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Topical Glucocorticoids Modulate the Lesion Interface After Cerebral Cortical Stab Wounds in Adult Rats.

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A lesion interface consisting of a glia limitans lined by a laminin-rich basal lamina forms within 3 weeks after leptomeningeal cells infiltrate into penetrating wounds in the adult mammalian CNS. This impedes axon regrowth. We have examined the effects of steroids on the formation of the lesion interface in the adult rat cerebral cortex. Topical glucocorticoids were applied on the surface of cortex encompassing stab wounds. Three weeks later, cryostat sections through the lesioned area were labeled with anti-laminin, anti-GFAP, anti-ED-1 and Nuclear Yellow. Steroid treatment attenuated all components of the lesion, including leptomeningeal cell infiltration. In vitro, steroid treatment did not alter laminin secretion but reduced cell proliferation in leptomeningeal cultures. These results suggest that steroids modulate lesion interface formation in the CNS in part by decreasing leptomeningeal cell proliferation.

Résumé

Chez les mammifères adultes, une lésion qui consistent en lame basale, et glia limitans forme après que les cellules provenant des leptomeninges (pie-mère et arachnoïde) pénetrent une déchirure dans le parenchyme du système nerveux centrale. Celle-ci empeche la régénerescence des axones. Nous avons étudié les effets d'un traitement de steroïde topique sur la guérison des plaies, dans le parenchyme cérébrale chez les rats mures. Nous avons créer des plaies dans les cerveaux des rats avec les lames de razoir. Trois semaines plus tard, nous avons trancher à travers les lésions en coupes sagittales minces dans le cryostat et nous les avons incubées avec des anti-corps contre le GFAP, le ED1 et le Nuclear Yellow. Les traitements ont diminué la formation de toutes les composantes de la plaie, surtout l'infiltration des cellules provenant des leptomeninges. Dans les épreuves où nous avons ajouté des glucocorticoïdes aux cultures de leptomeninges, nous avons constaté une diminution dans la prolifération des cellules mais non dans la production de laminin. Ces résultats nous mène à conclure que les glucocorticoïdes peuvent diminuer la formation d'une plaie en réduisant la multiplication des cellules provenant des leptomeninges.

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Publications resulting from my work while in the M.Sc. program at McGill University.

Li, M., David, S. (1996) Topical glucocorticoids modulate lesion interface formation after cerebral cortical stab wounds. Glia 18:306-318.

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ABSTRACTS

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PRIZES

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ANOVA	Analysis of variance
bFGF	Basic fibroblast growth factor
Ca^{+2}	Calcium ion
^oC	Degrees Celsius
CNS	Central Nervous System
CS-6-PG	Chondroitin sulfate-6-proteoglycan
DMEM	Dulbecco's modified Eagle's medium
dpl	Day post-lesioning
ECM	Extracellular matrix molecule
ED-1	Monoclonal antibody recognizing rat macrophages
e.g.	For example
EHS	Engelbreth-Holm-Swarm
EM	Electron microscopy
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
g	Grams
GFAP	Glial fibrillary acidic protein
^3H-	Tritiated
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
i.e.	That is
IgG	Immunoglobulin G
IL	Interleukin
kDa	Kilodaltons
kg	Kilograms
M	Molar
MAG	Myelin-associated glycoprotein
MEM	Minimal Eagle's medium
mg	Milligrams
Mg^{+2}	Magnesium ion
mm	Millimeters
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NGF	Nerve growth factor

NT-3	Neurotrophin-3
6-OHDA	6-hydroxydopamine
PDGF	Platelet derived growth factor
pH	Hydrogen ion potential
PNS	Peripheral nervous system
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TGF-ß	Transforming growth factor-ß
TNF	Tumour necrosis factor
µCi	Microcurie
µg	Microgram
µm	Micrometer
µl	Microliter

INTRODUCTION

The adult mammalian central nervous system (CNS) does not favor the regrowth of neurons. Cajal (Cajal, 1928) noted abortive regeneration at sites of CNS lesioning and theorized that failure of axonal regeneration in the adult CNS lies with a deficiency of trophic and tropic substances in the environment. The possibility of axonal regeneration in the adult mammalian central nervous system was demonstrated by placing peripheral nerve grafts into CNS lesions (David and Aguayo, 1981; Richardson et al., 1980; Tello, 1911). Many studies in the past 15 years have revealed that neurons in most regions of the adult mammalian CNS can regenerate given a permissive environment (Bray et al., 1987). Mature CNS neurons can grow long distances in grafts but stop soon after they encounter CNS tissue (Carter et al., 1989; David, et al., 1981; Richardson, et al., 1980; Vidal-Sanz et al., 1987; Cheng et al., 1996). In contrast, neonatal CNS tissue is able to support axon regrowth after corticospinal lesions (Kalil and Reh, 1979) or embryonic CNS grafts (Bregman et al., 1989; Iwashita et al., 1994). Therefore developmental changes in the non-neuronal CNS environment is detrimental to successful axon regrowth. Other experiments indicate that introduction into the spinal cord of purified cultured Schwann cells (Paino et al., 1994) or olfactory bulb ensheathing glia (Li and Raisman, 1994) increased axonal sprouting and regrowth. Attempts to characterize growth permissive or inhibitory components of the neuronal environment has resulted in valuable insights into the study of neuroregeneration. Briefly, the roles of oligodendrocytes, reactive astrocytes, and the

lesion interface in preventing CNS regeneration will be reviewed. Emphasis will be placed on the roles of the non-neuronal cells, particularly leptomeningeal cells, in forming the lesion interface, and on ways of modulating that environment. Steroids, used to reduce scar formation elsewhere in the body, are suggested as a vehicle to attenuate lesion interface formation in the CNS.

Oligodendrocytes and regeneration

The identification of myelin-associated molecules that are inhibitory to axon growth constitute important discoveries in the field of regeneration. These molecules were purified from extracts of myelin preparations that were found to inhibit neurite outgrowth in vitro. Their roles in preventing regeneration in vivo are being evaluated in ongoing experiments. The molecular identity of the most well-known inhibitor is still elusive however myelin-associated glycoprotein has been identified as the second major inhibitor.

Mature oligodendrocytes and CNS myelin are non-permissive substrates for neuron and fibroblast adhesion and spreading, and neurite growth in vitro (Schwab and Caroni, 1988). These inhibitory effects were found to be due to two immunogenically related proteins with molecular weights of 250 and 35 kDa (Caroni and Schwab, 1988). Treatment of young adult rats with corticospinal tract lesions with monoclonal antibody (IN-1) against this inhibitor resulted in increased axon sprouting at the lesion site and axon growth of up to 11 mm past the lesion site (Schnell and Schwab, 1990). When treatment with the IN-1 monoclonal antibody was combined with growth factor treatment, NT-3 in particular, axon growth of up to 20.3 mm could be

seen (Schnell et al., 1994). More recent experiments document some functional recovery as measured by improvements in specific reflex and locomotor functions after spinal cord injury (Bregman et al., 1995). However, in these experiments, less than 10% of the axons grow past the lesion site and those that grew, bypassed the lesion by detouring around the lesion edge rather than penetrating through the interface. This indicates that the lesion interface itself serves as a significant barrier to growing axons.

Myelin-associated glycoprotein (MAG) was also shown to have potent neurite growth inhibitory properties in culture (McKerracher et al., 1994). Immunodepletion of MAG from bovine CNS myelin increased neurite growth by 63%, and recombinant MAG also had potent neurite growth inhibitory activity in vitro (McKerracher et al., 1994), including growth cone collapse (Li et al., 1996). Experiments in MAG mutants revealed reduced growth inhibition and some limited corticospinal axon regeneration after lesioning as compared to wild type mice (Li et al., 1996). A function-blocking monoclonal antibody for MAG is being developed to allow further assessment of the importance of MAG as an inhibitor in vivo. Meanwhile the significance of MAG in vivo is suggested by improved peripheral nerve regeneration in MAG mutants crossbred with Ola mutants (Schafer et al., 1995). Peripheral nerve regeneration is markedly reduced in Ola mice. This reduction in regeneration is due to the poor clearance of myelin after injury. Interestingly, peripheral nerve myelin lacks the IN-1 inhibitor but contains MAG.

Another molecule whose expression is correlated with myelination is janusin (Bartsch et al., 1993). This glycoprotein is

found only in the CNS where it is secreted by differentiated oligodendrocytes and inhibits both neuron cell attachment and neurite outgrowth on laminin (Pesheva et al., 1993). Janusin copurifies with F3/11, a neuronal cell surface molecule and antibodies against F3/11 reverse the neurite growth inhibitory effects of janusin in vitro (Pesheva et al., 1993).

Axon growth inhibitory molecules are probably important for axon guidance in development. Postnatally in rats, IN-1 expressing oligodendrocytes appears first in the ascending dorsal columns. These IN-1+ pathways likely serve as "guardrails" to channel the later arriving descending corticospinal tract fibers. Elimination of the inhibitors by X-irradiation or application of the IN-1 antibody cause aberrant intermixing of corticospinal and sensory tracts (Schnell and Schwab, 1991). Inhibitory molecules associated with mature oligodendrocytes and myelin may serve in axon guidance in development, but after trauma, these molecules may have important inhibitory effects on axon regeneration. It is possible that the myelin debris left at the lesion site after CNS injury may prevent axon regrowth across the lesion site. However neutralization of myelin associated inhibitory molecules does not enable axons to cross the lesion site after CNS lesioning. Therefore the formation of a mature interface at the site of CNS wounds is likely to play an important role in impeding axon regeneration.

Reactive astrocytes in CNS regeneration

Astrocytes are another group of cells that are present at the site of CNS lesions. There are conflicting reports about the role of

astrocytes as to whether they are permissive or nonpermissive substrates for axon regeneration. A key factor in understanding the contribution of astrocytes as supporting cells is to recognize that they are a heterogeneous group of cells.

Astrocytes undergo many changes after CNS injury. Reactive changes include increased expression of GFAP (Bignami and Dahl, 1976) mitosis, hypertrophy and proliferation of cytoplasmic processes (Lindsay, 1986; Malhotra et al., 1990). Astrocytes can also express extracellular matrix molecules (Reichardt et al., 1989) and produce trophic factors (David, 1992; Eddleston and Mucke, 1993).

In the past, reactive astrocytes were thought to inhibit axon regeneration (Reier et al., 1983b) and therefore studies were done to elucidate the etiology behind the morphological changes in astrocytes after injury. Microglial cell infiltration and IL-1 secretion in stab wounds of the cerebral cortex were closely related temporally with a peak at two days which is then followed by reactive astrogliosis (Giulian et al., 1989). IL-1 (Giulian et al., 1988) and gamma-IFN (Yong et al., 1991) in the adult rodent, and several other cytokines in the neonatal mouse (Balasingham et al., 1994) have been implicated in the development of reactive astrocytes. Microglia were later found to secrete a yet unidentified neurotoxin in culture whereas astroglia produced growth promoting factors (Giulian and Corpuz, 1993). Thus astrogliosis may be a positive response to molecules injurious to neurons.

Recent work indicates however that not all reactive astrocytes have similar characteristics after CNS injury. Astrocytes are heterogeneous in their GFAP immunoreactivity, their proliferation,

their expression of ECM molecules and their abilities to promote neurite growth after injury depending on the type of injury, time course, and location relative to the lesion site (David, 1992). Astrocytes near the lesioned area but not comprising the lesion interface seem to be permissive for axon growth. Reactive astrocytes or astrocyte precursors from kainic acid lesioned adult rat striatum or surgical lesions of the cortex permit axon growth (Lindsay, 1979; Lindsay et al., 1982). In addition, astrocyte monolayers made reactive in culture by treatment with macrophage conditioned medium, or IL-1 beta still support excellent neurite growth (Miller et al., 1994) Similarly, frozen sections of transected, predegenerated adult rat optic nerve provide excellent substrates for neurite growth (David et al., 1990). These effects may be due to neurotrophic factors (David, 1992, Eddleston and Mucke, 1993) or adhesion molecules (Reichardt, et al., 1989) expressed by astrocytes.

While the above two-dimensional astrocyte conformations allowed neurite growth, a three-dimensional array of astrocytes may impede axon growth. Long-term cultured astrocytes grafted between cut ends of sciatic nerve, did not support axon growth in vivo (Kalderon, 1988) nor did tubes filled with cultured astrocytes in vitro (Fawcett et al., 1989). However, given that the growing axons in these experiments were ensheathed by Schwann cells, it is not clear whether the avoidance of the astrocyte filled tubes is due to the inhibitory properties of astrocytes or to an increased preference of the growing axons for Schwann cells. The latter cells are known to express a number of diffusable (Collier and Martin, 1993) as well as

cell surface and ECM molecules that can promote axon growth (Martini, 1994).

Sprouting has been associated with reactive astrocytes near the injury site in the septohippocampal system (Gage et al., 1988). The promotion of neurite growth by astrocytes in vitro (Manthorpe et al., 1983) and after injury in vivo (Liesi et al., 1984) was thought to be due to laminin. However there is evidence that laminin may not be solely responsible for the permissiveness of neurite growth on astrocytes (David, 1988). Laminin may be important but not essential for axon sprouting after lesioning as shown in adult rat optic nerve. Sprouting was found to colocalize to GFAP+ but laminin negative areas at the lesion interface (Giftochristos and David, 1988).

The lesion interface is composed of a glia limitans, basal lamina and meningeal cells (Reier et al., 1989). The fine interdigitating network of intensely GFAP+ astrocytes seen at the interface may be distinct from astrocytes farther away from the lesion by virtue of their proximity to leptomeningeal cells. One distinguishing feature is their ECM secretion. While astrocytes do not secrete laminin, fibronectin or type I collagen upon contact with intact or degenerating axons (Ard et al., 1993), a basal lamina immunoreactive for laminin and fibronectin is formed when astrocytes contact leptomeningeal cells in culture (Abnet et al., 1991). Another adhesion molecule, tenascin, is secreted at the interface between astrocytes and meningeal cells in culture and in cut end of transected optic nerve in the first two weeks after lesioning (Ajemian et al., 1994).

In addition to the changes in the expression of various ECM molecules, the ability of astrocytes to support neurite growth is

affected upon contact with leptomeningeal cells. In contrast to astrocytes, fibroblasts and leptomeningeal cells have been shown to be very poor substrates for neurite growth (Fallon, 1985; Noble et al., 1984). However when neurons are cultured on astrocytes plated on a monolayer of leptomeninges, neurite growth on astrocytes is reduced by 40% compared to neurons cultured on astrocytes alone (Ness and David, 1997). Rudge and Silver (Rudge and Silver, 1990) have also reported that neurite growth on reactive astrocytes harvested from the wall of the lesion onto nitrocellulose implants placed into adult rat cerebral cortex was poor. Since these cells are harvested from the wall of the lesion, the astrocytes are likely to be overlying a layer of meningeal cells. These observations suggest that astrocytes lining the lesion interface (i.e. comprising the glia limitans) are likely to be nonpermissive for axon growth.

These astrocytes have been shown to express tenascin and chondroitin-sulfate proteoglycans, chondroitin-6-sulfate proteoglycan in particular, which are molecules felt to confer the growth-inhibitory phenotype (McKeon et. al., 1991). In vivo, these astrocytes are typically found at boundaries during CNS development (Snow et al., 1990; Brittis et al., 1992; Pindzola et al., 1993) and at the site of CNS injury (Ajemian and David, 1991; Laywell et al., 1992), where they would be close to a basal lamina. Astrocytes alone can be isolated in culture however and still retain similar functional and antigenic properties (Groves et al., 1993; Meiners et al., 1995). These molecules may influence cell migration (Gates et. al., 1995) but chondroitin sulfate proteoglycan alone or with tenascin does not inhibit the sprouting of myelinated fibers after injury (Lips et al., 1995) or

correlate with growth cone collapsing activity (Fok-Seang et. al., 1995). This suggests that the local glial-pial interaction may provide molecular signals inducing the astrocytes to express inhibitory molecules limiting axon growth. Basic FGF, which is found in wounds (Finkelstein et al., 1988), upregulates tenascin and CS-6-PG on cultures of neonatal astrocytes (Meiners et al., 1993, 1995). When the interface is absent, axon growth can occur as when pioneer olfactory axons enter the brain where there are breaks in the laminin-positive meninges on the surface of the olfactory bulb primordium (Gong and Shipley, 1996) or when axons enter the dorsal root entry zone before the maturation of the glial dome (Pindzola et al., 1993).

Astrocytes as well as oligodendrocytes thus have roles in axon guidance during development and the pathological reappearance of these guidance or boundary molecules at a site of injury may contribute to the local impediment to axon regeneration.

The CNS lesion interface is a barrier to axonal regeneration.

An understanding of the lesion interface after injury has evolved with the techniques available to study it. At first conceptualized as a fibroblastic scar, strategies focused on diminishing its formation. Then the astrocytic scar gained importance and factors contributing to its formation were studied. In reality, the "scar" is an interface where a basal lamina is formed between two primary cell types, the pial cells and the glial cells. The presence of inflammatory cells in the wound may mediate the influx of pial and glial cells and subsequent deposition of extracellular matrix.

From the 1950's to the late 1970's, much effort was directed at reducing the fibroblastic "scar" formed after CNS lesioning. Enzymes, bacterial polysaccharide, systemic corticosteroids and occlusive sheaths were used with some success in reducing the cicatrix but not in promoting axonal regeneration either histologically or functionally (Guth et al., 1980). The lack of success might more probably be attributed to incomplete suppression of the lesion interface. Even a few leptomeningeal cells in a lesion cavity can induce the formation of the glia limitans (Eng et al., 1987). The cellular and molecular environment at the lesion interface was not fully evaluated in earlier studies since only light microscopic assessments were performed. More recent ultrastructural studies (Reier et al., 1989) and immunocytochemical techniques (Berry et al., 1983) have allowed a more thorough study of the lesion interface. The maturation of the glia limitans at the site of penetrating injury is coincident with the infiltration of leptomeningeal cells.

Components of the lesion interface.

Electron microscope studies have shown that the lesion interface is formed by a glia limitans, composed of one or more layers of reactive astrocytes which are lined by a basal lamina, with meningeal cells, macrophages and collagen fibrils within the lesion space (Reier et al., 1989). Immunocytochemical studies allowed further characterization of extracellular matrix deposition and astrocyte orientation at the lesion site (Berry et al., 1983; Matthewson and Berry, 1985). In the neonatal rat, there is very little glial fibrillary acidic protein (GFAP) induced by lesioning and no glia limitans formation

(Berry et al., 1983). In the first 2-4 days post-lesioning (dpl) in the adult rat, blood cells infiltrate into the lesion and astrocyte processes are directed toward the lesion (Matthewson et al., 1985). Fibroblasts then enter at 4-8 dpl after which astroglia produce fine parallel interdigitating processes that line the lesion walls by 8-12 days. The maximal general GFAP immunoreactivity is seen at 8 dpl but the accumulation of layers of parallel astrocyte foot processes (gliosis) at the lesion interface continues up to 20 dpl (Matthewson et al., 1985). In the CNS, macrophages tend to arrive later to lesion sites than in the PNS (Perry and Gordon, 1988). It is notable that the maximal GFAP response follows the infiltration of macrophages and leptomeningeal cells (Maxwell, 1990; Logan and Berry, 1993). Types I and III collagen immunoreactivity were seen corresponding to the fibrillar collagen deposition in the wound, and types IV and V collagen immunoreactivity were seen in the basement membranes (Berry et al., 1983).

Further details were provided by EM studies of basal lamina formation after spinal cord lesions (Carbonell and Boya, 1988). In this study an irregular basal lamina was seen at 10 dpl and the typical glia limitans was seen at 14 days. The meningeal cells were seen as a loose cellular network at 14 days and sometimes formed solid cell masses at 25 days. This study provides further evidence that the formation of the glia limitans is coincident with the invasion of leptomeningeal cells.

Meningeal cells induce the formation of the glia limitans.

Basal lamina is deposited when leptomeningeal cells contact astrocytes (Abnet et al., 1991; Ajemian et al., 1994; Berry et al., 1983;

Kusaka et al., 1985; Logan et al., 1994; Sievers et al., 1994). Evidence that leptomeningeal cells produce basal lamina constituents is provided by immunocytochemistry of primary meningeal cell cultures (Ajemian et al., 1994; David, 1988; Sievers et al., 1994). When meningeal cells and astrocytes are cocultured they segregate and later deposits of laminin, fibronectin (Abnet et al., 1991) and tenascin (Ajemian et al., 1994) are detected at the interface between the 2 cell types. Ultrastructural studies similarly reveal formation of basal lamina when mouse spinal cord tissue is grown in combination with its pia-arachnoid (Kusaka et al., 1985).

During development, both astrocytes and leptomeninges contribute to the deposition of extracellular matrix molecules at the interface between the two cell types (Sievers et al., 1994). Selective destruction of meningeal cells covering the hamster cerebellum with 6-hydroxydopamine resulted in decreased immunoreactivity for type IV collagen, laminin and fibronectin 24 hours later, which recovered if meningeal cells repopulated the cerebellar surface (Sievers et al., 1994).

Further evidence for the role of meningeal cells in the formation of the glia limitans and its impediment to axonal regeneration is provided by morphologic studies of fetal graft integration into adult rat CNS (Kruger et al., 1986; Reier and Houle, 1988). Three zones of graft/host interaction are seen. The floor of the graft completely fuses and integrates with host tissue. The deep portion of the walls show incompletely interdigitating reactive glial processes as if forming a glia limitans. The superficial region between graft and host is characterized by the formation of a basal

lamina from meningeal cell infiltration resulting in poor integration of the graft into the host tissue. Others have also noted gliosis along areas of mesodermal invasion in transplants and the subsequent impediment to axon regeneration (Reier et al., 1986; Reier et al., 1983a).

The above studies demonstrate the role of leptomeningeal cells in forming a glia limitans upon contact with astrocytes. The basal lamina is immunoreactive for glycoproteins, laminin, fibronectin, type IV collagen and other basement membrane constituents. These proteins are secreted by leptomeningeal cells and possibly astrocytes upon contact with leptomeningeal cells. These ECM molecules may act as an adhesive substrate that promotes the infiltration of leptomeningeal cells into the adult CNS lesion cavity. This notion is supported by the finding that only 10-15% of neonatal leptomeningeal cells in culture express ß-1 integrin receptors compared to 90% of adult leptomeningeal cells (unpublished observations from our lab). The ß-1 integrin is part of the heterodimer that binds to various ECM molecules including laminin, fibronectin, and collagen (Reichardt, et al., 1989).

Once leptomeningeal cells have entered the wound, they may respond to a number of mitogenic signals since these cells have receptors for FGF (Takahashi, 1991; Fayein, 1992), NGF (Yan, 1988; del Valle, 1992), PDGF-ß (Wang, 1990) insulin-like growth factor (Bondy, 1992; Brar, 1993; Couce, 1992; Stenvers, 1994) and IL-1 (Ban, 1991, 1994). They may also send molecular cues to surrounding cells, such as astrocytes, to proliferate or secrete matrix components through such signals as TGF-ß (Logan, 1992; Johnson, 1992), NGF (Yoshida and Gage,

1991) and insulin-like growth factor (Stylianopoulou, 1988), which have been localized to meningeal tissues.

Increases in the levels of several cytokines and growth factors in CNS wounds have been reported, e.g., TGF-β (Logan, et al., 1992b), IL-1, IL-2, IL-6 (Nieto-Sampedro and Berman, 1987; Nieto-Sampedro and Chandy, 1987; Taupin et al., 1993; Tchelingerian et al., 1993; Woodroofe et al., 1991), TNF (Taupin et al., 1993; Tchelingerian et al., 1993), and bFGF (Finklestein et al., 1988; Frautschy et al., 1991; Logan et al, 1992a).

Corticosteroids modulate wound healing

There are many similarities between the formation of the glia limitans and wound healing elsewhere in the body. In the skin, for example, wound healing occurs in three stages: inflammation, granulation tissue formation, and extracellular matrix molecule remodeling (Raghow, 1994). Inflammation involves platelet degranulation, clotting, cell recruitment and phagocytosis of debris. Granulation tissue formation involves growth factor release, cell proliferation and activation, and re-epithelialization. During ECM molecule remodeling, granulation tissue is dissolved and ECM molecules are turned over to provide strength to the wound (Raghow, 1994).

In disease, fibrosis is an extreme of wound healing and glucocorticoids have often been used to reduce scar formation. Glucocorticoids modulate all aspects of the wound healing process. They attenuate cytokine effects (Munck and Naray-Fejes-Toth, 1994); and decrease macrophage recruitment (Leibovich, 1975), epithelial

migration (William and Mertz, 1978), fibroblast proliferation (Bodor, 1991; Weber, 1992), collagen deposition (Goforth, 1980), and glycosaminoglycan synthesis (Sarnstrand et al., 1982). Glucocorticoids also modulate extracellular matrix secretion and synthesis as seen in an animal model of murine nephritis (Nakamura et al., 1992), in fibroblasts (Ekblom, 1993), in cytotrophoblasts (Guller et al., 1993), in a rat pancreatic acinar cell line (Stallmach et al., 1992) and in PC12 cells (Zhang et al., 1993).

In contrast to the previous steroid-induced changes, glucocorticoids inhibit lipid peroxidation after spinal cord injury via a non-glucocorticoid receptor mechanism (Bracken et al., 1990). The effect is thought to be independent of the glucocorticoid receptor since analogs that lack the 11-beta-hydroxyl functional group are also potent inhibitors of lipid peroxidation (Braughler et al., 1987). Prior animal studies have revealed that to achieve this effect, high systemic doses are required (Hall, 1992). However, systemic dexamethasone does not seem to alter wound healing (Giulian et al., 1989).

In our model, which is a model of penetrating injury as opposed to the compressive injuries studies by Hall, the glucocorticoid is applied topically instead of systemically since the effect is needed only locally as in skin wounds. We propose using steroids to modulate wound healing after CNS injury, primarily to prevent cell migration into the wound which the literature suggests is a prerequisite for the maturation of the glia limitans and basal lamina that are impediments to axon regeneration. Topical applications of Petroleum jelly have been used in culture to limit diffusion of growth factors and migration of cells in compartment cultures (Campenot, 1982), and to decrease

epithelialization after skin wounds (William and Mertz, 1978). In our experiments, having the steroid in ointment form allows both local delivery of the steroid and optimal reduction in cell migration.

Glucocorticoids do have multiple effects and can also decrease cytokine effects which may increase lesion interface formation (Munck and Naray-Fejes-Toth, 1994). Antibodies to TGF-ß have been shown by Logan et al., (1994) to reduce the formation of the basal lamina at the lesion interface after cortical stab wounds. The effects of blocking other cytokines and growth factors expressed at higher levels in injured CNS tissue are not known at present, but are currently being investigated.

Rationale

Wound healing in the CNS, like the skin involves mesenchymal cell migration and ECM deposition. In the CNS the lesion interface is an impenetrable barrier to axon regeneration because of the formation of a glia limitans and basal lamina. This glia limitans seems to be formed in response to leptomeningeal cell migration into the lesion cavity after penetrating injury to the CNS. Meningeal cell migration may be mediated by adhesion to extracellular matrix molecules deposited at the lesion site. Diminution of leptomeningeal cell migration and proliferation, and ECM deposition in the lesion interface at the site of CNS injury may hinder leptomeningeal cells from migrating into the lesion cavity. Decreasing the local inflammatory response may also be helpful. These effects can be mediated by glucocorticoids. By preventing the formation of the glia limitans and basal lamina, perhaps axons would be more likely to cross the lesion site after penetrating CNS injury. This strategy would of course have to be combined with other treatments such as antibodies against myelin inhibitors and growth factors in order to achieve improved axon regeneration.

Hypothesis

The hypothesis that will be tested is that topical glucocorticoid application will attenuate the formation of the glial-pial interface at the site of CNS lesions by affecting leptomeningeal cell infiltration and extracellular matrix deposition.

Objectives

<u>In vivo:</u>

1) To demonstrate that topical applications of glucocorticoids can attenuate the formation of the lesion interface after stab wounds to the cerebral cortex by examining the various components of the lesion interface using immunocytochemistry (e.g.. GFAP for the glia limitans, ED1 for macrophages, laminin for the basal lamina, Nuclear Yellow for the leptomeningeal cells). The following changes would be evaluated.

    a) the quality and total length of laminin immunoreactivity in the wound

    b) the quality of glia limitans formation

    c) the quantity of leptomeningeal cells in the wound

2) To correlate the light microscopic findings with electron microscopy, especially with regard to the influx of leptomeningeal cells since they have no specific cell surface marker.

3) To verify whether the astrocytes farther away from the interface would be affected by the glucocorticoid treatments. These GFAP[+] astrocytes would be counted and tested for GFAP content by Western blot.

4) To quantify the degree of macrophage invasion in and around the wound by counting ED1^{+} cells as a measure of the anti-inflammatory action of the steroids.

In vitro: to assess the cellular mechanisms of glucocorticoid-induced effects

1) To quantify leptomeningeal cell adhesion and spreading onto various extracellular matrix molecules, e.g. laminin, fibronectin and collagen by measuring the cell area using image analysis.

2) To quantify the influence of glucocorticoids on leptomeningeal cell secretion of laminin in culture by Western blot.

3) To quantify the influence of glucocorticoids on leptomeningeal cell proliferation by measuring ^{3}H-thymidine incorporation into the cells.

Topical Glucocorticoids Modulate the Lesion Interface after Cerebral Cortical Stab Wounds in Adult Rats

ABSTRACT: A lesion interface consisting of a glia limitans lined by a laminin-rich basal lamina and leptomeningeal cells, forms within 2-3 weeks after penetrating wounds to the adult mammalian central nervous system (CNS). This interface prevents the growth of axons across the lesion. We have examined the effects of topically applied steroids on the formation of such an interface after stab wounds to the adult rat cerebral cortex. Immediately after lesioning, the surface of cortex in the region of the wound was treated with a topical application of either 0.1% halcinonide, or 0.05% betamethasone dipropionate or their respective placebos. Cryostat sections through the lesioned area were obtained 3 weeks later, and assessed by immunofluorescence. Steroid treatment attenuated all components of the lesion. The continuous anti-laminin labeling along the lesion in untreated rats became patchy after steroid treatment. The number of leptomeningeal cells that infiltrated into the wound was reduced in the laminin-negative regions in steroid treated rats. In addition, astrocytic processes in the laminin-negative regions after steroid treatment were loosely arranged, as compared with the tightly packed parallel processes forming the glia limitans in laminin+ regions in controls. The mechanism of steroid-mediated attenuation of the lesion interface was examined in vitro. Betamethasone but not halcinonide reduced laminin secretion slightly in leptomeningeal cell cultures, but both steroids reduced cell proliferation. These results suggest that

steroids modulate the formation of the lesion interface after CNS injury, at least in part, by decreasing leptomeningeal cell proliferation. Such modulation of the lesion interface by steroids or other agents may permit the growth of axons across the lesion site, and thus could enhance the overall degree of axon regeneration if other factors such as neurotrophic support and neutralization of axon growth inhibitory molecules are optimized.

INTRODUCTION

The adult mammalian CNS environment does not favor the regrowth of axons. However, mature CNS neurons can regrow axons if provided with a permissive environment, such as, a peripheral nerve graft (David and Aguayo, 1981; Richardson et al., 1980), or immature CNS tissue (Bregman et al., 1989; Kalil and Reh, 1979; Keirstead et al., 1992 Reier et al., 1992). The environmental influences thought to play particularly important roles in the failure of axon regrowth in the adult CNS are, the presence of inhibitory molecules (Schwab et al., 1993) and the lack of appropriate levels of neurotrophic factors (Jelsma and Aguayo, 1995). Experiments combining blocking the NI-35/250 myelin-associated axon growth inhibitor (with the IN-1 monoclonal antibody) and injections of neurotrophins, resulted in local enhancement of regenerative sprouts and long distance regrowth of axons which did so by detouring around the lesion (Schnell et al., 1994). These and other studies on embryonic CNS tissue transplantation (Eng et al.,1987; Kruger et al., 1986) suggest that the

formation of a mature interface (fibroblastic/glial scar) at the site of CNS wounds impede axon regeneration across the lesion.

The lesion interface has been well characterized, particularly after stab wounds to the rodent cerebral cortex (Berry et al., 1983; Matthewson and Berry, 1985; Maxwell et al., 1990; Reier et al., 1989). At maturity the lesion interface comprises of a wall of astrocytes and their interdigitating processes (glia limitans), which is lined externally by a basal lamina and meningeal cells. The latter forms the fibrotic scar that fills the lesion cavity. The sequence of events that lead to the formation of this interface begins with the infiltration of monocytes and leptomeningeal cells into the wound (Logan and Berry, 1993; Maxwell, et al., 1990). Astrocytes and leptomeningeal cells secrete ECM molecules that form the basal lamina. In vitro studies on leptomeningeal cells and astrocytes (Abnet et al., 1991; Ajemian et al., 1994), and in vivo studies on the developing cerebellum (Sievers et al., 1994) suggest that the formation of the glia limitans and the basal lamina is associated with the presence of leptomeningeal cells. In this study we have examined whether the formation of the glia limitans and the basal lamina at the site of CNS lesions can be attenuated by reducing leptomeningeal cell infiltration into cerebral cortical stab wounds.

In other tissues in which wound healing has been studied extensively, e.g., the cornea (Bodor and Varga, 1990) and skin (William and Mertz, 1978), topical glucocorticoids decreased the formation of the fibrotic scar. These steroids act by modulating several aspects of the wound healing process, i.e., epithelialization, including cell migration (William and Mertz, 1978), fibroblast

proliferation (Bodor et al., 1991; Weber, 1992), collagen deposition (Goforth and Gudas, 1980) and macrophage recruitment (Leibovich and Ross, 1975). We therefore used topical glucocorticoids to modulate the formation of the lesion interface after cerebral cortical stab wounds in adult rats. Topical steroids have the advantage of acting locally and longer with a single application, as well as resulting in less systemic immunosuppression. Topical applications also have the advantage that steroids can be applied directly to leptomeningeal cells.

Here we report studies in which we examined the effects of topically applied glucocorticoids on the formation of the lesion interface after stab wounds to the adult rat cerebral cortex. We also examined the mechanism of action of these steroids on leptomeningeal cells in vitro.

MATERIALS AND METHODS

Cortical lesions: Adult female Sprague-Dawley rats (200-250 g) were anesthetized with intraperitoneal chloral hydrate (420 mg/kg). A left parietal craniotomy was performed, the dura opened and the cortex exposed. A parasaggittal stab wound 2mm long by 1.5 mm deep was made in the parietal cortex with a razor blade. Blood vessels were avoided. A single topical application of either betamethasone dipropionate 0.05% (Diprolene; Schering), halcinonide 0.1 % (Halog; Westwood-Squibb) or placebo ointments (also obtained from the respective companies) was made over the lesion. The ointments were extruded over the lesion site through a 1ml syringe with a 26-gauge needle and spread and kept in place with a piece of nitrocellulose

paper. The bone flap was then set in place and the skin wound closed. Another control group received only cortical lesions (untreated controls). In a pilot study 3 other steroids (halobitusol propionate, momitasone furate and triamcinolone acetonide) were also tried but were not as effective as the two steroids used in this study.

Preparation of tissue: Three weeks after lesioning, the animals were deeply anesthetized with chloral hydrate and perfused through the left ventricle first with 0.1M phosphate buffer (pH 7.4) and then with 4 % paraformaldehyde (Fisher) in 0.1M phosphate buffer. Serial sections (12 μm) through the entire lesioned area were cut on a cryostat and mounted on gelatin-coated glass slides.

Immunocytochemistry: Tissue sections from only the middle 1mm of the lesioned area (total length of lesion was 2mm) were processed for immunocytochemistry. Sections were incubated with 3% ovalbumin overnight at 4°C to block nonspecific binding followed by incubations with primary antibodies overnight at 4°C and then secondary antibodies plus Nuclear Yellow (0.001%, S769121 Hoechst) at room temperature for 1-2 hours. Primary antibodies used included monoclonal anti-GFAP (1:400 Sigma), polyclonal rabbit anti-laminin (1:200 Telios), or monoclonal anti-ED1 (1:100 Serotec). The secondary antibodies used were rhodamine-conjugated goat anti-mouse IgG (1:200 Hyclone) against all monoclonal antibodies and fluorescein-conjugated goat anti-rabbit IgG (1:400 Cappel) against polyclonals. Sections were washed in 0.1M phosphate buffer for 30 minutes between incubations with antibodies. The slides were mounted in

bicarbonate buffered glycerol (pH 9.0) containing phenylenediamine (Sigma) and viewed with a fluorescence microscope (Leitz Olympus microscope) equipped with N2 (rhodamine), L2 (fluorescein) and D (Nuclear Yellow) filters. Staining controls received only secondary antibodies and revealed no nonspecific staining.

Quantification and statistical Analysis

Laminin immunoreactivity: The depth of laminin deposition at the lesion interface relative to the depth of the original lesion was quantified as a percentage. The lesion depth was determined by the presence of autofluorescent cell debris deposited along the lesion. Because the laminin interface was discontinuous in steroid treated animals, the lengths of the laminin patches were added up and presented as a percentage of the total lesion length. Measurements were made from three sections per animal.

GFAP immunoreactivity: The number of intensely GFAP positive astrocytes in four fields (each one 200 x 200 μm) 300 μm lateral to the middle of the lesion was counted. The quantification was done from three sections per animal. The depth of laminin deposition and GFAP immunoreactivity were compared in the different experimental groups using the Wilcoxon Rank Sum Test, a non-parametric test of significance that parallels the unpaired t-test.

ED1 counts: The number of ED1 positive cells were counted in the middle of the lesion and in each of four (200 x 400 μm) fields lateral to the middle of the lesion.

Nuclear yellow counts: In the absence of a leptomeninges-specific marker, GFAP-negative cells with elongated nuclei distinctive

of leptomeningeal cells, were counted in laminin-positive and laminin-negative areas of the wound. The counts for each animal were normalized to the number of cells/500 μm length of the wound. These values were analysed for significance using the Student's t-test.

Western blotting: The lesioned cortex ($2mm^3$) was homogenized in 1.2 mls 0.1M phosphate buffer (pH 7.4) containing 50 μl each of 6M urea, 1% aprotinin, soybean trypsin inhibitor (50 μg/ml), and phenylmethylsulfonylflouride (5mM), all at 4°C. After homogenization, the samples were centrifuged, aliquoted and stored at -70°C for SDS-PAGE analysis. Samples were boiled in reducing buffer (distilled water, 0.5 M Tris-HCL pH 6.8, glycerol, 10% SDS, ß-mercaptoethanol, 0.1% Bromophenol blue), electrophoresed on a 5% acrylamide gel, and transblotted onto PVDF membrane (Bio-Rad). The PVDF membrane was then blocked with 10% skim milk in Tris-buffered saline for 1 hour at room temperature and washed with 0.05% TBS-tween-20 (Bio-Rad) for 3x5 minutes. The membrane was then incubated with a polyclonal anti-GFAP (1:500 obtained from M.Raff) at 4°C overnight and an additional one hour at room temperature. The membrane was washed then before incubation in alkaline phosphatase conjugated goat anti-rabbit IgG (Promega) for one hour at room temperature and developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indyl phosphate (Sigma). Incubation with primary antibody was omitted in control.

Electron Microscopy: Animals were perfused three weeks after lesioning with 0.5% paraformaldehyde, 1.5% glutaraldehyde in 0.08M

phosphate buffer containing 0.002% calcium chloride. The lesioned area was post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in plastic (Epon).

Meningeal cultures: The cortical leptomeninges from adult Sprague-Dawley rats were removed, cut into small pieces, and trypsinized in Ca^{+2}, Mg^{+2}-free Hank's Balanced Salt Solution (HBSS) containing 0.125% trypsin and 0.125% collagenase (Sigma) for 20-30 minutes. After neutralizing the trypsin, the cells were triturated with a Pasteur pipette, pelleted by centrifugation (500g for 10 minutes) and resuspended in Dulbecco's modified Eagle's medium containing 1% MEM-vitamins, 1% penicillin/streptomycin and 10% FBS (all from Gibco/BRL). The resuspended cells were plated in $25cm^2$ flasks (T-25) coated with poly-L-lysine (5 µg/ml, Sigma, 300 000 MW) and grown in a humid atmosphere of 5% CO2 at 37° C.

Cell spreading on various extracellular matrix (ECM) molecules: Adult leptomeningeal cultures were grown in T-25 flasks for two days before use. The flasks were washed twice with MEM-HEPES and trypsinized with 0.125% trypsin in HBSS. After 5-10 minutes the trypsin was neutralized and the cells triturated, centrifuged and resuspended in DMEM and 10% FBS. $2x10^4$ cells (per 100 µl) were plated onto 12 mm round glass coverslips coated with various ECM molecules. Round glass coverslips (12 mm) were incubated with PLL overnight at $37^O C$, washed and coated with 100 µl of laminin (EHS sarcoma, BRL), collagen (extracted from rat tail) or fibronectin (obtained from Dr. S. Carbonetto, McGill Univ.) at 50 µg/ml. After incubating coverslips

with either collagen overnight at room temperature, or laminin or fibronectin at 37°C for 4 hours, they were rinsed twice with HBSS and 100 μl of the meningeal cell suspension was plated on each. Twenty-five to thirty-five minutes after plating, the cells were washed with MEM-HEPES, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 30 min., and stained with Coomassie Blue for 5-10 min. Trypan Blue was used to assess the viability of cells in each experiment. The size (area) of 100 cells per substrate was determined using a Zeiss Axiomat inverted microscope and an Image-1 image analysis program (Universal Imaging Co.). The areas of the meningeal cells on different substrates were compared using the median test, which tests the null hypothesis that the medians of two experimental groups are equal.

Steroid effects on laminin secretion by leptomeningeal cells: Primary leptomeningeal cell cultures were prepared as described above, and plated in T-25 flasks. After 1 week the cells were replated into 24 well plates at 750 cells per well. After allowing the cells to attach overnight they were treated with 10^{-5} M halcinonide, or 10^{-5} M betamethasone. Control cultures were grown in DMEM and 10% FBS alone or containing 0.02% ethanol. The latter was used to solubilize the steroids obtained as a powder from the manufacturer. After 6 days of treatment, the cultures were solubilized with 2% SDS in Tris-buffered saline and protease inhibitors for 1-2 minutes. These samples were used for Western blotting as described above using a polyclonal anti-laminin antibody.

Steroid effects on leptomeningeal cell proliferation: Adult primary leptomeningeal cells, after two days in culture, were replated from flasks into a 24-well plate at a density of 500 cells/well. These cultures were treated with either steroid at 10^{-5}M or DMEM and 10% FBS, alone or containing 0.02% ethanol for one week. For the last 24 hours, 2 μCi/ml of ^{3}H-thymidine was added to the cultures. After washing, the cultures were solubilized and ^{3}H-thymidine incorporation measured with a scintillation counter. This experiment was done in duplicate with three wells per experimental group. The statistical analysis was done using the ANOVA and the Tukey-Kramer post-test.

RESULTS

Lesions were made in a total of 36 animals for the immunofluorescence study. Twenty-eight of these were analysed. Two animals in the control group, five in the halcinonide treated, one in the halcinonide placebo treated, and two of the betamethasone treated animals were excluded because of infections or large cavitations. Six animals in each of the control, halcinonide and halcinonide placebo groups, three in the betamethasone and seven in the betamethasone placebo groups were analysed. All animals were examined 3 weeks post-lesion.

Steroids reduce laminin deposition at the lesion interface: Laminin immunoreactivity was used as a marker of the lesion interface since laminin deposition is correlated with the establishment of a mature basal lamina, which is completed by 3 weeks post-lesioning (Maxwell

et. al., 1990). In control animals, i.e., lesioned but untreated and placebo treated rats, the laminin immunoreactivity was continuous along most of the depth of the lesion, extending deep into the cerebral cortex (Fig. 1A). In contrast, in halcinonide treated animals, laminin immunoreactivity appeared discontinuous along the lesion and did not extend as deeply into the cortex as in the controls (Fig. 1 B, C). Application of betamethasone resulted in an even greater patchy laminin-like immunoreactivity, much of which appeared to be superficially located near the surface of the cortex (Fig. 2 A, B). Some of the laminin immunoreactivity may have been associated with blood vessels. The extent of the laminin immunoreactivity was quantified by adding the lengths of all the laminin-positive patches along the lesion. Steroid treated animals had statistically significant reductions in the total length of laminin deposited along the lesion interface as compared to lesioned but untreated controls (p<0.01), and to their respective placebos (p<0.05). In halcinonide and betamethasone treated animals there was a 24% and 69% diminution, respectively, compared with untreated controls (Fig. 3 A,B), and a 18% and 63% reduction, respectively, from placebos. In the untreated control and placebo treated groups the laminin deposition along the lesion interface extend from the surface of the cortex to 82% and 78% of the length of the lesion, respectively. These differences are not statistically significant. Thus both qualitatively and quantitatively laminin-like immunoreactivity was decreased by topically applied steroids.

Steroids decrease the infiltration of leptomeningeal cells into the wound: To assess whether the decrease in laminin immunoreactivity in the lesions was associated with a decrease in leptomeningeal cells within the wound, the number of leptomeningeal cells in laminin-positive areas and laminin-negative areas were counted. Because of the lack of leptomeninges-specific antibody markers, leptomeningeal cells were identified at the light microscopic level by the shape of their characteristically elongated nuclei visualized by Nuclear Yellow labeling. Elongated nuclei of endothelial cells could be distinguished from those of leptomeningeal cells by their configuration around blood vessels. Nuclei of other cells such as astrocytes, and macrophages were excluded based on their nuclear morphology and labeling with antibody specific markers (i.e., anti-GFAP and ED1). The mean number of leptomeningeal cells in laminin-negative areas of the wound is 82% less than in laminin-positive areas (Fig. 4 and 5A-D). These results were further confirmed by electron microscopy (Fig. 6A,C). In the steroid treated animals the leptomeningeal cells were mainly found closer to the cortical surface, rather than in the deeper portions of the cortical lesion. In addition, electron microscopy also revealed that a well developed basal lamina and collagen deposition extended deep within the lesion in untreated and placebo treated animals, but was lacking in regions devoid of leptomeningeal cells in animals treated with steroids (Fig. 6 A-E).

Steroids attenuate glia limitans formation: The de novo glia limitans that forms after penetrating wounds can also be identified by the characteristically reorganized linear arrangement of the astrocytic

processes. In the control group, this interface consisted of intensely GFAP immunoreactive, tightly packed, astrocyte processes running parallel to the lesion (Fig. 7A). After steroid treatment, the astrocyte processes were not as extensive and appeared as a loose arrangement of GFAP+ astrocyte processes at the lesion interface (Fig. 7B). This was particularly noticeable in areas along the lesion that lacked laminin-like immunoreactivity.

In untreated lesioned control animals the reactive gliosis at the ultrastructural level was minimal and confined largely to the immediate area of the lesion (Fig. 6A,B). This was likely due to the late post-lesion survival time (i.e., 3 weeks), and the thin razor blade lesion. The latter factor may also account for the minimal necrosis, and the relatively normal appearance of the neuropil located adjacent to the lesion (Fig. 6A,B). In steroid treated rats, the astrocytic processes at the lesion interface contained fewer intermediate filaments (Fig. 6C,E).

Steroids do not alter GFAP immunoreactivity or GFAP content: Besides examining the GFAP immunoreactivity along the wall of the lesion cavity, we also assessed the effects of steroids on the astrocyte response in the region adjacent to the lesion by evaluating (I) the number of GFAP+ astrocytes in an area 300 μm lateral to the mid-portion of the lesion, and (ii) estimating the difference in GFAP content on Western blots. Because the primary aim of this study was to examine the effects of steroids on the maturation of the lesion interface that is completed three weeks after injury, this post-lesion time point was examined. The number of GFAP+ astrocytes was reduced

with both steroid and placebo treatments. The mean astrocyte counts were: 46 (SEM ± 4) for lesioned controls; 33 (SEM ± 3) for halcinonide placebo; 29 (SEM ± 2) for halcinonide; 26 (SEM ± 2) for betamethasone placebo; and 21(SEM ± 4) for betamethasone. The mean number of GFAP+ astrocytes in an unoperated animal was 26. Although the number of GFAP+ astrocytes in the steroid treated groups (betamethasone and halcinonide) was not significantly different from their respective placebo groups, these groups were significantly different from the untreated lesion control group, p<0.01 and p<0.05 respectively. The difference between the untreated lesioned control and placebo groups also reached statistical significance (p<0.05). We attribute this to the occlusive effect of the ointment to molecules that induce reactive changes in astrocytes.

The synthesis of GFAP in the various treatment groups was further assessed by Western blotting. Glial fibrillary acidic protein was increased in the lesioned untreated cortex as compared with the normal unlesioned cortex (Fig. 8), however, neither the steroid nor placebo treatments altered this lesion-induced increase in GFAP (Fig. 8).

Steroids do not influence macrophage number in the lesion: Macrophages were visualized with the monoclonal antibody ED1, an intracellular macrophage antigen. Halcinonide treated animals often had accumulations of inflammatory cells superficially, over the surface of the stab wound, making it difficult to count cells accurately in this region. Because of this, macrophage counts were made within an area 400x400 micron across the middle of the lesion. Qualitatively,

the macrophages appeared to be more localized to the immediate area of the lesion in the controls and placebos, but were spread out more in the steroid treated groups (Fig. 9 A,B). However, the total number of ED1+ cells was not significantly different between the various experimental groups.

Spreading of leptomeningeal cells on ECM substrates: The spreading of adult leptomeningeal cells was quantified 30 minutes after plating on substrates coated with either laminin, fibronectin or type I collagen. These cells were significantly larger on laminin (median=714.66 μm) as compared with fibronectin (median=473.99 μm), collagen (median=276.07 μm) and PLL (median=229.28 μm), p<0.05 for laminin and fibronectin and p< 0.15 for collagen (Fig. 10).

Steroids slightly reduce laminin secretion by leptomeningeal cells in vitro: Since laminin was a preferred substrate for attachment and spreading, the effect of the two steroids on the secretion of laminin by leptomeningeal cells in vitro was assessed by Western blotting. Under the in vitro conditions used, betamethasone treatment slightly reduced laminin produced by leptomeningeal cells (Fig.10). In contrast halcinonide did not affect laminin secretion by these cells (Fig. 11). Amido black staining of identical blots confirmed even loading of the lanes (data not shown).

Steroids diminish leptomeningeal cell proliferation: Proliferation of leptomeningeal cells in vitro was assessed by measuring ^{3}H-thymidine uptake. Steroids significantly decreased this uptake into

leptomeningeal cells as compared with controls (Fig. 12). Halcinonide treatment resulted in a 31% (p<0.05) reduction from controls, while betamethasone treatment reduced the counts by 61% (p<0.01).

DISCUSSION

The lesion interface that forms after penetrating injuries to the adult CNS, is an impediment to axon regeneration (Kruger et al., 1986; McKeon et al., 1991; Reier et al., 1989; Schnell and Schwab, 1990; Schnell et al., 1994). At maturity, this interface is composed of a fibrotic/glial "scar," consisting of: (I) leptomeningeal cells, macrophages and collagen; (ii) the newly formed glia limitans that lines the wall of the lesion, and (iii) a basal lamina that separates the two (Berry, et al., 1983; Eng et al., 1987; Maxwell, et al., 1990; Reier, et al., 1989). In this study, we examined whether the formation of this interface after cortical stab wounds can be attenuated by the application of topical glucocorticoids. We have found that topical applications of glucocorticoids to cerebral cortical stab wounds (I) reduce the usually continuous laminin immunoreactivity at the lesion interface, which represents the basal lamina, to a patchy appearance; (ii) alter the orientation of astrocytes that form the glia limitans, to form a loose network of astrocytes in the laminin-negative areas of the wound; and iii) reduce the number of cells resembling leptomeningeal cells, that infiltrate into the wound. In addition, the in vitro studies suggest that the steroids tested may mediate these effects largely by reducing leptomeningeal cell proliferation.

The topical application of steroids used in this study differs, in the dosage, route of administration, the cells targeted and the rationale for use, from the methylprednisolone treatments used clinically for spinal cord injuries (Bracken et al., 1990). In the latter, large doses of the steroid (bolus of 30 mg per kg body weight, followed by 5.4mg per kg body weight for 23 hours) are given intravenously within 8 hours after injury (Bracken et al., 1990). Systemic glucocorticoids at such high doses are thought to mediate their effects primarily (though not exclusively) by scavenging free radicals and preventing lipid peroxidation (Hall, 1992), thus reducing neuronal damage. The low concentrations of steroids we have applied topically (total of approximately 0.1 - 0.2mg) is unlikely to affect lipid peroxidation, but to have other receptor-mediated effects such as reducing proliferation and migration of cells. Our aim was to limit these effects of the steroids locally to the surface of the cortex, where they are likely to influence primarily the most superficially located cells, i.e., the leptomeningeal cells.

Steroid effects on laminin deposition and leptomeningeal cell infiltration into the wound: Although much is known about the formation of reactive astrocytes, i.e., the glial component of the "scar" after CNS injury (reviewed by Eddleston and Mucke, 1993), the factors underlying the formation of the fibroblastic component of the "scar" after penetrating CNS injuries are not as well understood. The infiltration of leptomeningeal cells into penetrating CNS wounds results not only in the formation of a fibrotic scar, but may also result in the transformation of astrocytes near the lesion to form the glia

limitans, and to the deposition of the basal lamina. Evidence supporting this comes from studies in which the appearance of the leptomeninges covering the developing rat cerebellum was transiently blocked by treatment with 6-hydroxydopamine (6-OHDA) (Sievers, et al., 1994). This resulted in a delay in the formation of the glia limitans and basal lamina. In addition, leptomeningeal cells fail to infiltrate into stab wounds in the neonatal rat cerebral cortex and this is associated with the absence of fibroblastic/glial scar (Maxwell, et al., 1990). Our earlier studies have shown that leptomeningeal cells infiltrate to cover the wall of a cerebral cortical wound within a few days after lesioning (Giftochristos and David, 1988) and to cover the cut end of the adult rat optic nerve within 24 hours (Ajemian et al., 1994). In other work we attempted to kill leptomeningeal cells with local applications of 6-OHDA, but were unsuccessful because of its toxic effects on cortical tissue (unpublished observations). In this study we used topically applied steroids to block leptomeningeal cell infiltration into cortical wounds. Topical steroids have been shown to decrease cell migration and epithelialization (William, et al., 1978) in skin and corneal wounds. Different cell types have been shown to attach, spread and migrate on various ECM molecules if they possess the appropriate receptor systems. Since laminin fibronectin and collagen are found in the wound cavity, we carried out the functional studies to assess the spreading of leptomeningeal cells on these ECM molecules. Because our results show that these cells have the appropriate receptor systems to attach and spread on laminin, we assessed the effects of the steroid treatments on expression of laminin. We have shown that a continuous basal lamina immunoreactive for laminin,

does not form after topical steroid treatment. We quantified the numbers of cells that resemble leptomeningeal cells that infiltrated into the wound, identifying them by their distinct nuclear morphology and absence of GFAP and ED1 staining. In areas showing steroid-induced loss of laminin immunoreactivity, there was a significant reduction in the number of leptomeningeal cells. These results were also confirmed by electron microscopy.

As mentioned earlier, steroids are known to modulate the proliferation of fibroblasts (Bodor, et al., 1991; Hien et al., 1994; Weber, 1992), and inflammatory cells (Alamwi et al., 1991; Ganter et al., 1992); decrease ECM molecules (Ekblom, 1993; Goforth and Gudas, 1980; Guller et al., 1993; Nakamura et al., 1992) and ECM molecule receptor production (Stallmach et al., 1992; Zhang et al., 1993); decrease cell migration (Hien et al., 1994); and decrease cytokine expression (Munck and Naray-Fejes-Toth, 1994). Therefore, the reduction in leptomeningeal cell infiltration into cerebral cortical wounds by the topically applied steroids may be mediated via the effects of the steroids on the secretion of ECM molecules required for effective cell migration into the wound, and/or on reduction of leptomeningeal cell proliferation. To elucidate which of these two mechanisms might underlie the in vivo changes that we observed, we assessed the effects of these steroids on laminin secretion and cell proliferation in vitro. Laminin was chosen because our in vitro studies showed that leptomeningeal cells attach and spread best on a laminin substrate as compared with fibronectin or collagen, thus suggesting that these cells are likely to use a laminin-mediated mechanism to migrate into the wound. Under our culture conditions one of the steroids

(betamethasone) slightly decreased laminin secretion by these cells as detected by Western blots. In contrast, leptomeningeal cell proliferation was significantly reduced by both steroids. As with the in vivo findings, betamethasone had a more potent effect on leptomeningeal cell proliferation than halcinonide. These findings suggest that steroids may decrease leptomeningeal cell infiltration into the wound at least in part by reducing cell proliferation.

The macrophages that appear in CNS wounds (Giulian et al., 1989) release cytokines that could attract leptomeningeal cells into the wound. The diffuse distribution of macrophages in the lesioned area after steroid treatment may therefore contribute to the reduction in leptomeningeal cells into the wound. Glucocorticoids are known to inhibit microglial cell proliferation (Ganter, et al., 1992). However, with the topical applications used in this study, the number of ED1+ cells in the area adjacent to and including the lesion were unaffected. Glucocorticoids also reduce the levels of certain cytokines (Munck and Naray-Fejes-Toth, 1994), which may have influenced the infiltration of leptomeningeal cells in our experiments. Increases in the levels of several cytokines and growth factors in CNS wounds have been reported, e.g., TGF-ß (Logan, et al., 1992b), IL-1, IL-2, IL-6 (Nieto-Sampedro and Berman, 1987; Nieto-Sampedro and Chandy, 1987; Taupin et al., 1993; Tchelingerian et al., 1993; Woodroofe et al., 1991), TNF (Taupin et al., 1993; Tchelingerian et al., 1993), and bFGF (Finklestein et al., 1988; Frautschy et al., 1991; Logan et al, 1992a). Antibodies to TGF-ß have been shown by Logan et al., (1994) to reduce the formation of the basal lamina at the lesion interface after cortical stab wounds. The effects of blocking other cytokines and growth factors expressed

at higher levels in injured CNS tissue are not known at present, but are currently being investigated.

Astrocyte responses 21 days after steroid treatment: Reactive gliosis after CNS injury may be induced by cytokines released by macrophages at the lesion. Exogenous cytokines introduced into the CNS can also induce similar changes in astrocytes (Balasingham, et al.,1994; Giulian et al., 1988; Watts et al., 1989; Yong, et al., 1991). Since steroids can attenuate certain cytokine effects (Munck and Naray-Fejes-Toth, 1994), we quantified the number of reactive astrocytes at a distance of 300 μm away from the wall of the lesion, and estimated by Western blots the changes in GFAP content in 2 mm^3 blocks of tissue containing the lesion. Although the Western blots did not show any differences between any of the lesioned groups, the number of intensely GFAP+ astrocytes in both the steroid and placebo treated groups were significantly decreased as compared with the lesioned but untreated control group. This suggests that the occlusive effects of the placebo ointments may have a protective effect, perhaps by reducing the spread of inflammatory mediators released by macrophages that accumulate on the surface of the lesioned cortex or by mast cells in the periosteum and dura (Johnson et al., 1992). The blocking effects of the placebo ointments may be similar to the ability of Petroleum jelly to limit the diffusion of growth factors and the migration of cells in compartment cultures (Campenot, 1982), and to diminish epithelialization after skin wounds (William and Mertz, 1978). The lack of any difference between the steroid and placebo

groups, however, suggests that under these treatment conditions, steroids do not influence astrocyte reactivity.

Although the steroids did not significantly alter the number of astrocyte at a distance of 300 μm from the wall of the lesion, as compared with the placebos, it did alter the arrangement of astrocytes and their processes along the walls of the lesion. In control animals, 3 weeks after lesioning, astrocyte processes were tightly packed and oriented parallel to the lesion to form the new glia limitans. After steroid treatment, the astrocytes particularly in laminin-negative regions were arranged in a loose meshwork. These laminin-negative areas also contained fewer leptomeningeal cells, and in these regions the astrocytic processes appeared to span the wound. These areas are therefore potential sites through which axon growth may occur. The cortical stab wound model, however, is not an appropriate model to study axon regeneration, because of the way axons are organized within the cortex. In some preliminary work in which anti-neurofilament labeling was done after such cortical stab wounds, excellent neurofilament labeling was obtained, but because of the thinness of the lesion, it was difficult to determine whether the axonal labeling stopped at the lesion or extended across it (data not shown). Anterograde labeling of axons, preferably of a long fiber tract, is needed to effectively assess axon regeneration. Such studies are currently being undertaken. Despite the steroid-induced attenuation of the lesion interface, axonal regeneration under these experimental conditions may also be influenced by reactive astrocyte-derived inhibitory molecules (Groves et al., 1993; Le Roux and Rey, 1996; McKeon et al., 1991; Meiners et al., 1995; Pindzola et al., 1993). How

such astrocyte-derived inhibitors may be influenced by the steroid treatments is also currently under investigation.

Strategies such as these, designed to reduce the infiltration of leptomeningeal cells into CNS wounds, may modulate the lesion interface to permit axon regeneration across it. Successful long distance regeneration of axons, however, will require appropriate neurotrophic support and neutralization of axon growth inhibitory molecules.

Figure 1: (A) Laminin-like immunoreactivity 3 weeks after cerebral cortical stab injury in control untreated lesion (A), halcinonide-placebo (B) and halcinonide (C) treated rats. The anti-laminin labeling is continuous along much of the length of the lesion in untreated (A) and placebo treated (B) rats. In contrast, the labeling is patchy and discontinuous after halcinonide application (C). Bar=100 μm.

A   B   C

Figure 2: (A) There is marked laminin deposition at the lesion site in a betamethasone -placebo treated animal. However, laminin-like immunoreactivity at the lesion interface is markedly diminished after betamethasone application (B). Bar=100 μm

Figure 3: Total length of laminin-like immunoreactivity expressed as a percentage of the lesion depth (which was estimated by the depth of the autofluorescent hemosiderin deposits). (A) Total length of laminin deposition in halcinonide treated animals is significantly less than either control ($p < 0.01$) or placebo ($p < 0.05$). (B) Total length of laminin deposition in the betamethasone treated animals is also significantly less than either control ($p < 0.01$) or placebo ($p < 0.05$).

A

B

Figure 4: The number of leptomeningeal-like cells per 500 μm length of the lesion in laminin-positive (LAM+) and laminin-negative (LAM-) areas in control and steroid treated animals. (p<0.008)
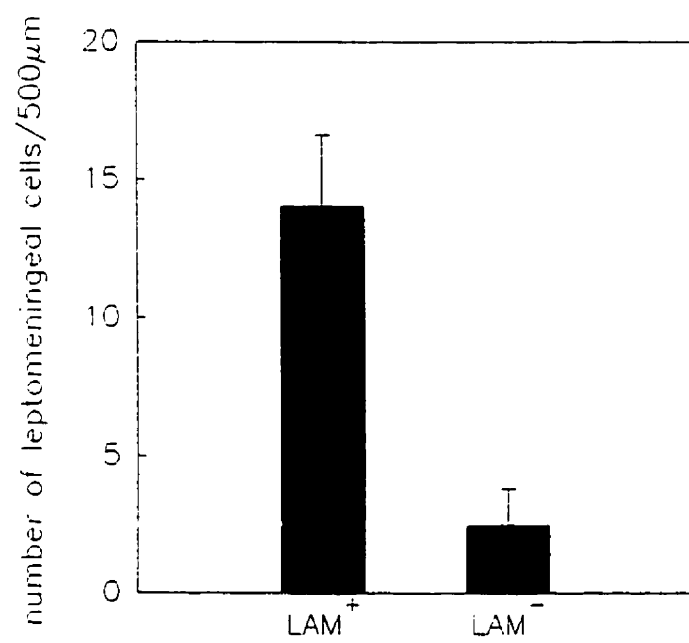
Figure 5: Laminin+ (A) and laminin-negative (C) patches in control and halcinonide treated rats, respectively. B and D show the Nuclear Yellow labeled cells in the same field as in A and C, respectively. Note the increased cellularity associated with the laminin+ region of the lesion (B). Bar=50 μm.
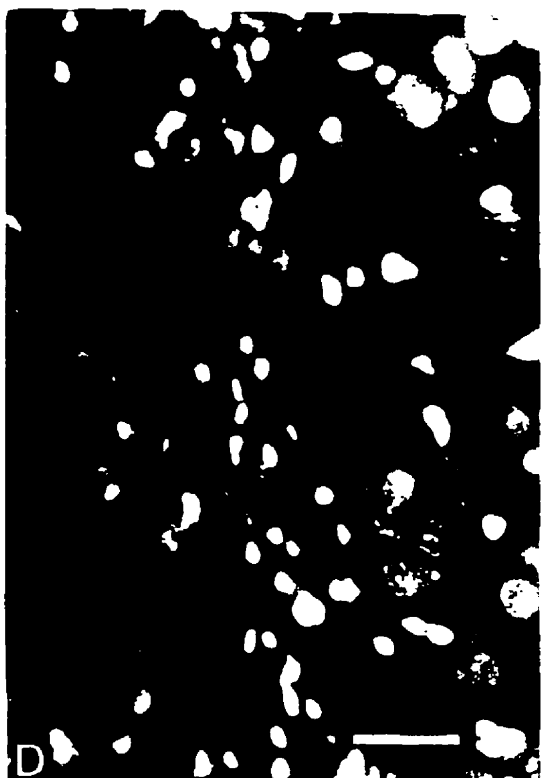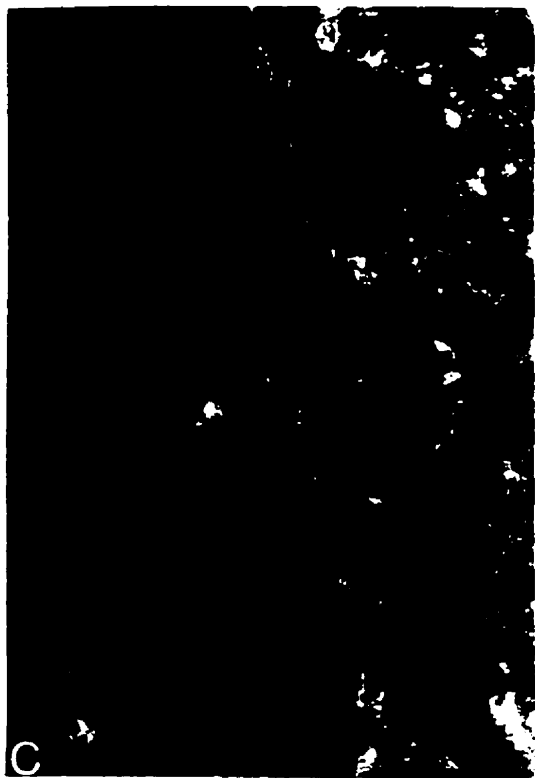
Figure 6: Electron micrographs through the area of the lesion in a control untreated rat (A,B), and after betamethasone application (C-E). Note the presence of an elongated leptomeningeal cell in the untreated lesion (arrow in A). Higher magnification of this area is shown in B. Note the basal lamina between the leptomeningeal cell and glia limitans (arrow in B). Leptomeningeal cells are not seen in the area of the lesion shown in C from a betamethasone treatment rat. However, macrophages can be seen at the top and bottom of this micrograph (C). The lesion extends between the two macrophages. (D) Higher magnification of the area near the upper arrow in C shows macrophages containing inclusion bodies, and convoluted basal lamina (arrows). (E) Higher magnification of the area near the lower arrow in C. Note the absence of basal lamina and leptomeningeal cells in this micrograph. Astrocyte processes also have fewer glial filaments as compared to the untreated lesion shown in A and B. Bar=3 μm in A & C; 0.5 μm in B, D, E.
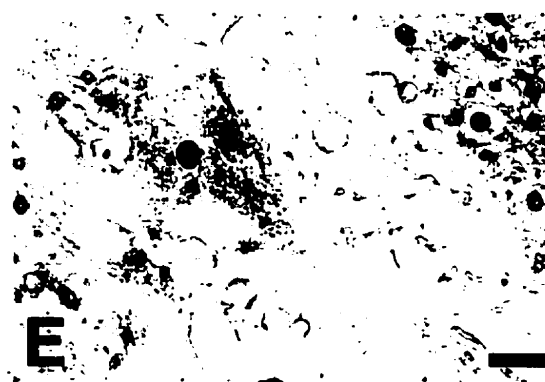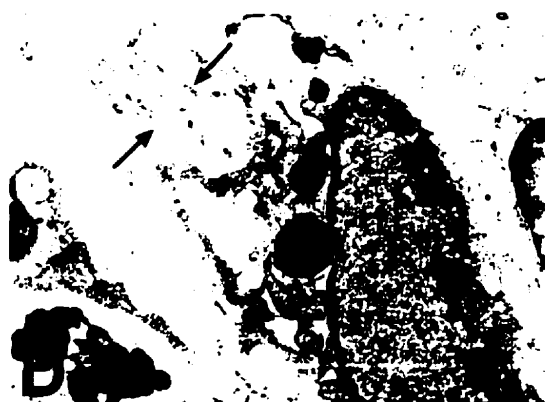
Figure 7: (A) Anti-GFAP staining of a control untreated animal shows tightly packed astrocyte processes oriented parallel to the lesion. (B) Corticosteroid application results in a loosely arranged network of astrocytes at the lesion interface. Bar=50 μm.

Figure 8: Anti-GFAP Western blot of tissue obtained from normal unlesioned (lane 1), lesioned untreated (lane 2), lesioned placebo treated (lane 3), and halcinonide treated (lane 4) animals.

1   2   3   4

Figure 9: (A)   ED1+ macrophages are localized to the lesion interface in control animals. (B) These cells are more dispersed near the lesion after steroid treatment.   Bar=50 μm.
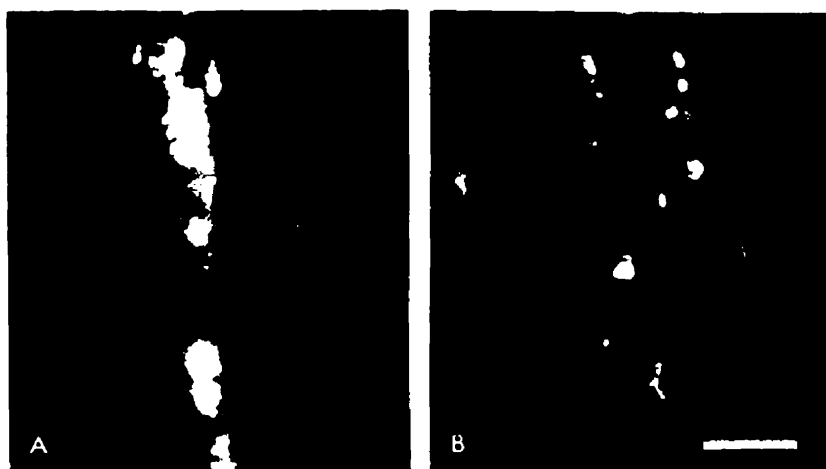
Figure 10: Spreading of leptomeningeal cells on various ECM substrates. The percentage of cells at or greater than a certain area (vertical axis) are expressed as a function of the size of the cell in microns (horizontal axis). Laminin (A) is a more adhesive substrates for meningeal cells, compared to fibronectin (B), collagen (C), and poly-L-lysine (D).
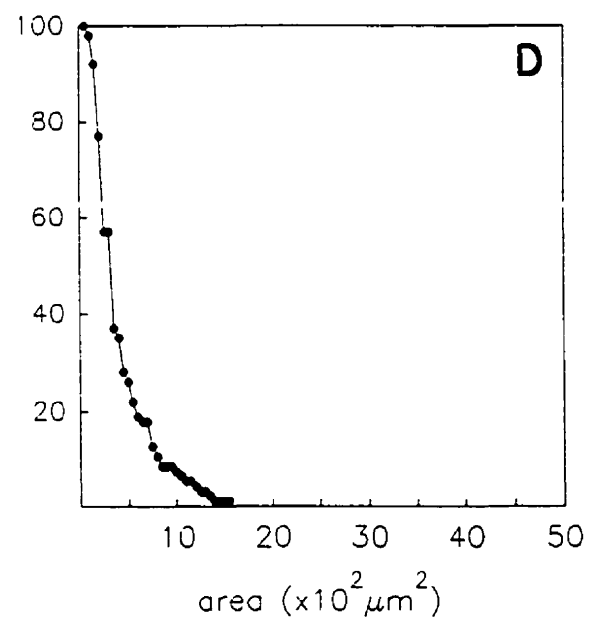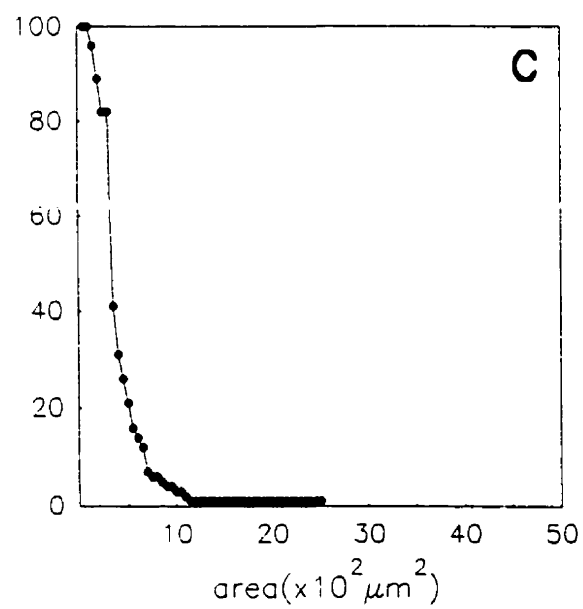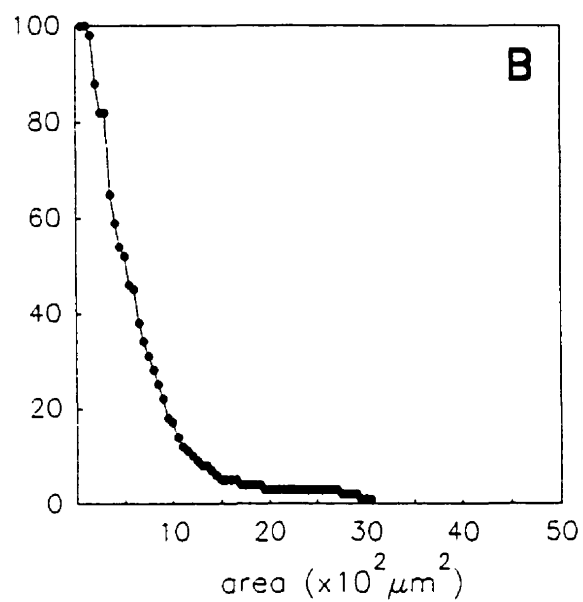
Figure 11: Anti-laminin Western blot of cultures of adult rat leptomeningeal cells treated with control medium (lane1), halcinonide (lane 2), and betamethasone (lane 3). Note that the level of laminin is slightly reduced after betamethasone treatment. The staining of the lower molecular weight bands in lanes 1 and 2 are likely break-down products of laminin.

Figure 12:   Leptomeningeal cell proliferation in vitro is reduced by 31% by halcinonide, and 61%   by betamethasone,   compared to treatment with control medium.

Summary

1) In this thesis I have quantified the basal lamina, leptomeningeal cell, astrocyte and macrophage components of the mature lesion interface, three weeks after stab wound lesioning in the adult rat CNS, using immunocytochemical techniques. The lesion interface comprises leptomeningeal cells lined by a laminin-immunoreactive basal lamina, a GFAP$^+$ glia limitans, and ED1$^+$ macrophages.

2) I have demonstrated the ultrastructure of the normal mature lesion interface with electron microscopy. There is a tightly interdigitating glia limitans that forms adjacent to the basal lamina and together they delimit the CNS environment from the infiltrating leptomeningeal cells and few macrophages.

3) I have shown that topical steroid treatment with halcinonide or a more potent steroid, betamethasone, results in a disruption in the formation of the basal lamina, the glia limitans, and in greater dispersion of macrophages as visualized by immunofluorescence. The areas of disruption in the basal lamina correspond to areas where the numbers of elongated cell nuclei, most likely leptomeningeal cells, are reduced.

4) Electron microscopy reveals that steroid treatments reduce leptomeningeal cell infiltration and basal lamina formation after lesioning.

5) The parenchymal astrocyte and macrophage responses three weeks after stab wounding are diminished.

6) Placebo ointments of both steroids seem to have an occlusive effect such that the numbers of reactive astrocytes in the brain parenchyma are significantly different from untreated controls.

7) Leptomeningeal cells spread better on laminin than fibronectin, collagen, or poly-L-lysine substrates.

8) Under our culture conditions, betamethasone slightly reduced laminin secretion by leptomeningeal cells but halcinonide did not affect laminin secretion.

9) Both steroids reduce leptomeningeal cell proliferation in culture in proportion to the potency of the steroid.

Conclusions

Topical glucocorticoids attenuate the formation of all components of the mature lesion interface including the glia limitans, the basal lamina and the leptomeningeal cell infiltration. Though the reduction in leptomeningeal cell infiltration into the wound may be multifactorial, our in vitro experiments suggest that it may be mediated at least in part by steroid reduction of leptomeningeal cell proliferation. The literature would support that the reduction in leptomeningeal cell infiltration into the wound would correlate with the attenuation of basal lamina and glia limitans formation which we demonstrate through our in vivo experiments. Where the lesion interface is diminished, the brain parenchyma seems integrated. In other models, this continuity of CNS tissue would encourage axonal regeneration, for example in embryonic transplants (Kruger et al., 1986).

Although glucocorticoids can mediate the desired reduction in leptomeningeal cell infiltration, it still remains to be shown that the attenuation of the interface can increase axon regeneration. Other growth promoting strategies such as neutralization of myelin-associated inhibitors and the addition of growth factors must be combined with the attenuation of the lesion interface.

While it is apparent that glucocorticoids decrease lesion interface formation, glucocorticoids have many effects, some of which may be detrimental, as seen in the pilot experiments where certain animals had focal infections or hematomas. Further work of this nature would focus on diminishing leptomeningeal cell infiltration by

affecting cell adhesion, migration or proliferation into the wound. This would entail identifying specific molecular cues that promote leptomeningeal cell invasion and counteracting them with inhibitors such as antibodies.

Currently in the lab, antibodies against bFGF have been used successfully in diminishing some ECM components of the interface (Wang and David, 1996).

Future Directions

1) Testing the effects of steroids on the modulation of selected cytokines in vitro and in vivo.

2) Using antibodies against selected cytokines and demonstrating their effects on the formation of the lesion interface.

3) On a broader scale, strategies to attenuate lesion interface formation might include:

a) antibodies against cytokines,

b) development of soluble cytokine receptors,

c) use of macrophage deactivating agents,

d) use of function-blocking antibodies against ECM molecules and adhesion molecules.

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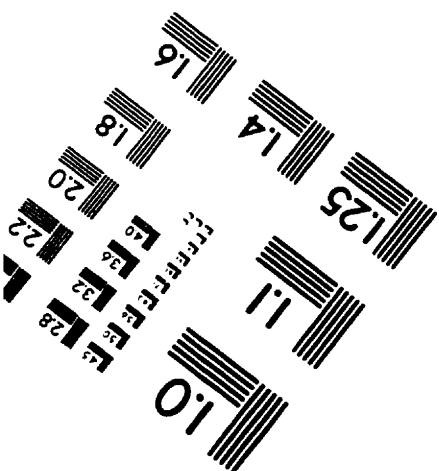vivo microdialysis: evidence of a role for microglia in cytokine production, J. Neuroimmunol 33 :227-236.
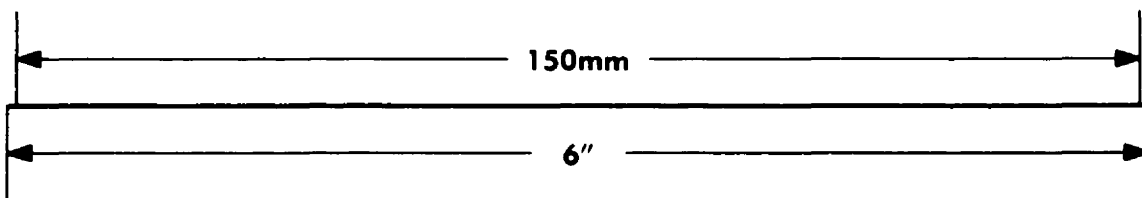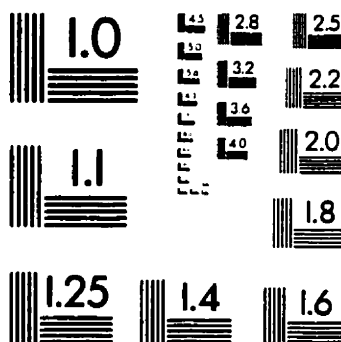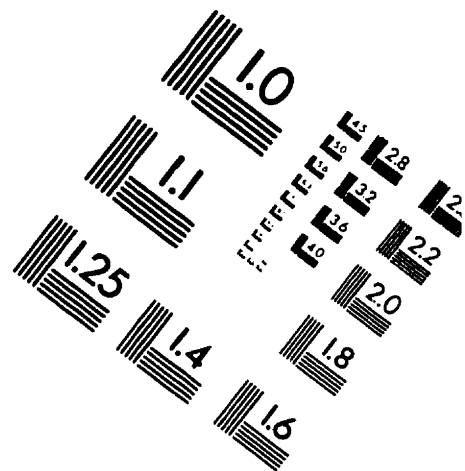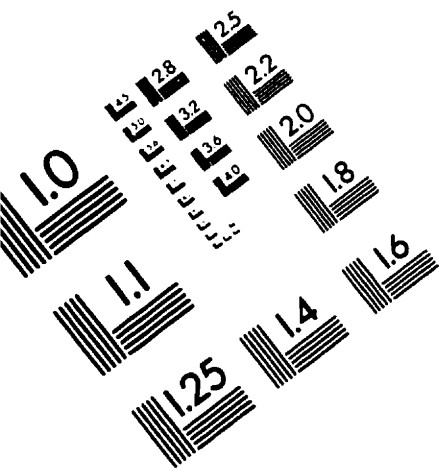
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IMAGE EVALUATION
TEST TARGET (QA-3)

150mm

6"