channel it is going through (Huck et al., 1984). This could be examined *in vivo* or *in vitro* using specific glutamate channel inhibitors combined with L-AAA.

A better characterization of the ECM expressed at the glial scar should be examined. Molecules such as CSPG (Davies et al., 1997; Fitch and Silver, 1997; McKeon et al., 1991; Pindzola et al., 1993), tenascin (McKeon et al., 1991; Zhang et al., 1997), fibronectin (Liesi et al., 1984; McKeon et al., 1991; Wang et al., 1997), collagen IV (Stichel et al., 1999) and laminin (Li and David, 1996; Liesi et al., 1984; Logan et al., 1999; Stichel and Muller, 1994) are all reported to

be increased after CNS injury. The effect of ablating or removing GFAP-IR astrocytes on the expression ECMs should be examined.

The use of L-AAA as a method of examining the glial scar and it role in CNS regeneration is warrented. Experiments should be initiated in which specific neuronal tracts are axotomized and L-AAA is applied at the injury site to remove the glial scar. These experiments would use similar neuronal tracing techniques as Keirstead et. al. (1995) in which the treatment and axotomy is combined with double label retrograde neuronal tracing techniques to assess if any injured axons are able to re-grow across the injury site. The severing of specific descending neuronal tracts, such as the rubrospinal or the corticospinal tract, would also be coupled with treatment of L-AAA and anterograde injections into the specific neuronal nuclei. If injured fibres were to grow after treatment, then their route of growth could easily be determined. However these experiments only provide information if removing the glial scar aids in axonal growth. If there is not any axonal growth observed after treatment then the reason for this remains unclear. To overcome these potential problems, a model could be used in which CNS growth is observed after injury.

There are a few treatments in which CNS axonal growth is observed after injury. For example, after CNS axotomy, disruption or removal of myelin in the chick and rat facilitates limited axonal growth after a spinal cord injury (Dyer et al., 1998; Keirstead et al., 1995). Cervically injured rubrospinal tract axons have shown limited growth into the permissive environment of a PN transplant (Kobayashi et al., 1997; Ramon y Cajal, 1928; Richardson et al., 1984). These techniques could be combined with L-AAA treatment in order to examine any changes in

regenerative responses. A decrease in neuronal growth after L-AAA treatment combined with a PN graft or myelin inhibition would indicate that the astroglial scar is providing some beneficial role in promoting a CNS regenerative response. If the opposite is seen, an increase in axonal growth observed after a combined treatment, then there is stronger evidence for the astroglial scar inhibiting axonal regrowth after injury. These studies would give further evidence for the role of the glial scar has in CNS regeneration.

It is still unknown how L-AAA enters the astrocyte and by which channel. These experiments could be accomplished by using specific channel blockers (Gegelashvili and Schousboe, 1997) combined with L-AAA to study the specific uptake route of this chemical. The changes in expression of ECMs associated with the scar after astrocyte ablation is still unknown. For example, after L-AAA treatment to the spinal cord, the changes in expression of ECMs such as CSPG, Laminin and collagen IV could be examined. The astrogliotic scar also has been suggested to be an attempt to reform the BBB after a penetrating injury (Schwab and Bartholdi, 1996). Studies should be considered to examine the speed at which the BBB is able to reform with the application of L-AAA when compared to vehicle injections in order to elucidate the function astrocytes have in reforming the BBB. Finally, L-AAA could be combined with other regeneration or growth techniques to examine the role the astroglial scar has in preventing axonal regeneration.

Using L-AAA to remove astrocytes from the CNS is not only limited to regeneration studies. L-AAA could be used to examine the role astrocytes may have in forming the BBB during development. For example, when the DREZ is forming, it is uncertain if astrocytes are

responsible for keeping Schwann cells out of the CNS (Golding and Cohen, 1997). Once the BBB is formed it is uncertain what role astrocytes have in gating the passage of cells through the BBB (Raivich et al., 1998). These studies would help elucidate the role astrocytes have in the non-pathological CNS.

The ability to remove reactive astrocytes from the CNS in a variety of conditions leads to a variety of potential avenues for research. This research should provide a better understanding of the function astrocytes have in a variety of situations ranging from the formation of the BBB, their role injury induced scar formation and their putative role in CNS regeneration.

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