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CNS Injury, Glial Scars, and Inflammation:

Inhibitory extracellular matrices and regeneration failure

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Abstract

Spinal cord and brain injuries lead to complex cellular and molecular interactions within the central nervous system in an attempt to repair the initial tissue damage. Many studies have illustrated the importance of the glial cell response to injury, and the influences of inflammation and wound healing processes on the overall morbidity and permanent disability that result. The abortive attempts of neuronal regeneration after spinal cord injury are influenced by inflammatory cell activation, reactive astrogliosis and the production of both growth promoting and inhibitory extracellular molecules. Despite the historical perspective that the glial scar was a mechanical barrier to regeneration, inhibitory molecules in the forming scar and methods to overcome them have suggested molecular modification strategies to allow neuronal growth and functional regeneration. Unlike myelin associated inhibitory molecules, which remain at largely static levels before and after central nervous system trauma, inhibitory extracellular matrix molecules are dramatically upregulated during the inflammatory stages after injury providing a window of opportunity for the delivery of candidate therapeutic interventions. While high dose methylprednisolone steroid therapy alone has not proved to be the solution to this difficult clinical problem, other strategies for modulating inflammation and changing the make up of inhibitory molecules in the extracellular matrix are providing robust evidence that rehabilitation after spinal cord and brain injury has the potential to significantly change the outcome for what was once thought to be permanent disability.

Keywords

Astrocytes; glial scar; spinal injury; regeneration; inflammation; proteoglycan; extracellular matrix

INTRODUCTION

Meaningful rehabilitation after spinal cord injury encompasses many aspects of acute and chronic patient care. The underlying basic science of wound healing in the central nervous system (CNS) involves fundamental concepts of cellular structure and function, communication between glial cells and neuronal processes, and complex molecular interactions between cells and their extracellular environments. Understanding the basic functions of glial cells in the brain and spinal cord and their interactions with inflammatory

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cells and injured axons will allow the development of treatment strategies that can promote regeneration after injury (Fitch and Silver, 2007).

The glial cells of the CNS (astrocytes, oligodendrocytes and their precursors, and microglia) supply both structural and physiological support, and also respond to injury or disease. Oligodendrocytes provide myelin sheaths for enhanced axonal transmission (Wood and Bunge, 1984) but they or their progenitors can also remyelinate under certain circumstances (Chang et al., 2000; Nishiyama, 2007; Trotter, 2005; Wilson et al., 2006). Microglial cells are the resident immune system phagocytic cells within the brain and spinal cord (Kim and de Vellis, 2005), and they also respond rapidly for neural protection or healing after injury (Davalos et al., 2005; Wu et al., 2007b). Astrocytes are important for neurotransmitter regulation (Schousboe and Westergaarde, 1995), ion homeostasis (Walz, 1989), blood brain barrier maintenance (Wolburg and Risau, 1995), and the production of extracellular matrix molecules destined for the basal lamina and perineuronal net (Ard et al., 1993; Bernstein et al., 1985; Canning et al., 1996; Grierson et al., 1990; Massey et al., 2006; McKeon et al., 1991; Smith-Thomas et al., 1994; Tom et al., 2004a). After injury, they are also the major cell type responsible for walling off areas of damage to protect the fragile brain tissue from further erosion (Fitch et al., 1999; Myer et al., 2006). The formation of a mechanically obstructive glial scar composed of astrocytes and connective tissue elements was at one time thought to explain the failure of regeneration within the CNS after injury (Windle and Chambers, 1950). However, subsequent work has demonstrated that the glial scar is far more than just a physical barrier, as the role of glial cells in preventing successful regeneration involves complex cellular and molecular interactions (Fitch and Silver, 2001). Astrocytes, oligodendrocyte progenitor cells, microglia, and peripheral inflammatory cells with their proposed roles in the failure of functional regeneration in the CNS will be discussed in this article.

NONPERMISSIVE ENVIRONMENT FOR REGENERATION IN THE CNS

Growth Potential of CNS Neurons

Despite early claims that axons in the adult CNS were incapable of regeneration (Le Gros Clark, 1943), experimental research has demonstrated that CNS axons are capable of at least small amounts of regeneration. Sprouting within the lesion site (Liu and Chambers, 1958), (Guth et al., 1985), and even short distance sprouting from surviving axons (Freund et al., 2006; Goshgarian, 2003; Li and Raisman, 1994; Taylor et al., 2006) have been seen in several different experimental models. However, at the lesion site itself newly formed growth cones quickly become dystrophic (Li and Raisman, 1995; Misgeld and Kerschensteiner, 2006; Misgeld et al., 2007). They do not leave the immediate site of injury and appear incapable, when left to their own devices, of producing any long-distance regeneration.

Recent work has shown that a gradient of inhibitory extracellular matrix molecules can convert an actively extending axonal growth cone into a dystrophic ending that fails to continue long-distance regeneration. These sprouting tips of severed axons then remain stalled in place without further functional growth. Interestingly, these dystrophic growth cones are actually dynamic structures with continuing turnover of membrane components and are extremely dynamic *in vitro* and *in vivo* (Kerschensteiner et al., 2005; Tom et al., 2004b). Time-lapse video of these dystrophic endings illustrates repeated episodes of endocytosis and efforts to send out membrane veils (Tom, et al., 2004b). The failure of these dystrophic growth cones to continue growing to produce long-distance regeneration appears to be related to the inhibitory and nonpermissive nature of the injured brain and spinal cord.

In contrast to the CNS, the peripheral nervous system (PNS) is growth supporting for regeneration, especially after a crush injury (Lazar et al., 2004). Segments of peripheral nerves with the associated PNS environment can even encourage remarkable long-distance

regeneration of many types of CNS axons when such grafts are inserted centrally (David and Aguayo, 1981; Richardson et al., 1980). These classic transplantation experiments proved that adult CNS axons do have an intrinsic capacity for lengthy regeneration, albeit perhaps less than optimal growth (see article by Filbin, *et al.*, in this issue). However, the vast majority of regenerating axons fail to continue upon re-entering the CNS environment, which further demonstrates the inhibitory and nonpermissive nature of the CNS. The injury responses of the two branches of the sensory dorsal root ganglion (DRG) neuron further demonstrate the dichotomy between CNS and PNS regeneration, as successful long-distance regeneration easily occurs after injury to the peripheral branch but does not occur with attempted growth of the central branch into the CNS (Golding et al., 1997; Kliot et al., 1990).

Molecules Within the Glial Scar Contribute to Regenerative Failure

The formation of a histologically apparent glial scar, composed of astrocytes and connective tissue elements, is one of the most studied but poorly understood barriers to regeneration of CNS axons. Traditionally, thought to be a simple mechanical barrier (Windle and Chambers, 1950), later studies suggested that regeneration still fails even when a dense glial scar does not form (Guth et al., 1986). Multiple models have now demonstrated that the molecular composition of the scar and the production of inhibitory molecules by astrocytes are contributing factors for regenerative failure (Busch and Silver, 2007; Fawcett, 2006; Fitch and Silver, 1997a; Fitch and Silver, 2000; Liu et al., 2006; McGraw et al., 2001; Silver and Miller, 2004; Yiu and He, 2006; Zhang et al., 2006). Reactive astrocytes within the glial scar have been shown to upregulate molecules such as tenascin (Apostolova et al., 2006; Brodkey et al., 1995; McKeon et al., 1995), Semaphorin 3 (Pasterkamp et al., 2001), ephrin-B2 (Bundesen et al., 2003), slit proteins (Hagino et al., 2003), and a host of chondroitin sulfate proteoglycans (Jones et al., 2003a; McKeon, et al., 1995; Rhodes and Fawcett, 2004). While not all of these candidates have been fully characterized in terms of their contributions to regenerative failure, a substantial body of work has been accumulated to illustrate the inhibitory effects of scar formation within the CNS. Compelling evidence that the lesion environment itself is critical for CNS regenerative failure came from experiments where adult dorsal root ganglion neurons were micro-transplanted into undamaged or pre-lesioned white matter tracts (Davies et al., 1997; Davies et al., 1999). This careful micro-transplantation avoids a vigorous inflammatory response, which allows dorsal root ganglion neurons immediate access either to a relatively intact host glial environment or one that is undergoing Wallerian degeneration. These sensory axons regenerate rapidly and over long-distances within adult white matter tracts of the brain and spinal cord. As the rapidly growing adult neurons reach an area of CNS damage, with associated inflammatory infiltrates and inhibitory molecules, the axons convert into a dystrophic state and are unable to continue (Davies, et al., 1999). These findings highlight the importance of the glial scar in the creation of an inhibitory environment for unsuccessful regeneration within the CNS.

Increases in proteoglycan molecules have been found in the CNS in many different injury models. The NG2 proteoglycan is increased after CNS injury by oligodendrocyte precursor cells (Levine, 1994; Rhodes et al., 2006), phosphacan is upregulated within glial scars (McKeon, et al., 1995), and phosphacan, brevican, versican, and neurocan are all produced within the injured spinal cord (Jones, et al., 2003a). Chondroitin sulfate proteoglycans are found in the brain after stab wound (Fitch and Silver, 1997a), in the spinal cord after injury to the dorsal root (Pindzola et al., 1993), and in the spinal cord following injury (Fitch and Silver, 1997a; Jones, et al., 2003a; Jones et al., 2003b). The rapid and long lasting upregulation of proteoglycans within the vicinity of the glial scar has implicated them in the creation of nonpermissive growth environments in the CNS, similar to their role in boundary formation within the CNS during development (Fitch and Silver, 1997b; Grimpe and Silver, 2004; Silver, 1994; Snow et al., 1990). It is now well established that proteoglycans associated with reactive

astrocytes clearly inhibit neurite outgrowth *in vitro* (Bovolenta et al., 1993; Canning et al., 1993; Dou and Levine, 1994; McKeon, et al., 1991; Snow et al., 1990; Tom, et al., 2004b), and these molecules also appear to play a key role in creating an environment that is not appropriate for successful long-distance regeneration of adult neurons after injury *in vivo*, since their modification allows successful regeneration to occur (Bradbury et al., 2002; Houle et al., 2006; Steinmetz et al., 2005).

TRIGGERS FOR THE PRODUCTION OF INHIBITORY EXTRACELLULAR MATRIX

Unlike myelin-associated inhibitory molecules, which are present at relatively static levels before and after CNS injury (see Zheng, *et al.*, in this issue), inhibitory matrices such as proteoglycans are upregulated in areas of gliosis following traumatic injuries in the brain and spinal cord. They are typically enhanced in regions of BBB breakdown (Fitch and Silver, 1997a; Rhodes, et al., 2006), but new evidence also suggests they are upregulated in the perineuronal net in areas distant from the lesion in synaptic centers denervated by the injury (Massey, et al., 2006). In order to appropriately manipulate the extracellular matrix, we must first consider those triggers that initiate the increased production of inhibitory molecules.

The cellular hypertrophy and increases in glial fibrillary acidic protein (GFAP) that are the hallmarks of astrocyte reactivity occur rapidly after CNS injury. While the specific triggers remain unclear, there are a number of hypotheses about factors that contribute to astrocyte activation and gliosis. Degeneration of severed axon tracts appears to lead to gliosis, even in regions remote from the site of trauma in the brain or spinal cord (Barrett et al., 1981; Fitch and Silver, 1997a; Massey, et al., 2006; Murray et al., 1990; Steward and Trimmer, 1997). Cytokines or other molecules that may trigger gliosis include TNF-alpha (Rostworowski et al., 1997), endothelin-1 (Hama et al., 1997), IL-1 (Giulian and Lachman, 1985), IL-6 (Chiang et al., 1994), thrombin (Nishino et al., 1993), and CNTF (Kahn et al., 1995). Some of these may originate as soluble serum factors, or they can be directly produced by astrocytes, activated microglia, or peripheral macrophages. Oligodendrocyte precursor cells also become activated in response to similar triggers such as platelets, macrophages, and inflammation-associated cytokines (Rhodes, et al., 2006).

Traditional definitions of astrocyte activation are based on morphology, numbers, and increases in GFAP expression and/or detection. However, it is perhaps more important to consider which triggers lead not only to "gliosis" but to the production of inhibitory molecules such as proteoglycans that create a nonpermissive environment for axons attempting to regenerate. Some have suggested that degenerating axons and their dying terminals may be a trigger for such extracellular matrix production by astrocytes (see Massey, *et al.*, in this issue). Evidence also suggests that the inflammatory infiltration and activation associated with CNS injury is associated with production of inhibitory molecules, as CSPGs are associated with breakdown of the blood brain barrier and infiltrating macrophages present within a lesion site (Fitch, et al., 1999; Fitch and Silver, 1997a), a phenomenon that is also seen with activation of oligodendrocyte precursor cells after injury (Rhodes, et al., 2006). Pro-inflammatory molecules that activate microglial cells and macrophages can trigger astrocyte gliosis and upregulation of proteoglycans (Fitch, et al., 1999), upregulation of NG2 proteoglycan by oligodendrocyte precursor cells (Rhodes, et al., 2006), and cytokines associated with inflammation can also influence astrocyte extracellular matrix production (DiProspero et al., 1997).

It is important to consider inflammation within the CNS as a potential source of cytokines and other signaling molecules that can lead to upregulation of inhibitory molecules after an injury. Microglial cells from the CNS and activated macrophages from the periphery both respond to trauma in the brain and spinal cord, and this inflammatory response may contribute to secondary tissue damage after the primary insult (Blight, 1994; Fitch, et al., 1999). This cascade

of secondary damage, progressive cavitation, and glial scarring was demonstrated in an *in vivo* model of inflammation using microinjection of zymosan, a non-toxic, specific phagocytic activator of the macrophage mannose receptor and the beta-glucan site of the CR3 integrin receptor (Fitch, et al., 1999). The resulting inflammation leads to significant axotomy and increases in astrocyte cavity size, while control injections of latex microspheres do not induce this damaging inflammatory cascade. Upregulation of inhibitory proteoglycans is also found at the interfaces between developing cavities and surrounding reactive astrocytes (Fitch and Silver, 1997a; MacLaren, 1996) further demonstrating the association of inflammation with upregulation of these molecules (Fitch, et al., 1999). The biological function of these inhibitory molecules when surrounding necrotic cavitation is unknown, but it is possible that these proteoglycans may be protective for the surrounding viable tissue to prevent further damage from the cavity progression that results from ongoing inflammatory processes (Fitch and Silver, 2007; Myer, et al., 2006).

Interestingly, inflammation within the CNS has been suggested to provide neuroprotective benefits during the healing process of the brain and spinal cord depending on the activation state of the cells (Klusman and Schwab, 1997; Lotan and Schwartz, 1994; Schwartz and Yoles, 2006). In addition to pro-inflammatory cytokines, macrophages can secrete factors that are growth promoting, such as NGF and NT-3 (Elkabes et al., 1996), thrombospondin (Chamak et al., 1994), and IL-1 (Giulian et al., 1994). Even zymosan, a trigger of robust inflammation, when placed in the vitreous chamber of the eye can stimulate regenerating optic nerve fibers (Leon et al., 2000) and when placed into the DRG before root crush appears essential to the success of DRG regeneration into the spinal cord when combined with modification of the extracellular matrix in the CNS compartment (Steinmetz, et al., 2005). Apparently intense inflammatory states created just outside of the CNS illustrate that in certain circumstances inflammation can promote enhanced regeneration by triggering a conditioning-like effect within the neuron (Calvo et al., 2005; Lund et al., 2002; McQuarrie and Jacob, 1991; Wu et al., 2007a). However, fulminant inflammation centrally tends to create cavities (Fitch, et al., 1999) and it is unclear whether further increasing inflammatory activity, even after attempting to modify immune cell activity, will lead to the desired functional regeneration within the brain and spinal cord (Kigerl et al., 2007; Kigerl et al., 2006; Lazarov-Spiegler et al., 1996; Schwartz and Yoles, 2006).

MODIFICATION OF **E**XTRACELLULAR **M**ATRICES TO **E**NHANCE **R**EGENERATION

Strategies designed to enhance clinical outcomes and rehabilitation after spinal cord injury continues to be a topic of great interest, in particular because no treatment protocols with proven efficacy are currently in clinical use. While there was once great interest in the use of high-dose methylprednisolone for spinal cord injury (Bracken et al., 1990; Bracken et al., 1992), methodological problems with these studies and controversies surrounding subsequent trials have led to criticism and discontent with the routine use of this treatment strategy (Coleman et al., 2000; Hurlbert, 2001). Steroids were also previously thought to be helpful for patients with clinically significant head injuries until the recent randomized CRASH trial with over 10,000 enrolled patients demonstrated increased mortality when using methylprednisolone (Roberts et al., 2004). Modification of inflammatory processes and the resulting inhibitory extracellular matrices following trauma are likely to be important for improving clinical outcomes, but it appears that steroid treatment alone is not sufficient to provide reproducible and meaningful clinical recovery in human patients for spinal cord or brain injury (George et al., 1995; Pointillart et al., 2000; Prendergast et al., 1994; Roberts, et al., 2004; Tsutsumi et al., 2006).

Some other strategies for encouraging CNS neurons to regenerate are to increase local levels of growth factors in an effort to stimulate axonal growth. Genetically engineered fibroblasts

secreting NGF can encourage axons to regenerate into the center of an injury when implanted in this region (Jones, et al., 2003b; Kawaja and Gage, 1991; Tuszynski et al., 1997), although this sprouting remains within the area of trophic support and does not lead to long distance functional regeneration. Intrathecally delivered NT-3, NGF, and GDNF can promote axonal regeneration across the dorsal root entry zone (Ramer et al., 2002; Ramer et al., 2000), and glial overexpression of NGF can stimulate regeneration of nociceptive axons from the dorsal roots which in some circumstances may lead to thermal hyperalgesia (Romero et al., 2000; Tang et al., 2004). Combination therapy such as treatment with NT-3 and cAMP together to stimulate neuronal cell bodies has been shown to allow regeneration beyond the lesion site of spinal injuries (Lu et al., 2004). The use of multiple simultaneous approaches to this complex problem may prove important when constructing future strategies for improving rehabilitation and functional recovery.

One important component to consider including in such combination approaches to therapy is modification of the inhibitory extracellular matrix molecules produced after injury. *In vitro* experiments have demonstrated that proteoglycans associated with reactive astrocytes can be modified to allow increased growth of axons by removing or preventing the production of sugar epitopes on the proteoglycan molecules (Bradbury, et al., 2002; Grimpe and Silver, 2004; McKeon, et al., 1995; McKeon, et al., 1991; Steinmetz, et al., 2005), and an *in vivo* transgenic model to express a chondroitin-sulfate proteoglycan degrading enzyme in astrocytes demonstrates a local efficacy for reductions in proteoglycans to enhance CNS axon growth after injury (Cafferty et al., 2007). Neutralizing a heparan/chondroitin sulphate proteoglycan expressed after brain injury with a blocking antibody will allow neurite outgrowth and prevent growth cone collapse *in vitro* (Bovolenta et al., 1997). Injured corticospinal fibers and uninjured serotonergic fibers increase sprouting after enzymatic treatment to remove sugar epitopes (Barritt et al., 2006), and *in vivo* studies have revealed remarkable long-distance regeneration of adult axons through CNS white matter tracts after enzymatic treatment to digest these proteoglycan side chains (Steinmetz, et al., 2005). The dorsal root entry zone is an important transition between the PNS and CNS where regenerating axons abruptly stop and fail to enter the CNS (Golding, et al., 1997; Klot, et al., 1990; Perkins et al., 1980; Tower, 1931). This inhibitory interface can be overcome with a combination strategy that utilizes zymosan, a specific inflammatory activator of macrophages, and enzymatic digestion of inhibitory proteoglycans with chondroitinase ABC (Steinmetz, et al., 2005). Neither of these treatments alone were successful *in vitro* or *in vivo*, but together they led to robust regeneration of nerve fibers with electrophysiologically proven synapse formation. This is another example of a promising combination therapy, where a specific inflammatory environment is created outside the CNS using macrophage activators and the extracellular matrix in the CNS is modified to counteract the associated proteoglycan inhibitors.

Other recent therapeutic approaches have used the concept of combining different approaches to promote regeneration. Using an antibody to the NG2 proteoglycan along with a conditioning lesion to increase axon growth has recently been shown to enhance the regeneration of sensory fibers in the dorsal columns (Tan et al., 2006). A different combination strategy has resulted in functional regeneration of motor abilities when extracellular inhibitors are modified along with the use of PNS nerve bridges. Combining PNS nerve bridges with chondroitinase ABC to digest the sugar epitopes of chondroitin sulfate proteoglycans allows regenerating axons to bypass the spinal cord lesion and also allows axonal exodus from the conduit (Houle, et al., 2006). This results in reformation of functional synaptic connections with denervated interneurons and motor neurons below the level of the lesion. These animals had improved forelimb swing during locomotion, enhanced ability to use the forelimbs, and improved performance on a horizontal rope. All of these behavioral gains were diminished after transection of the PNS nerve bridge (Houle, et al., 2006). These findings emphasize that modification of inhibitory astrocyte molecules associated with certain inflammatory states after

CNS injury are important considerations for combination strategies in spinal cord injury and rehabilitation.

CONCLUSION

Efforts are ongoing to further elucidate the critical components of CNS injury responses that prevent meaningful regeneration of injured nerve tracts. A key aspect of this response is the production of inhibitory extracellular matrix molecules via complex interactions between glial cells and inflammatory infiltrates, and the subsequent inhibitory effects on growing axons that leads to abortive regeneration and the formation of dystrophic neurons (see Figure 1). Recent work highlights the importance of a combination approach to therapy, and a successful strategy to improve patient rehabilitation after spinal injury will need to incorporate modification of inflammatory states, neutralization of inhibitory extracellular molecules, and stimulation of growth promoting factors by a variety of approaches.

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Figure 1. Wound healing, secondary damage, and abortive regeneration in the central nervous system

The lesion cavity of a central nervous system injury expands as inflammatory cells interact with the surrounding reactive astrocytes and other reactive glial cells. This region of glial scarring is associated with upregulation of inhibitory extracellular matrix molecules, such as proteoglycans, that are distributed in an increasing concentration gradient from the lesion penumbra to the lesion center. This intense inflammatory response leads to a cascade of secondary damage to axons initially spared from direct trauma, and demyelination of adjacent axons that are not readily re-myelinated by adult oligodendrocytes and precursor cells. The gradient of inhibitory molecules upregulated in the areas of intense inflammation provides an environment that is nonpermissive for regeneration, and dystrophic neurons develop the classically-described sterile end-balls with clubbed endings that are characteristic of abortive attempts at regeneration.