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CHEMOKINES AND INFLAMMATION IN WOUND HEALING FOLLOWING SPINAL CORD CONTUSION INJURY IN THE MOUSE

DISSERTATION

Presented in Partial Fulfillment of the Requirement for the Degree

Doctor of Philosophy in the Graduate School of

The Ohio State University

By

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* * * * *

The Ohio State University
2002

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ABSTRACT

A three-step approach has been used to study the role of β chemokines such as monocyte chemoattractant protein-1 (MCP-1) in the pathophysiology of spinal cord injury (SCI). Initially, a mouse model of spinal contusion injury using a computer-controlled electromagnetic spinal cord injury device (ESCID) was characterized in C57Bl/6 mice with behavioral and histopathological outcome assessment. The results demonstrate that graded contusion injuries can be produced reliably in mice using the ESCID and behavioral and histological outcome measures correlate with the severity of injury.

We then applied this model in transgenic mice with a depletion of CCR2, a principal receptor for MCP-1 to determine the specific role of binding of MCP-1 to this receptor in the inflammatory process after contusion injury. CCR2 deficient mice resulted in significantly reduced Mac-1 immunoreactivity restricted to the lesion epicenter at 7 days postinjury. The regions devoid of Mac-1 immunoreactivity corresponded to areas of reduced myelin degradation at this time. These results indicate that chemokines acting through CCR2 contribute to the early recruitment of monocytes to the lesion epicenter following SCI.

In addition, we used this mouse model to further characterize the general patterns of chemokine expression, cellular reactions, and scar formation in two
commonly used inbred mouse strains: C57Bl/6 and 129X1/SvJ. We find that 129X1/SvJ mice exhibit differences in the patterns of macrophage and astrocyte response at the core lesion area of the epicenter beginning at 14 days postinjury which correspond to enhanced axon growth and laminin deposition. Studies of chemokine expression after injury in these two mouse strains demonstrate a primary role for MCP-1 in early macrophage recruitment and suggest a secondary role of other β chemokines such as MCP-3, MIP-1α, or MIP-1β in the cellular response after injury.

Taken together, the results indicate that the inflammatory responses following spinal contusion injury are precisely regulated by the expression of specific chemokines. These work represent an important step to elucidate the complex cellular and molecular mechanisms following SCI and will have important implications for developing strategies to improve the pathophysiological outcome after injury.
DEDICATION

To my deceased mother and father for their constant love and great expectations. And to my husband Fan Gui, and my daughter Cynthia, whose love and support makes everything seem possible.
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With so many to thank I would like to begin with my advisor, Dr. Lyn Jakeman, who has supported and supervised all of my research work since I came to the lab. She contributed a great amount of time to my training even before she became my advisor and taught me a lot on how to think and write scientifically, or how to pursue a question in a scientific manner. She was always accessible to talk with my data and share with my exciting and discouraging moments. I am grateful for all of them.

Gracious thanks to Dr. Bradford Stokes, who was my advisor and now my co-advisor. His constant optimism and encouragement made me work in a comfortable environment. He was such a great man who not only cared about my research but also my family and was always available to discuss those issues when they came up.

This dissertation would not be made possible without the technical assistance from the members of Dr. Stokes’ lab over the years. I give special thanks to Zhen Guan, the superb “neurosurgeon” in the lab, who taught me all the tricks on mouse surgery; to Ping Wei for teaching me all staining procedures and immunohistochemistry; to Fengqin Yin for her surgical assistance. They have

v

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dad passed away and never loss confidence on me.
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PUBLICATIONS


FIELDS OF STUDY

Major Field: Physiology
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Lesion morphometry from LFB stained sections. (A) Total cross-sectional (CS) area at the epicenter was decreased following 0.5mm and 0.8mm displacement vs. laminectomy (LA) control (*p < 0.001). Significant differences were also found between the 0.3mm and 0.5mm, and between the 0.3mm and 0.8mm injury groups (*p < 0.001). (B) The amount of spared white matter reported as the % of total CS area of the cord revealed significant differences between all three injury groups and laminectomy controls (*p < 0.001), and between each of the injury groups (*p < 0.001 vs. 0.3mm, *p < 0.001 vs. 0.5mm group). (C) Lesion length was significant greater when the injury level was higher (*p < 0.001 vs. 0.3mm group, *p < 0.001 vs. 0.5mm group).

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GFAP-positive astrocytes (C) except for small foci surrounding some blood vessels (*). The extracellular matrix stained most densely with antisera against fibronectin (F). Laminin immunoreactivity (E) was observed in association with vessels (arrowheads), but was also found in thickened cables (arrows). Collagen IV (G) antisera stained thin tendrils, many of which appeared to be oriented perpendicular to the plane of the section (arrowheads). Scale bars: A. 100μm. B-G. 20μm

Photomicrographs of the immunohistochemistry studies showed different zones of the lesion area 9 weeks after 0.5mm injury. A discrepancy in spatial extent between LFB stained tissue (A and D) and adjacent GFAP stained tissue (B&E) revealed a transition region (outlined in C-I) of reactive gliosis at the edge of the lesion. While the central core area was occupied by dense fibronectic scar tissue (F), the transition area was characterized by a high density of demyelinated axons (D and G), reactive astrocytes (E), reactive microglia (H), and laminin (C). In contrast, fibronectin (F), and collagen IV (I) were less prominent in this region than in the nearby central core area. Scale bars: A-B. 100μm. C-I. 40μm

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Distribution of Mac-1 proportional area measures at intervals spanning the rostro-caudal extent of the lesion at 7 dpi. Differences in Mac-1 immunoreactivity were restricted to the center of the lesion and 0.2 mm caudal to the epicenter (t-test; *=p<0.05; **=p<0.01). solid bars correspond to CCR2 (+/+) specimens and open bars correspond to CCR2 (-/-) specimens
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Sample images from RPA using probe sets to identify chemokine (A) and chemokine receptor (B) mRNAs. Total RNA extracted from one spinal cord (20 μg) was loaded per lane.

Quantitative analysis of chemokine and chemokine receptor mRNA expression obtained from RPA. Bars represent mean ± SEM of the densitometric ratio of protected species, calculated relative to L32 density. Black bars represent CCR2 (+/+) ; n=7 at 1 dpi; n=5 at 7 dpi. Open bars are CCR2 (-/-); n=12 at 1 dpi, n=9 at 7 dpi. Two way ANOVA was used to determine the effect of dpi and genotype on group means (legend, upper right of each panel; int.= interaction effect). Asterisks above each pair of bars represent the results of t-tests to compare densitometric ratio for each chemokine at 1 or 7 dpi. * p<0.05; ** p<0.01; ***p<0.001.

LFB analysis of the peripheral white matter rim at the lesion epicenter at 7 dpi (A, B) and 14 dpi (C, D). A, C. Digital photomicrographs of LFB stained sections from CCR2 (+/+) and (-/-) specimens. Note the residual LFB stain in the center of the CCR2 (-/-) specimen at 7 dpi only. Residual or spared white matter in the peripheral rim was identified and manually outlined as indicated for quantitative analysis. Scale = 200
B.D. White matter (LFB staining) in the peripheral rim was expressed as a percentage of the Cross-sectional Area (CS Area) at the lesion epicenter. CCR2 (+/-) mice showed a non-significant trend toward decreased tissue sparing at 7 and 14 days as compared with (+/+). Values represent mean ± SEM: 7dpo (+/+, n=9; +/-, n=13; p=0.07); 14dpo (+/+, n=3; +/-, n=4; p=0.13).

Lesion morphometry from LFB stained section in C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice at 3, 7, 14, 28, and 63 dpi. (A) Total cross-sectional area at the epicenter was significantly decreased over time (**p < 0.01). The rate of tissue shrinkage was greater in C57Bl/6 mice than in 129X1/SvJ mice (**p < 0.01). (B) The total spared white matter was also decreased over time (**p < 0.01) and less was spared in C57Bl/6 mice (*p < 0.05). (C) The amount of spared white matter reported as the % of total cross-sectional area of the cord was not significantly changed over time and not different between two mouse strains.

Lesion morphometry from LFB stained section in C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice at 3, 7, 14, 28, and 63 dpi. (A) Total cross-sectional area at the epicenter was significantly decreased over time (**p < 0.01). The rate of tissue shrinkage was greater in C57Bl/6 mice than in 129X1/SvJ mice (**p < 0.01). (B) The total spared white matter was also decreased over time (**p < 0.01) and less was spared in C57Bl/6 mice (*p < 0.05). (C) The amount of spared white matter reported as the % of total cross-sectional area of the cord was not significantly changed over time and not different between two mouse strains.

Representative photomicrographs of sections stained with Luxol fast blue (LFB) at the injury epicenter at 3 (A-B), 7 (C-D), 14 (E-F), and 28 (G-H) days post injury (dpi) in C57Bl/6 (A, C, E, G) and 129X1/SvJ (B, D, F, H) mice. Scale bars, 200 μm.

Representative photomicrographs of Mac-1 immunoreactivity at the lesion epicenter of C57Bl/6 mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), and 28 days (G-H) after the 0.5mm contusion injury. A few round phagocytic profiles are found within the gray matter region and surrounding white matter at 3 dpi (A and B). By 7dpi, large round macrophages occupied most of the cross-sectional area of the spinal cord with intense Mac-1 immunoreactivity (D). By 14 dpi, the intensity of Mac-1 staining was less than that seen at 7 dpi, and was associated with large round cells in the center of the lesion (F). Reactive microglia profiles can be found within the peripheral tissue rim (E). By 28 dpi, large round cells were still present in the center of the lesion, but were very lightly stained by Mac-1 (H). Scale bars A, C, E, and G, 200 μm; B, D, F, and H, 50 μm.

Representative photomicrographs of Mac-1 immunoreactivity at the lesion epicenter of 129X1/SvJ mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), 28 days (G-H), and 63 days (I-J) after the 0.5mm contusion injury. The pattern and distribution of Mac-1 immunoreactivity at 3 and 7 dpi were similar as seen in the C57Bl/6 mice. By 14 dpi, there were less Mac-1 stained round cells in the center of the lesion (F), and the intensity of Mac-1 staining was less than that seen at 7 dpi. Reactive microglia profiles can also be found within the peripheral tissue rim with
strong Mac-1 immunoreactivity (E). By 28 dpi, there were nests of round cells lightly stained with Mac-1 in the center of the lesion (H). Mac-1 immunoreactivity associated with peripheral reactive microglia decreased compared to that at 14 dpi (G). By 63 dpi, overall Mac-1 immunoreactivity was low, and revealed the same pattern of staining as seen at 28 dpi (I-J). Scale bars A, C, E, G, and I. 200 μm; B, D, F, H, and J = 50 μm

Representative photomicrographs of F4/80 immunoreactivity at the center of the lesion at the epicenter of C57Bl/6 (A, C, E, G, I) and 129X1/SvJ (B, D, F, H, J) mice at 3, 7, 14, 28, and 63 days after the 0.5mm contusion injury. The pattern and distribution of F4/80 immunoreactivity at 3 (A-B) and 7 (C-D) dpi were similar as revealed by Mac-1 immunostaining and between two strains of mice. By 14 dpi, F4/80 immunoreactivity remained robust and was associated with large packed round cells in the lesion center in C57Bl/6 mice (E). While in 129X1/SvJ mice, there were less F4/80 stained round cells in the center of the lesion (F). By 28 dpi in C57Bl/6 mice (G), those large round cells remained in the lesion center and were still stained strongly with F4/80, while in 129X1/SvJ mice, there was much less F4/80 stained cells (H) and the intensity of F4/80 staining appeared less than that seen in C57Bl/6 mice. The differences in F4/80 staining pattern between C57Bl/6 (I) and 129X1/SvJ (J) mice were also clearly seen at 63 dpi. Scale bars. 50 μm

Quantitative comparisons of Mac-1 (A) and F4/80 (B) immunoreactivity at the center of the lesion of the injury epicenter in C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice at different time points post injury. Two way ANOVA revealed significant strain differences in both staining. At 14 and 63 dpi, Mac-1 and F4/80 stained macrophages were significantly less in 129X1/SvJ mice (***p < 0.001 vs C57Bl/6 mice). At 28 dpi, F4/80 stained macrophages were also were significantly less in 129X1/SvJ mice (***p < 0.001 vs C57Bl/6 mice)

Representative photomicrographs of GFAP immunoreactivity at the lesion epicenter of C57Bl/6 mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), and 28 days (G-H) after the 0.5mm contusion injury. At 3dpi, GFAP stained astrocytes showed hypertrophied morphology in both gray and white matter. By 7 dpi, GFAP+ reactive astrocytes were most prominent in the peripheral rim. In the lesion center, GFAP staining was greatly reduced and only seen associated with some blood vessels (D). From 14 dpi and thereafter, the center of the lesion remained unstained by GFAP (E-H). Scale bars A, C, E, and G. 200 μm; B, D, F, and H. 50 μm

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Representative photomicrographs of GFAP immunoreactivity at the lesion epicenter of 129X1/SvJ mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), 28 (G-H), and 63 (I-J) days after the 0.5mm contusion injury. At 3 and 7 dpi, GFAP staining was similar as seen in C57Bl/6 mice. By 14 dpi, unlike in C57Bl/6 mice, there were increased GFAP stained astrocytes seen in the center of the lesion. These GFAP stained astrocytes remained in the lesion center by 28 and 63 dpi. Scale bars A, C, E, G, and I, 200 µm; B, D, F, H, and J, 50 µm.

Quantitative comparisons of GFAP (A) and neurofilament (B) immunoreactivity at the center of the lesion of the injury epicenter in C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice. *p < 0.05. **p < 0.01. ***p < 0.001 vs C57Bl/6 mice at same time interval.

Representative photomicrographs of neurofilament immunoreactivity at the lesion epicenter of C57Bl/6 mice at 3 day (A-B), 7 days (C-D), 14 days (E), and 28 days (F) after the 0.5mm contusion injury. Note a few axons in the center of the lesion since 7 dpi. Scale bars A and C, 200 µm; B, D, E, and F, 50 µm.

Representative photomicrographs of neurofilament immunoreactivity at the lesion epicenter of 129X1/SvJ mice at 3 day (A-B), 7 days (C-D), 14 days (E), and 28 days (F) after the 0.5mm contusion injury. Note few axons were left in the center of the lesion at 7 dpi (D), but obvious more axon profiles by 14 (E) and 28 (F) days after injury. Scale bars A and C, 200 µm; B, D, E, and F, 50 µm.

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Representative photomicrographs of laminin immunoreactivity at the lesion epicenter of 129X1/SvJ mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), and 28 days (G-H) after the 0.5mm contusion injury. Note that the laminin immunoreactivity in the center of the lesion remained intense even by 14 (F) and 28 (H) dpi. Scale bars A, C, E, and G, 200 µm; B, D, F, and H, 50 µm.
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Sample images from RPA using probe sets to identify chemokine mRNAs in the spinal cords of C57Bl/6 (B) and 129X1/SvJ (W) mice.

Time course of MIP-2 (A) and IP-10 (B) mRNA expression in the injured spinal cords of C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice after the 0.5mm contusion injury. Bars represent mean ± SEM of the densitometric ratio of protected species, calculated relative to L32 density. Two way ANOVA was used to determine the effect of dpi and genotype on group means (legend, upper right of each panel). Asterisks above each bar represent the results of Dunnett post-hoc test to compare values at each time point with matched control (* p < 0.05; ** p < 0.01). Strain differences at each time point were revealed by Bonferroni's post-hoc test (^^ p < 0.01).

Time course of MCP-1 (A) and MCP-3 (B) mRNA expression in the injured spinal cords of C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice after the 0.5mm contusion injury. Bars represent mean ± SEM of the densitometric ratio of protected species, calculated relative to L32 density. Two way ANOVA was used to determine the effect of dpi and genotype on group means (legend, upper right of each panel). Asterisks above each bar represent the results of Dunnett post-hoc test to compare values at each time point with matched control (* p < 0.05; ** p < 0.01).

Time course of MIP-1α (A) and MIP-1β (B) mRNA expression in the injured spinal cords of C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice after the 0.5mm contusion injury. Bars represent mean ± SEM of the densitometric ratio of protected species, calculated relative to L32 density. Two way ANOVA was used to determine the effect of dpi and genotype on group means (legend, upper right of each panel). Asterisks above each bar represent the results of Dunnett post-hoc test to compare values at each time point with matched control (* p < 0.05; ** p < 0.01). Strain differences at each time point were revealed by Bonferroni's post-hoc test (^ p < 0.05).
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<td>Biomechanical parameters as predictors of behavioral and histological outcomes at 9 weeks postinjury. The correlation between open field locomotion (BBB score) and biomechanical parameters (displacement, peak force, and maximum impulse/momentum) or histological outcomes represent Spearman correlation coefficient (r_s). All other values represent Pearson correlation coefficient (r). Significance is indicated where p &lt; 0.05*, 0.01**, 0.001*** ........................................................................................................64</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>CCR2</td>
<td>CC chemokine receptor 2</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSPG</td>
<td>chondroitin sulfate proteoglycan</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ESCID</td>
<td>electromagnetic spinal cord injury device</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>IP-10</td>
<td>interferon-γ-inducible protein-10</td>
</tr>
<tr>
<td>LFB</td>
<td>Luxol fast blue</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccaride</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MCP-3</td>
<td>monocyte chemoattractant protein-3</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIP-2</td>
<td>macrophage inflammatory protein-2</td>
</tr>
<tr>
<td>MIP-1α (β)</td>
<td>macrophage inflammatory protein-1α (β)</td>
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<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>RPA</td>
<td>Rnase protection assay</td>
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<tr>
<td>SCI</td>
<td>spinal cord injury</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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INTRODUCTION

Background

Traumatic injury to the adult mammalian spinal cord results in immediate physical disruption of the spinal cord parenchyma, followed by a sequence of secondary events, which extend the region of tissue destruction beyond the primary injury site (Balentine, 1978; Blight, 1996; Schwab and Bartholdi, 1996). Reactive processes that occur within minutes or hours and continue for days or weeks, collectively referred to as secondary injury, are believed to be responsible for progressive tissue damage and permanent neurological deficits (Young, 1993; Dumont et al., 2001). Ischemia (Ducker et al., 1978; Holtz et al., 1989; Senter and Venes, 1978), intracellular calcium accumulation (Balentine and Spector, 1977; Moriya et al., 1994; Stokes et al., 1983), lipid peroxidation (Hall and Braughler, 1989; Braughler and Hall, 1982), excitotoxicity (Liu et al., 1991; Panter et al., 1990), and inflammation (Dusart and Schwab, 1994; Popovich et al., 1997) are among the proposed mechanisms that contribute to secondary injury.

Although a complex secondary cascade is implicated through experimental studies of various spinal cord injury (SCI) models, many features of the histopathological evolution of the lesion site are similar across models (Schwab and Bartholdi, 1996). Shortly after injury, the injured spinal cords show hemorrhagic petechiae and edema that progress over several hours to neuronal cell death and apoptosis (Balentine, 1978; Dusart and Schwab.
1994; Shuman et al., 1997; Beattie et al., 2000). Several weeks after contusion injury, the progressive necrosis often leads to development of large fluid-filled cavities or fibrotic scar tissues that extend rostrally and caudally over several segments of the cord (Reier et al., 1988; Bunge et al., 1993; Dusart and Schwab, 1994). Distal axons in the surrounding white matter undergo Wallerian degeneration, and apoptosis is observed in rostral and caudal white matter for several months (Schwab and Bartholdi, 1996; Crowe et al., 1997).

Despite the degenerative processes described above, there is also clear evidence that the spinal cord possesses an intrinsic capacity for repair (Beattie et al., 1997; Reier et al., 1983; Fitch and Silver, 1999; West et al., 2001). Glial cell proliferation, abortive axonal regeneration, partial remyelination, and neovascularization are all well observed phenomenon after traumatic injury to the spinal cord (Blight, 1991b; Guth et al., 1985; Loy et al., 2002; Zhang and Guth, 1997). Thus, the pathophysiological sequelae after SCI is best characterized by both degenerative and reparative processes (Guth et al., 1999; McTigue et al., 2000). However, unlike the peripheral nervous system or other organs, the wound healing response is far less complete and degenerative events predominate after SCI resulting in permanent loss of function (Means and Anderson, 1983; Reier et al., 1983).

Clinical attempts to find effective therapies to manage spinal cord injury in humans are guided by an understanding of the underlying pathophysiology. The key to understanding the complex processes is to elucidate the critical events that regulate the balance between these destruction and repair. More than a decade ago, the Second National Spinal Cord Injury Study (NASCIS2) demonstrated that high doses of the...
corticosteroid methylprednisolone (MP) were effective in improving motor and sensory functions if given to patients within 8 hours after SCI (Bracken et al., 1990). While the mechanisms of action of MP are not completely understood, the results suggest that there is an interval during which many secondary events may happen and can be modulated. The inflammatory response that is initiated in response to injury is such an event that plays an essential role in both tissue destruction and repair (Dusart and Schwab, 1994; Guth et al., 1994; Popovich et al., 1997; Young, 1993; Zhang et al., 1997).

**Inflammatory Response in CNS Injury**

Traumatic injury to the CNS is followed by an inflammatory cascade that begins within hours and peaks within several days (Carlson et al., 1998; Dusart and Schwab, 1994; Popovich et al., 1997). This cascade is initiated by immediate endothelial disruption and induces changes in vascular permeability, edema, the release of inflammatory mediators, and cellular reactions which are comprised of activation of resident glial cells and recruitment of peripheral inflammatory cells (Blight, 1985; Popovich et al., 1993; Schnell et al., 1999).

The CNS had long been considered to be an immunologically privileged site because of observations involving the lack of lymphatic vessels, the physical sequestration by the blood-brain barrier and delayed rejection of tumor allografts (Streilein, 1995). Nevertheless, studies on inflammation in immune-mediated pathological states, such as multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), demonstrate that the CNS is of immune competence (Fabry et al., 1994; Hickey and Kimura, 1988; Hickey, 1991; Hickey et al., 1991). In fact, the inflammatory response is
a universal event that is observed not only in MS and EAE, the typical inflammatory disease in the CNS, but also in various acute and chronic neurological diseases, such as stroke. Alzheimer's disease, HIV-related dementia, as well as head and spinal cord trauma (Perry et al., 1995; Perry et al., 1997; Popovich et al., 1997).

Although the responses in the CNS, including inflammatory cell infiltration, cytokine production, and upregulation of major histocompatibility complex (MHC) molecules, are similar to those of the acute inflammatory response seen in peripheral tissues, there are clear differences. In general, an equivalent stimulus leads to a much weaker or delayed response in the CNS parenchyma. For example, lipopolysaccharide (LPS), a potent inflammatory agent, is capable of eliciting a strong inflammatory response in the periphery (rapid neutrophil influx followed by monocyte entry). In contrast, following the injection of LPS into the mouse brain, the brain fails to recruit significant numbers of neutrophils and recruits monocytes only in a delayed fashion (Andersson et al., 1992). Interleukin-1β (IL-1β), another pro-inflammatory cytokine, also only causes minimal recruitment of neutrophils when injected into the rat brain (Anthony et al., 1997). In Wallerian degeneration of peripheral nerves, there is very pronounced recruitment of monocytes (Beuche and Friede, 1986; Griffin et al., 1992). In contrast, either retrograde or anterograde degeneration in the CNS at sites distant from region of trauma results in only transient activation of resident microglia but minimal monocyte recruitment (Perry et al., 1994; Graeber et al., 1998). The peak macrophage response is also delayed in the lesioned spinal cord or optic nerve as compared with the sciatic nerve (Dusart and Schwab, 1994; Perry et al., 1994; Perry et al., 1987). The differences between the inflammatory response observed in peripheral tissues and in the CNS...
CNS following different types of challenge demonstrate that the inflammatory response is a highly modified event in the CNS (Perry et al., 1995).

An effective and self-limited inflammatory response is vital for the tissue repair in the periphery. In the example of a skin wound, the blood-derived leukocytes are recruited to the lesion site to remove necrotic cellular debris by phagocytosis, release enzymes that loosen the extracellular matrix, and secrete cytokines and growth factors that promote the proliferation of cells essential for reestablishment of a normal tissue environment (Kirsner and Eaglstein, 1993; Waldorf and Fewkes, 1995). However, in the case of SCI, this inflammatory response often fails to bring about complete functional recovery. Instead, the cavitation and scar formation that occurs at the injury site serves to create an environment hostile for axon regeneration (David and Aguayo, 1981; Guth et al., 1980; Fitch and Silver, 1999; Schwab and Bartholdi, 1996). Currently, there are two opposing yet seemingly related theories on the role of inflammation in wound healing after CNS injury. One proposes that regeneration failure and incomplete wound healing in the lesion site are attributed to a restricted inflammatory reaction in the CNS involving insufficient recruitment and activation of macrophages (Lazarov-Spiegler et al., 1998; Schwartz et al., 1999). Evidence that supports this notion includes the finding that the transplantation of activated macrophages into injured CNS results in axonal regrowth and partial functional recovery (Lazarov-Spiegler et al., 1996; Lazarov-Spiegler et al., 1998; Rabchevsky and Streit, 1997). Other work suggests that uncoordinated and non-self-limited inflammatory responses of the injured spinal cord resulting in the continued release of cytotoxic substances are responsible for progressive necrosis and abortive axonal regeneration (Giulian et al., 1993; Zhang et al., 1997). Neuroprotection and
enhanced cellular repair and functional recovery with steroidal and nonsteroidal anti-inflammatory agents support this view (Behrmann et al., 1994; Guth et al., 1994; Hall et al., 1986; Oudega et al., 1999).

The controversial role of the inflammatory response in wound healing after SCI is largely dependant on the diverse effects exerted by its cellular components, namely infiltrating neutrophils, monocytes and lymphocytes, and resident microglia and astrocytes (Toews et al., 1998). Spinal cord injury induced by mechanical, immunological, or excitotoxic insults commonly elicits an inflammatory cellular response that is biased toward a pronounced macrophage and microglial reaction (Andersson et al., 1991; Carlson et al., 1998; Giulian, 1987; Popovich et al., 1997). Thus, the cellular and functional consequences of the macrophage response are an area of intense study in the search of approaches to improve functional repair after SCI (Blight, 1992; Giulian and Robertson, 1990; Perry and Gordon, 1988; Popovich et al., 1999).

The response of macrophages to a traumatic spinal cord injury is marked by the recruitment of peripheral blood-borne monocytes and activation of resident microglia (Dusart and Schwab, 1994; Popovich et al., 1997). In rodents, endogenous microglia are activated within hours after insult and monocytes begin to enter the injury site beginning at about 24 hours post injury, recruited by chemoattractants that are produced within the tissue parenchyma (Springer, 1994; McTigue et al., 1998). Following SCI in the rat, the accumulation of macrophages reaches a peak at 5-8 days and these cells remain in the epicenter chronically (Carlson et al., 1998; Popovich et al., 1997; Streit et al., 1998).

Microglia are highly sensitive to alterations in the CNS parenchyma and respond rapidly to acute CNS injury by undergoing morphological and phenotypic changes.
When activated in the adult CNS, microglial cells transform from a ramified (resting state) morphology to a hypertrophied, less process-bearing (activated state) morphology, and finally to a rounded (ameboid) macrophage-like morphology (Streit et al., 1988; Streit et al., 1999). In addition to morphological changes, activation of microglia is also accompanied by upregulation of surface markers such as complement receptors (CR3) (Graeber et al., 1988), Fc receptors (Perry and Gordon, 1988), MHC class II molecules (Popovich et al., 1993), CD4 antigen (Perry and Gordon, 1987) and production of cytokines and growth factors (Bartholdi and Schwab, 1997; Streit et al., 1998). This pattern suggests that the rapid microglial response is important in the initiation of a full inflammatory reaction. Once differentiated to a full phagocytic state, microglia are histologically indistinguishable with macrophages derived from blood monocytes. They are referred to collectively as brain macrophages and share several common phenotypic markers and functional attributes (Jordan and Thomas, 1988; Perry and Gordon, 1987; Perry and Gordon, 1988; Perry et al., 1993).

The role of macrophages in SCI remain enigmatic, in part because of the functional diversity of these cells (Nathan, 1987). On one hand, macrophages play a beneficial role in axonal regeneration by facilitating the removal of myelin, and other toxic cell components (Avellino et al., 1995; Scheidt et al., 1986). Thus, the delayed infiltration of macrophages in the CNS relative to the periphery may contribute to the failure of regeneration after CNS injury by preventing myelin clearance (George and Griffin, 1994; Perry et al., 1987). In addition, through the release of cytokines and growth factors (e.g., IL-1, TNF-α, TGF-β, bFGF), microglia and macrophages can promote neuronal survival, angiogenesis, and remyelination (Banati and Graeber, 1994; Rabchevsky and Streit.
Microglia and macrophages also can increase the permissiveness of the tissue environment to neuritic sprouting after injury by secreting permissive extracellular matrix molecules such as fibronectin, or modifying inhibitory cell surface properties at the injury sites as shown in vitro (David et al., 1990; Nathan, 1987).

However, macrophages within the CNS may also be deleterious to spared tissue following SCI. These cells produce neurotoxic molecules, including quinolinic acid (Blight et al., 1995; Popovich et al., 1994) and nitric oxide (Grzybicki et al., 1998; Yamanaka et al., 1998), which can contribute to secondary or bystander damage to tissues and cells that survive the initial wave of necrotic and apoptotic cell death (Blight, 1992). Approaches attempted to deplete or functionally inhibit macrophages have been shown to enhance tissue sparing and promote functional recovery in guinea pig and rat models of SCI (Blight, 1994; Giulian and Robertson, 1990; Popovich et al., 1999).

Therefore, the reactive response of macrophages seems to be a critical event that affects the histopathological outcomes after SCI. Clear elucidation of beneficial and detrimental roles of the macrophage response in the context of SCI represents an important step in the development of effective therapies to enhance wound repair, limit further destruction, and promote regeneration. For this reason, it is of considerable interest to identify the signaling molecules and other mediators that regulate the recruitment of macrophages following SCI and examine their roles in the evolution of complex histopathology.

The regulatory factors that govern the characteristic inflammatory response in the CNS have been long sought. The release of pro-inflammatory cytokines, and other chemoattractants from the site of injury induce adhesion molecules expression on both
endothelium and leukocytes, and recruit leukocytes into the lesion site (Winn et al., 1998; Benveniste, 1992). However, the selective recruitment of macrophages in the CNS seems not to be attributed to absence of an adhesion molecule or impaired cytokine expression. For example, adhesion molecules are readily induced following pro-inflammatory challenges, e.g. by injection of LPS into the brain, where there is no appreciable neutrophil recruitment (Bell and Perry, 1995). Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1β are also expressed rapidly in response to ischemia (Liu et al., 1994a), traumatic injury to the brain and spinal cord (Fan et al., 1995; Feuerstein et al., 1994; Yakovlev and Faden, 1994), and in response to stimuli such as LPS which gives rise to minimal neutrophil infiltration (Higgins and Olschowka, 1991; Quan et al., 1994). These studies suggest that the factors conferring the differences in leukocyte recruitment may lie downstream of cytokine production and adhesion molecule expression.

Differential expression of chemokines (chemoattractive cytokines) represents a putative signal for selective leukocyte infiltration after injury or disease (Adams and Lloyd, 1997; Glabinski et al., 1995; Ransohoff, 1997; Rollins, 1997; von Tscharner et al., 1986). Studies by Bell et al. demonstrated that following the micro-injection of MIP-2 (a CXC chemokine, see below) there was a marked recruitment of neutrophils to the brain, and the injection of MCP-1 (a CC chemokine) provoked monocyte recruitment (Bell et al., 1996b). Selective leukocyte recruitment to the CNS parenchyma in response to chemokine expression has also been demonstrated by overexpression of certain chemokines in transgenic mice (Tani et al., 1996; Bell et al., 1996a; Fuentes et al., 1995). Therefore, the more restricted inflammatory reaction in the CNS as described above may
be related to the differences in the expression pattern or levels of appropriate chemokines and chemokines appear to be the most important mediators of leukocyte recruitment.

**Chemokines in CNS Inflammation**

Chemokines are a family of small, pleiotropic secreted peptides that play a variety of roles in intercellular signaling, especially important for leukocyte migration and inflammatory processes (Rollins, 1997; Oppenheim et al., 1991). The chemokines, which comprise more than 50 members at present, are divided into four groups based on the positions of cysteine residues, in which two of the principal groups are C-X-C, or \( \alpha \) chemokine family and C-C, or \( \beta \) chemokine family. The \( \alpha \)-chemokines are further divided into two subfamilies: the ELR-CXC chemokines that contain a receptor binding glutamate-leucine-arginine (ELR) motif and non ELR-CXC chemokines (Strieter et al., 1995). The ELR-CXC chemokines, such as human interleukin-8 (IL-8) and growth-related oncogene \( \alpha \) (GRO-\( \alpha \)), show specificity for the recruitment of neutrophils, and the non ELR-CXC chemokines, such as \( \gamma \)-interferon-inducible-protein 10 (IP-10) are chemoattractive for activated T cells (Baggiolini, 1998). The \( \beta \)-chemokines commonly attract monocytes, lymphocytes, eosinophils and basophils.

Chemokines act through specific receptors that belong to the superfamily of seven-transmembrane domain receptors that signal through GTP-binding proteins (Kelvin et al., 1993). There are five receptors for the \( \alpha \) chemokines (CXCR1-5) and eleven receptors for the \( \beta \) chemokines (CCR1-11) that have been identified (Bajetto et al., 2001). In terms of their receptor binding properties, the chemokines are promiscuous which means that
multiple chemokines can bind to a single receptor and multiple receptors can recognize individual chemokines.

With regard to the CNS, some chemokines are thought to be essential for normal development and homeostasis (Bacon and Harrison, 2000; Rezaie et al., 2002). The evidence to support this view is from many in vitro and in situ hybridization studies showing that there are chemokines and chemokine receptors that are constitutively expressed in the CNS by microglial cells, astrocytes, and neurons (Coughlan et al., 2000; Horuk et al., 1997). The pattern of chemokine expression has been investigated in a variety of different CNS pathologies, such as MS and EAE, stroke, Alzheimer’s disease, HIV encephalitis, and trauma. The results indicate that they also play a key role in the pathogenesis of these diseases (Asensio and Campbell, 1999; Bajetto et al., 2001; Huang et al., 2001; Mennicken et al., 1999; Ransohoff, 1997).

In terms of CNS trauma, the expression profiles of selected chemokines has been examined in sites of neural trauma following a variety of insults (Ransohoff, 1997). Traumatic brain injury models that have been used include penetrating mechanical injury by stab wound (Berman et al., 1996; Glabinski et al., 1996; Ghirnikar et al., 1996), or aspiration (Hausmann et al., 1998; Muessel et al., 2000), and non-penetrating closed head injury (Zhang et al., 2001), or cryolesion (Grzybicki et al., 1998). The pattern of chemokine expression in the injured spinal cord has been studied in a spinal cord contusion injury model in the rat (McTigue et al., 1998) and a hemisection injury model in the mouse (Bartholdi and Schwab, 1997). Elevated chemokine expression profiles were revealed in all these settings and the mRNA expression pattern has uniformly shown the most robust increase in the expression of monocyte chemoattractant protein-1.
MCP-1) (Ransohoff, 1997). The selective expression of MCP-1 occurs before the onset of monocyte and macrophage accumulation and thus implies a major functional role for MCP-1 in monocyte recruitment in the response to CNS injury (Rollins, 1996).

Studies on the cellular source of the expressed chemokines reveal that parenchymal cells are competent to express chemokines under certain circumstances (Ransohoff et al., 1993; Glabinski et al., 1996). Astrocytes, for example, express MCP-1, IP-10, and GRO-α in acute EAE in mice (Ransohoff et al., 1993; Glabinski et al., 1997). These cells also express MCP-1 following a cortical stab wound in mice (Glabinski et al., 1996). Microglia and neurons are also possible sources of selective chemokine expression (Coughlan et al., 2000; Gong et al., 1997; Kishino et al., 1997). Thus, activated cells within the neural parenchyma may be critical for the initial recruitment of monocytes to the site of SCI.

Although the roles of selective chemokines such as MCP-1 in post-traumatic inflammation have been implicated through expression analyses, the direct cause-and-effect relationship between chemokines and pathology has not been demonstrated. In recent years, genetic approaches involving the use of targeted gene expression or deletion techniques appear to provide a powerful tool for defining the role of signaling molecules in the mechanisms of secondary injury and repair after CNS injury (Jakeman et al., 2001; Zhang et al., 1998). However there are not many such studies in SCI, partly because of the lack of reliable and reproducible injury models in the mouse.
Rationale and Experimental Goals

A variety of experimental models of SCI have been developed to address specific aspects of secondary injury and repair (Anderson and Stokes, 1992; Blight, 1996; Lighthall and Anderson, 1994). Experimental contusion injury reproduces spinal cord trauma most closely to the clinical setting (Bunge et al., 1997; Metz et al., 2000), and has been widely used for investigation of injury pathophysiology. Several animal species have been used in the development of contusion injury models, from larger mammals (Bresnahan, 1978; Griffiths, 1976; Yeo et al., 1975; Ford, 1983), to small rodents (Blight, 1991a; Gale et al., 1985; Thompson et al., 1992; Behrmann et al., 1992).

In recent years, mice have been increasingly used for neurotrauma research. This is in part because most genetic strategies involve the use of mice (Steward et al., 1999; Jakeman et al., 2001). The availability of naturally-occurring and genetically engineered mutations in this species makes them optimal experimental animals used to investigate specific components of the injury process, and therefore appropriate for studies aimed at evaluating the mechanisms of secondary injury and repair after SCI.

The objective of the studies that comprise this thesis is to investigate the role of β chemokines such as MCP-1 in the pathophysiology of SCI. It is my hypothesis that β chemokines and MCP-1 in particular regulate macrophage recruitment and activation during post-traumatic inflammation and contribute to histopathological sequelae of SCI. These studies apply a mouse model of spinal cord contusion injury to define the role of MCP-1 and identify other chemokines potentially involved in traumatic SCI. Information about signals that lead to macrophage recruitment and activation in sites of injury will constitute an essential foundation for understanding the post-traumatic...
inflammatory response. Eventually, this will lead to focused interventions to manipulate inflammation to improve the functional outcome of SCI.

A reliable trauma model with predictable and reproducible outcome measures is prerequisite for the investigation of the involvement of individual chemokines and cellular elements in the outcome of SCI (Anderson and Stokes, 1992; Blight, 1996; Lighthall and Anderson, 1994). We have developed a mouse model of spinal cord contusion injury (Jakeman et al., 2000). Chapter one characterizes the behavioral and histological outcomes following contusion injury in the C57Bl/6 mouse, a commonly used inbred mouse strain. Groups of mice injured with three different severities were tested by comparing biomechanical parameters with behavioral and histological outcome measures to determine whether long-term functional outcomes are proportional to the biomechanical changes.

To help in defining the role of MCP-1 in inflammatory response after SCI, in Chapter two, the macrophage response following a moderate contusion injury in mice lacking CCR2, the principal receptor for MCP-1, are evaluated and compared with wild-type mice. The transgenic approach will address the specific effects of CCR2 deletion after injury.

Chapter three describes the time course of cellular infiltration and scar formation following moderate contusion injury in two inbred mouse strains: C57Bl/6 and 129X1/SvJ. The latter strain of mice has been shown to have a defect in inflammatory recruitment in a peripheral inflammation model (White et al., 2002). Immunohistochemical methods are used to identify macrophage activation and infiltration, reactive astrogliosis, axonal growth, and extracellular matrix deposition after
injury. Comparison of the strain differences in the cellular reactions and histopathological sequelae reveals intricate interrelationships between these processes.

Chapter four further examines the patterns of chemokine expression following spinal contusion injury in the mouse. The pattern of chemokine expression in the two mouse strains will be analyzed as a function of post-injury survival time to reveal any correlation with inflammatory cellular reactions studied in Chapter three. The characteristic expression profiles in the two mouse strains suggest hypothetical roles for selective chemokines in the inflammatory response after SCI.
GENERAL METHODOLOGY

Experimental Spinal Cord Contusion Injury

Adult female mice (10-15 weeks of age; 18-24g) were used throughout these studies. All procedures involving experimental animals were performed in accordance with the Ohio State University Institutional Animal Care and Use Committee.

Spinal cord contusion injury was performed using an electromagnetic spinal cord injury device (ESCID) modified from the one that has been used in the rat (Jakeman et al., 2000). This device creates a single, controlled, and rapid compression of the exposed dorsal surface of the spinal cord following a partial laminectomy (Behrmann et al., 1992; Bresnahan et al., 1987; Stokes et al., 1995).

Mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), and were given prophylactic gentocin subcutaneously (1 mg/kg). Lubricant ointment (Gentak; Akorn, Inc.) was applied to the eyes to prevent drying. Using aseptic procedures, a single level laminectomy (1.5mm x 1.7mm) was prepared at the T\textsubscript{9} vertebral level (Jakeman et al., 2000). The mice were then placed onto a platform fixed on a two-axis machine tool table (Stokes et al., 1992). The mice were stabilized under the ESCID with scissors action forceps positioned along the lateral processes of T\textsubscript{8} and dorsal process of T\textsubscript{10} (Jakeman et al., 2000). The tip of the impounder (1.35mm diameter) was then lowered slowly onto the center of exposed dura overlying the spinal
cord until 1500 dynes of force was recorded by the force transducer located along the vertical shaft. Approximately 3 seconds after the force transducer reached this pre-load force, mice in the contusion injury groups were injured by a rapid displacement of the impounder for a predetermined distance over a 23 ms period (10 ms peak displacement duration). Injury severity was defined by the distance of displacement and has been shown to predict the lesion size and the magnitude of neurological impairment (Stokes et al., 1995; Jakeman et al., 2000). In Chapter one, three distances of displacement were used: 0.3, 0.5, and 0.8mm. From Chapter two to four, 0.5mm displacement was chosen as the injury level. This level of injury is considered to be of moderate severity and produces immediate hind limb paralysis followed by incomplete recovery over the first two weeks post injury. A description of hemorrhage or bruise at the injury site was recorded, and the wounds were sutured in layers.

Post Operative Care

After surgery, the muscle and overlying skin were sutured and topical antibiotic was applied to the skin incision site and 2cc of lactated Ringer's solution was given subcutaneously. Each mouse was housed singly until fully recovered from anesthesia. The mice were returned to standard housing (2-3/cage) the following day and provided with softened rodent chow and water. High caloric nutrient paste (Nutrical: Evsco, Inc.) was provided ad lib. as a dietary supplement until the mice regained ≥ 90% of their pre-operative weight. Bladder expression was performed twice daily and gentocin (p.o.. 50-80 mg/day) was given until the return of bladder control. Ascorbic acid was provided in the drinking water (1 mg/ml) throughout the duration of the study to maintain urine
acidity. Urine pH was measured weekly to monitor potential urinary infection. Post-operative analgesics (butorphenol, 10mg/kg) were available if needed, as determined by behavioral signs of pain such as vocalization, crouching, or piloerection. However, these symptoms were not observed in the course of these experiments.

**Tissue Preparation for Histopathology and Immunohistochemistry**

Following spinal cord injury, mice were sacrificed at different time points as specified in each chapter. For histology and immunohistochemistry, mice received an overdose of ketamine/xylazine anesthesia and perfused transcardially with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS (pH 7.4). The spinal cords were dissected and post-fixed for 2 hrs. and then transferred to 0.2 M phosphate buffer (PB) overnight at 4°C. Tissues were cryoprotected in 30% sucrose overnight at 4°C and frozen on dry ice. The frozen spinal cords were cut transversely and blocked into 2mm segments, and embedded into TissueTek (OCT compound, Fisher Scientific). Serial 10 μm thick cryostat sections through the entire injury site were mounted on Superfrost Plus slides (Fisher Scientific) slides.

**Histopathology**

One of every 10 slides (each slide contained 10μm sections spaced 100μm apart) was stained with Luxol fast blue (LFB) to identify myelinated white matter and residual tissue sparing. For each specimen, the lesion epicenter was defined as the tissue section with the least spared white matter, which has the smallest cross-sectional area of LFB staining in the peripheral rim (Behrmann et al., 1992; Jakeman et al., 2000). White
matter was considered as spared if the LFB staining was grossly normal in appearance and density (lacking cysts, degeneration).

The total cross-sectional area of the spinal cord and the lesion boundary were measured on digitized images of the epicenter using a computer-assisted image analysis program (MCID; Imaging Research Inc., Ontario, Canada) (Popovich et al., 1997) while viewing sections under the light microscope (20X). The amount of spared white matter was reported as in mm$^2$ and as a percentage (%) of the total cross-sectional area of the cord at the epicenter (Means et al., 1981; Basso et al., 1996a; Behrmann et al., 1992; Bresnahan et al., 1987). Lesion length (Chapter one) was determined to the nearest 100µm by examining the LFB stained sections rostrally and caudally from the epicenter until sections with normal distribution and density of LFB staining were seen.

**Immunohistochemistry**

In order to document the cellular responses and/or extracellular matrix deposition after injury, sections on the adjacent slide series were stained immunohistochemically using a panel of antibodies. For immunohistochemical detection of activated microglia and macrophages, slides were stained with monoclonal rat anti-mouse antibodies raised against either complement receptor CR3 (Mac-1, Serotec Ltd., Oxford, England; 1:200) or Fc antigen (F4/80, Serotec Ltd., Oxford, England; 1:100). The activation of astrocytes was detected by using rabbit polyclonal antisera raised against glial fibrillary acidic protein (GFAP, DAKO Corp., Carpinteria, CA; 1:30,000). Other antibodies used to detect cellular and extracellular components in the lesion site include: the 200kd neurofilament protein (NF-H, Chemicon, Temecula, CA; 1:6000), laminin (Sigma
Immunochemicals, St. Louis, MO; 1:6000), fibronectin (Sigma Immunochemicals, St.
Louis, MO; 1:500), and collagen IV (Chemicon, Temecula, CA; 1:2000). For all
immunohistochemistry, slides were dried at room temperature for 2 hours. After rinsing
in PBS, the sections were pretreated with 4% bovine serum albumin (BSA) in PBS. For
fibronectin staining, sections were incubated with 0.0025% trypsin in 0.1% CaCl_2 for 5
min at 37°C to expose antigen followed by 2% BSA in PBS and 0.1% Triton X-100 for 1
hour. Primary antibodies were applied at 4°C overnight, the sections were rinsed with
PBS, and secondary biotinylated antibodies were applied for 2 hrs. Endogenous
peroxidase activity was quenched in 6% hydrogen peroxide in methanol. Slides were
then incubated in Elite ABC or ABC complex (Vector Labs, Burlingame, CA) for 1 hr.
After washing in PBS, the staining was visualized with DAB or SG (GFAP staining) as
the chromogen (Vector Labs).

**Proportional Area Measurements**

For quantitative image analyses of cellular or extracellular matrix immunoreactivity,
proportional area measurements were carried out using the MCID imaging system.
Images from equally spaced sections at 100 μm intervals surrounding the epicenter were
digitized, and the optical density detection threshold was adjusted to differentiate positive
staining from background. The cross-sectional area of the spinal cord tissue section (*scan
area*) was drawn on digitized, spatially calibrated images (SONY 970 color CCD).
Target regions were then assigned which directly corresponded with positive staining in
each section. *Target area* was measured as the area of tissue occupied by positive

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immunohistochemically stained profiles within the scan area. Propriotional area was computed as the fraction of the area of positive staining divided by the scan area.

**RNA Preparation (Chapters 2 & 4)**

For analysis of chemokine mRNA levels, the mice were sacrificed by decapitation under ketamine/xylazine anesthesia. Approximately a 2cm segment of spinal cord including the lesion site were rapidly dissected and were snap frozen in liquid nitrogen and stored at -70°C. Total cellular RNA was prepared from frozen spinal cord tissue using Trizol extraction according to the manufacturer’s instructions (Gibco BRL. MD). RNA concentration was determined spectrophotometrically. A total of 20 μg of total RNA was used per sample.

**RNase Protection Assay (Chapters 2 & 4)**

Quantification of chemokines (Chapters 2 & 4) and chemokine receptors (Chapter 2) in injured mouse spinal cords was done by RNase protection assay (RPA) following in vitro transcription (PharMingen. San Diego. CA). The chemokine probe set was a gift from Dr. Iain L. Campbell at the Scripps Research Institute. La Jolla. CA (Asensio and Campbell. 1997). The chemokine receptor probe set was obtained from PharMingen (MCR-5). Protected fragments labeled with 32P were visualized and quantified by autoradiography using a PhosphorImager (Molecular Dynamics. Sunnyville. CA).
Statistical Analysis

All analyses were performed using GraphPad Prism3 software (GraphPad Software, Inc., San Diego, CA). Significance level was set at $p < 0.05$. 

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CHAPTER 1

BEHAVIORAL AND HISTOLOGICAL OUTCOMES FOLLOWING GRADED SPINAL CORD CONTUSION INJURY IN THE C57BL/6 MOUSE

Introduction

There are few clinical interventions currently available to apply to the devastating problem of SCI. Recent research in this field has revealed a complex pathophysiology initiated by trauma to the spinal cord. The response to injury is characterized by destructive and reparative cellular processes that together contribute to permanent loss of function (Balentine, 1978; Beattie et al., 1997; Dusart and Schwab, 1993; Schwab and Bartholdi, 1996; Zhang et al., 1997). However, investigators have not yet elucidated the contribution of most of the secondary biochemical pathways to functional outcome measures. Recent developments in genetic and transgenic research hold great promise for improving understanding of secondary injury processes and for identifying potential sites of clinical intervention (Jakeman et al., 2001; Steward et al., 1999; Zhang et al., 1996).

Specific aspects of secondary injury and repair can be addressed using experimental animal models (Anderson and Stokes, 1992; Stokes et al., 1995; Beattie et al., 1988; Blight, 1996; Lighthall and Anderson, 1994). For example, rapid contusion injury
models produce a closed spinal cord trauma that mimics many of the mechanical and cellular events that are associated with vertebral impact injuries (Allen, 1911; Panjabi and Wrathall, 1988; Wrathall et al., 1985; Noble and Wrathall, 1985; Stokes et al., 1995). The resulting histopathology shares many features of that seen in clinical specimens (Bunge et al., 1997; Metz et al., 2000). As with all animal models, reliability of the injury process and reproducibility of chronic outcome measures are critical requirements (Ford, 1983; Gruner, 1992; Wrathall et al., 1985; Stokes et al., 1995). These characteristics become more difficult to attain as the microsurgical approaches and outcome measures are adapted to smaller species.

We have recently described a murine spinal cord contusion injury model, produced with the use of ESCID (Jakeman et al., 2000). This device creates a single, controlled and rapid compression of the exposed dorsal surface of the spinal cord following a partial laminectomy (Behrmann et al., 1992; Bresnahan et al., 1987; Stokes et al., 1992). Particular features of this approach include the instantaneous measurement of biomechanical variables at the time of impact, simplicity of operation, and the precise control of the displacement profile as the independent variable that determines severity of injury (Stokes et al., 1995). The present study was performed to test the hypotheses that reliable injuries of predictable severity could be produced with the ESCID and that behavioral and histological outcome measures would correlate with the severity of injury. The range of injury severities was achieved by the distance of a single rapid displacement of the cord at the site of dorsal laminectomy (T4). Behavioral outcome measures, including open field locomotion (Basso et al., 1995), grid walking (Behrmann et al., 1992; Kunkel-Bagden et al., 1992; Bresnahan et al., 1987), and footprint analysis
(Kunkel-Bagden et al., 1993; Stokes and Reier, 1992) were used to assess recovery of function. Histological measures from sections through the lesion site were used to evaluate the extent of tissue damage at nine weeks post injury. Finally, the characteristics of the resulting scar tissue were defined using immunohistochemical markers to identify cells and extracellular matrix molecules in the chronic lesion site. The results show that graded contusion injury could be produced reliably in mice by using the ESCID injury device. The low variability in outcome measures provides the potential for detailed examination of the role of cellular events associated with closed contusion injury.

Methods

Subjects

A total of 31 adult female C57Bl/6 mice (10-15 week old; 19-24 g; Taconic Farms, Germantown, NY) were randomly assigned to one of five groups to examine chronic behavioral and histological outcomes: laminectomy control (n=7), 0.3mm displacement (n=4), 0.5mm displacement (n=8), 0.8mm displacement (n=8), or complete transection (n=4). The mice were housed 2-3 per cage and maintained in a 12 h light/dark cycle with free access to water and rodent chow before surgery.

Surgical Procedures

The processes of spinal cord contusion injuries were described in detail in the general methodology section. For the laminectomy control mice, the surgical sites were closed with chromic gut suture (muscle) and 5-0 Lycra (skin) without further
intervention. Contusion was produced by rapid displacement of the spinal cord to a peak distance of 0.3, 0.5, or 0.8mm in three injury groups of mice.

To determine the contribution of a very small proportion of residual fibers in the severe injury group to functional recovery, one group of mice received complete spinal cord transection. The exposed dura was cut using a pair of fine iridectomy scissors, and the spinal cord transected with microscissors. A sterile glass aspiration tube was used to remove a 2mm segment of the spinal cord. After hemostasis was achieved, the resulting cavity was explored carefully with a small glass probe to confirm the absence of residual tissue. The cavity was filled with gelfoam to ensure that functional recovery could not be attributed to regeneration across the injury site.

**Behavioral Assessment**

The mice were tested in three behavioral paradigms to determine if injury severity would correspond to behavioral performance. The day before surgery, the mice were tested in the open field and on a wire mesh grid, and forelimb and hindlimb footprints were recorded in the runway to evaluate the normal performance with which to compare deficits after injury.

**Open Field Locomotion.** A plastic wading pool with textured floor (91 cm diameter, 18 cm wall height) was used for this test in a quiet room with normal lighting. For acclimation, the mice were exposed to the testing environment daily for at least one week prior to surgery. Acclimation was complete when the mice moved freely about the pool without signs of fear, such as crouching, frequent defecation and urination, or vocalization (Basso et al., 1995). The mice were then tested alone at 1, 3, and 7 days.
post-operative and weekly thereafter. Testing continued for 9 weeks to evaluate chronic functional recovery (1-6 weeks) and post-injury training effects (6-9 weeks). The recovery of overground hindlimb locomotion was evaluated using the Basso, Beattie, Bresnahan (BBB) locomotor rating scale (Basso et al., 1995; Basso et al., 1996a; Basso et al., 1996b). A team of two experienced examiners evaluated each animal over a 4-minute period and provided an operationally defined score (0-21) for each hindlimb. The scores from the two hindlimbs were averaged at each time point.

**Grid Walking.** This test was adapted from one used for rats (Behrmann et al., 1992; Bresnahan et al., 1987; Grill et al., 1997; Kunkel-Bagden et al., 1992; Stokes and Reier, 1992) to evaluate the ability of the mice to locomote over a wire mesh grid (2.5 x 2.5 cm grid spaces, 28 x 35 cm total area). Only those mice that demonstrated consistent plantar stepping in the open field (BBB scores ≥11) were tested on the grid. Each mouse was videotaped for 3 minutes while on the grid during which a minimum of 30 sec of walking was required. One foot miss was counted when the hindlimb paw protruded entirely through the grid with all toes and heel extended below the wire surface. The total number of foot misses and total walking time were recorded from the videotapes by two independent examiners. The grid walking test was performed on the day prior to surgery and days 42 and 63 post-operative for mice in the laminectomy and 0.5mm injury groups and on days 15, 31, 42 and 63 for the 0.3mm injury group.

**Footprint Analysis.** To examine the step patterns of the forelimbs and hindlimbs during forward locomotion, mice with consistent plantar stepping were required to traverse a straight, well-lit runway (3 feet long, 2 inches wide) to receive a food treat in a darkened box at the far end. The bottom of the runway was lined with white paper and
the fore- and hindpaws were inked with dyes of red and black, respectively. A minimum of 2 nonstop passes was required. Paw position and toe drags were determined from a minimum of five step cycles per trial. A complete step cycle was defined as the distance from one pair of forelimb prints to the next. Forelimb-hindlimb matching was counted if one hindlimb step preceded each forelimb step in that cycle. The matching ratio was calculated as the number of matching cycles/total step cycles. One toe drag was counted when the ink streak from a hindpaw was longer than one paw length and was located between two hindlimb footprints on the same side. The base of support was defined as the distance between the heel spots of the two hindlimbs. Footprint analysis was performed on the day prior to surgery and days 42 and 63 post-operative.

**Training.** The animals were not trained prior to injury in order to evaluate residual function for the first six weeks post-injury in the absence of acquired compensatory mechanisms. In addition, no training on the grid or the runway was required to obtain the pre-operative data. Beginning at day 42 post-injury, the animals were trained daily on the grid and runway for three weeks to determine if post-injury training would affect their performances on these tasks. On the grid, the mice were trained twice a day (5-10 min per trial) to navigate the grid. They were encouraged to walk during training and testing by providing motivational rewards including food treats or a sample of their home cage bedding. The mice were also trained to proceed down the runway (5-10 trials per day) until they were able to pass the full length of the runway nonstop over several consecutive trials.
Tissue Preparation and Histopathology

Mice were sacrificed after the final behavioral assessment at 63 days postoperative. Tissue preparation and general histological (LFB staining with white matter sparing and lesion length measurement) and immunohistochemical methods were described in detail in the general methodology section. Proportional area measurements were used for quantitative image analyses of fibronectin immunoreactivity as described in the general methodology. Fibronectin stained sections were analyzed at 500μm intervals from the epicenter to the rostral and caudal extent of the lesion.

Statistical Analysis

Behavioral recovery patterns in the open field after injury (BBB scores) were compared between groups using Kruskal-Wallis nonparametric analysis of variance. Between-group comparisons were also made by the analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test at each time point post-injury. Group differences for injury biomechanical parameters (displacement, peak force, and maximum impulse/momentum), foot misses/sec on grid walking, base of support and toe drags from footprint analysis, and histological measures (total cross-sectional area, white matter sparing, lesion length, and proportional area of fibronectin immunoreactivity) were evaluated by one-way ANOVA followed by Bonferroni’s post-hoc test. Correlation analyses between open field locomotion (final BBB scores at 63 days post-operative) and biomechanical parameters or histological outcomes were performed using Spearman Rank Order correlation. The relationships between injury biomechanical parameters and
parametric behavioral (grid walk) and histological outcomes were determined by Pearson Product Moment correlation.

Results

Survival and Post-operative Recovery

All mice survived and remained active and healthy throughout the period of study. No cases of vertebral fracture, wound infection, autophagy, or bladder infection were observed. Weekly measurement of the urine pH did not indicate bladder infection in any subjects. Laminectomy animals showed no obvious deficits after recovery from anesthesia. Mice that received 0.3mm displacement injury regained reflex bladder function within the first week post-operative, while those in the 0.5mm displacement injury group required 21-28 days to recover this ability. In contrast, mice with 0.8mm contusion injury or complete transection did not regain bladder function and required manual expression daily throughout the duration of the study. The average weights of mice at the time of sacrifice were not different between groups and did not differ from the pre-operative weights.

Graded Injury Levels and Chronic Outcome Assessment: Injury Biomechanics

The mean values of maximal displacement, peak force, and impulse/momentum in the three contusion injury groups are shown in Table 1. Peak displacement amplitudes were significantly different across groups and between each group. The peak force of impact and maximum impulse/momentum increased with increasing displacement distance. Post-hoc tests revealed significant differences for peak force between 0.3 and
0.5mm \((p < 0.05)\), 0.3 and 0.8mm \((p < 0.001)\), and 0.5 and 0.8mm \((p < 0.001)\) groups. and for impulse/momentum only between 0.3 and 0.8mm \((p < 0.01)\) groups. The Pearson Rank Order analyses revealed significant correlation among displacement, peak force, and impulse/momentum values (Table 2). Records of displacement and force of impact for individual injuries closest to the group mean are illustrated in Figs. 1A and 1B.

**Behavioral Recovery**

**Open Field Locomotion.** The recovery of hindlimb locomotor function was evaluated over 9 weeks using the BBB rating scale. As described previously (Jakeman et al., 2000), the BBB rating scale provided a generalized and repeatable measure of hindlimb function in the open field, although there were some modifications as applied to the mouse. In particular, the speed and the size of the mouse hindpaws make precise discrimination of coordination and toe clearance (BBB score = 12-21) difficult to evaluate. We used the presence or absence of a clear rhythmic gait throughout a full forward run across the width of the pool to define matching of forelimb to hindlimb and alternation of hindlimb steps (Jakeman et al., 2000). These modifications had little impact on the present study, due in part to the separation of the moderate (0.5mm injury) and mild (0.3mm injury) groups below and above the range of scores where these issues are most crucial.

The time course of locomotor recovery for the five groups is shown in Fig. 1C. The laminectomy mice exhibited normal locomotion throughout the testing period (BBB score = 21). Mice in the 0.3mm group exhibited deficits in coordination (BBB score = 10-12) on the first day after injury. During the first two weeks, this group recovered
forelimb-hindlimb coordination, toe clearance, parallel paw position and normal tail position. By 14 days post injury, all BBB scores in 0.3mm group were 21 and not different from laminectomy or pre-operative values.

The 0.5mm group attained an intermediate level of functional recovery. The hindlimbs were paralyzed immediately after injury, and the mice recovered extensive movement of all hindlimb joints within the first week. By two weeks after injury, they exhibited consistent plantar stepping with no or occasional to frequent coordination (BBB score = 11-13). There was no further functional improvement in this group, so that the final mean BBB score was 12.4 ± 1.1 (mean ± SEM).

The mice in the 0.8mm injury group demonstrated flaccid paralysis with no or little hindlimb movement throughout the first week post-injury. These mice subsequently recovered slight or extensive joint movements during the following 2-9 weeks. Mice with this injury were never able to step with weight support. Their average BBB score at day 63 post-operative was 3.8 ± 1.0 (mean ± SEM).

The complete transection group showed little recovery through the testing period. During the first week, the mice were completely paralyzed. Beginning at 14 days post injury, they recovered some slight joint movements. In general, the degree of joint movement and number of hindlimb joints that showed movements in the transection group mice were slightly less than in the 0.8mm group mice. Their average BBB score at 63 days post-injury was 2.3 ± 0.4.

Kruskal-Wallis nonparametric analysis of variance revealed significant differences in outcomes for the five groups across time (p < 0.0001). However, Dunn's non-parametric post-hoc tests did not reveal any significant comparisons, presumably due to the lack of
power associated with the small group sizes. Bonferroni’s post-hoc test revealed significant differences between each of the three injury groups at each individual time point ($p < 0.001$). Significant differences were also found between the laminectomy and the 0.5mm group, between the laminectomy and the 0.8mm group, between the complete transection and the 0.3mm group, and between the complete transection and the 0.5mm group at all time points ($p < 0.001$). The 0.3mm group was significantly different from the laminectomy control only at 1, 3, and 7 days post injury ($p < 0.001$). The 0.8mm group did not differ from the complete transection group except at 14, 35, and 49 days post injury ($p < 0.05$ to 0.01).

**Grid Walking.** Mice from the 0.3mm and 0.5mm injury group recovered stepping ability and were tested for locomotor performance on the wire mesh grid. For the 0.3mm group (Fig. 2A), all the mice were tested beginning at 15 days post injury, when their BBB scores had recovered to 21. At all times after injury, the foot misses/sec were significantly higher than their pre-operative level ($p < 0.01$ at day 15, and $p < 0.05$ at day 31, 42, and 63). These deficits on grid walking did not change over time ($p > 0.05$ between days post injury). The 0.5mm group mice were tested on the wire grid at days 42 and 63 post injury (Fig. 2B). There were significant group differences between the 0.3mm and 0.5mm groups ($p < 0.0001$) with increased deficits seen in 0.5mm group at both time points.

**Footprint Analysis.** Footprint recordings were taken from all mice in the 0.3mm and 0.5mm injury groups as well as the laminectomy control group at 42 and 63 days post injury. Representative footprint tracings from each group are shown in Fig. 3. The laminectomy control animals showed consistent forelimb-hindlimb coordination, no toe
drags, no paw rotation, and hindlimbs consistently placed lateral to the forelimbs (Fig. 3A). Mice from the 0.3mm group showed good forelimb-hindlimb coordination on all passes (Fig. 3B). After injury, the hindlimb prints were often superimposed on those of the forelimbs, rather than lateral as seen with laminectomy controls. Toe drags were rarely seen in the 0.3mm group. Footprints obtained from the 0.5mm injury group revealed ink streaks extending from both the hindlimbs indicating toe drags, paw rotation, and frequent loss of spatial coordination between the forelimb and hindlimb prints (Fig. 3C).

After the 0.3mm or 0.5mm injury, the hindlimb base of support was significantly decreased ($p < 0.001$) for both injury groups compared to the laminectomy control (Fig. 4A). There were no significant differences between the injury groups for this measure, suggesting that the absolute base of support was not dependent on the severity of injury. Significantly more toe drags per step cycle were seen in the 0.5mm group than the 0.3mm group (Fig. 4B).

In order to provide a quantitative measure of the deficits in forelimb/hindlimb patterns that were evident on the footprint records, we developed an index that reflects the proportion of coordinated, or forelimb/hindlimb matching step cycles (Fig. 4C). The index was calculated by counting the number of complete forelimb step cycles that were in spatial register (1:1 correspondence) with hindlimb prints. This value was then divided by the total number of forelimb step cycles in two full runway records. All laminectomy control mice and half of the 0.3mm injury mice had a perfect matching index value of 1.0. In contrast, there was wide variation in this measure of coordination in the mice with the 0.5mm injury. Some mice demonstrated good coordination for the entire series of
step cycles from two runway passes, while most at this injury level showed deficits during one or both of the passes.

**Training.** The animals that had consistent stepping by 42 days post-operative (laminectomy and the 0.3mm and 0.5mm injury groups) received daily training on the grid and runway for three weeks. There were no significant differences in the number of foot misses on grid walking before and after training for either group. The base of support, the number of toe drags, and the forelimb-hindlimb matching ratio from footprint analyses did not reveal any differences between 42 and 63 days post-operative during which training was performed.

**Histopathology**

All mice were sacrificed at 63 days post-operative. Transverse LFB stained tissues were examined under light microscopy. At the epicenter, the rapid displacement injury caused the tissue to form a central core lesion area surrounded by a rim of spared white matter (Fig. 5). In the 0.3mm group (Fig. 5B), tissue shrinkage was minimal. These specimens showed the greatest area of spared white matter and also frequently contained small regions of spared gray matter. Small cavities were present in the central gray matter area of each of these specimens. These cavities were typically lined with small cells and macrophages and resembled cysts that are found in other mammals after injury (Balentine. 1978; Noble and Wrathall. 1985; Means and Anderson. 1983; Reier et al., 1983). In the 0.5mm group (Fig. 5C), tissue shrinkage around the impact site was obvious. At the epicenter, the spared white matter formed a narrow rim surrounding the central lesion area. Microcysts were found in the surrounding white matter and within
the lesion scar, but there were no large cavities in these specimens. The spared white matter in the 0.8mm group could only be identified in small regions within the ventral funiculi when examined at higher magnification (Fig. 5D). In those mice with complete transection, serial sections through the gelfoam site were examined at higher magnification, and no spared myelin was identified with the LFB stain (not shown).

Significant differences in total cross-sectional cord area were found between the 0.5mm and 0.8mm groups and laminectomy or 0.3mm injury groups (Fig. 6A). The amount of spared white matter decreased with increased injury severity (Fig. 6B). The spared white matter in the 0.8mm group was only 2.5% of the total cross-sectional area and did not differ from the complete transection group, which had no spared white matter. The lesion length was determined by examining the tissue in serial 100μm divisions (Fig. 6C). The average lesion lengths were significantly different ($p < 0.0001$) between groups. The lesion length was greater with more severe injuries ($p < 0.001$ between each group in Bonferroni's post-hoc test).

The cellular and extracellular components of the lesion site were examined using a panel of immunohistochemical markers. In all contusion injuries, the central core of the lesion was characterized by a matrix of cellular and extracellular tissue and a scarcity of axons as identified with antisera raised against the 200kd neurofilament proteins (Figs. 7A and 7B). Within the central matrix was an accumulation of F4/80 positive cells that morphologically exhibited the features of phagocytic macrophages (Fig. 7D) (Streit et al., 1988). In contrast, GFAP-positive astrocyte processes remained principally at the edge of the lesion, with only a few profiles seen around blood vessels in the center of the scar (Fig. 7C). Except for the cavities seen in the 0.3mm injury group, the central scar area
was filled with extracellular matrix proteins, including fibronectin (Fig. 7F) and collagen-
IV (Fig. 7G). Laminin immunoreactivity was mainly found associated with large and
small blood vessels and in thick tendrils within the lesion site (Fig. 7E). No differences
in cellular components were observed with different injury severities.

Histological and immunohistochemical staining of adjacent sections revealed a
distinct transition area between the tissue that composed the central scar (Fig. 7) and the
spared, myelinated rim of tissue along the periphery of the spinal cord (Figs. 5 and 8A).
This transition zone (Fig. 8), which was particularly obvious following the moderate
(0.5mm) injury, resembled a penumbra region of mismatch between the LFB stain (Figs.
8A and 8D) and the borders of GFAP positivity (Figs. 8B and 8E) or neurofilament
immunostaining (Fig. 8G). The mismatch of neurofilament and glial immunoreactivity
and LFB stains suggest a region of hypomyelinated axons intermingled with reactive
astrocytes and microglial cells (Fig. 8H), while both fibronectin- (Fig. 8F), and collagen
IV- (Fig. 8I) immunoreactivity were intermediate in staining density between that found
in the scar and the lower levels in the surrounding spared white matter.

We hypothesized that there would be differences in the distribution of the scar tissue
in three injury groups. Since the lesion area was completely filled with a dense
fibronectin positive matrix, we quantitatively analyzed fibronectin immunoreactivity at
epicenter, rostral and caudal extent to the epicenter (Fig. 9). Proportional area measures
were compared across groups at each location. One way ANOVA revealed significant
group differences in fibronectin-positive proportional area at the epicenter ($p < 0.0001$),
0.5mm rostral ($p < 0.0001$) and caudal ($p = 0.0002$) to the epicenter.

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**Correlation Analysis**

The biomechanical parameters of the contusion injury were correlated with behavioral and histological outcomes (Table 2). The Spearman Rank Order correlation analysis revealed a significant negative correlation between each of the biomechanical parameters (displacement, peak force, and maximum impulse/momentum) and the final BBB scores. However, only displacement correlated with the outcome on the grid walk test, perhaps because the most severe animals could not be tested with this measure. All three biomechanical parameters also correlated significantly with histological outcome measures (white matter sparing, lesion length, and proportional area of fibronectin immunoreactivity). The BBB scores and grid walking test were strongly correlated with white matter sparing, lesion length, and fibronectin-positive scar area. The three histological outcome measures were significantly correlated with one another.

**Discussion**

The extension of a contusion injury model to the mouse provides opportunities for investigating the effects of genetic variation on a number of relevant outcome measures (Jakeman et al., 2001; Steward et al., 1999). We have shown previously that a controlled contusion injury can be produced in the mouse using the ESCID (Jakeman et al., 2000). The present studies demonstrate that contusion injuries resulting in mild, moderate or severe behavioral deficits and corresponding histopathological consequences can be produced with low variability in this strain and species. Three distinct contusion injury groups were prepared by defining the amplitude of displacement at 0.3mm, 0.5mm, or 0.8mm. Significant differences in performance on three behavioral tests and quantitative
measures of histological outcome distinguished the groups. These data provide a multifaceted description of the behavioral and histological consequences of controlled contusion injury in a murine model.

**Modeling Considerations**

Suitable models of spinal contusion injury require a standardized injury procedure as well as reproducible functional and morphological outcome measures (Blight, 1996; Bresnahan et al., 1987; Stokes and Horner, 1996; Wrathall et al., 1985). Use of the ESCID for mouse spinal cord contusions provides several advantages for performing consistent injuries. In particular, the establishment of a fixed pre-load or 'touch' force ensures that the impact is initiated from a standardized starting point of minimal compression (Stokes et al., 1992). The computer-controlled displacement sequence and the electromagnetic shaker produce a constant impact pattern that is precisely duplicated in each specimen (Jakeman et al., 2000). Finally, the success of this approach in the mouse also depends on surgical procedures that achieve rigid stability of the vertebral column prior to injury and intensive post-operative care. Together, these factors contribute to low variability as well as a 100% survival rate, even in the most severe injury group in this mouse model.

**Graded Injury Severity**

An extensive literature of contusion injury modeling in the rat indicates that the amount of histological damage and the extent of functional deficits are directly related to severity of the injury as determined by the investigator (Wrathall et al., 1985; Behrmann
et al., 1992; Bresnahan et al., 1987; Gale et al., 1985; Basso et al., 1996a). In most cases, changes in the chosen independent variable (e.g. mass or height of a weight dropped onto the spinal cord) produce corresponding changes in functional and histological outcome. The resulting outcomes are typically referred to as mild, moderate or severe with regard to the extent of tissue damage and functional deficits.

In the present study, we hypothesized that variations of 0.2-0.3mm in displacement would result in different functional and histological outcomes in the mouse model. The results demonstrate that graded outcomes can be produced by setting the amplitude of displacement of the mouse spinal cord at 0.3mm, 0.5mm or 0.8mm. Animals from these groups showed distinct patterns of locomotor recovery and significant differences in histological measurements, including lesion length, white matter sparing, and fibrous scar tissue volume. Therefore, adjusting displacement amplitude is an effective means to produce graded injury outcomes. Other recent studies have confirmed that graded injury outcomes can also be achieved in mice using a weight-drop (Kuhn and Wrathall, 1998) or compression (Farooque, 2000) injury. However, both of those models lack the biomechanical measures that permit direct monitoring of variations during the injury sequence.

The functional recovery patterns for the contusion groups were also compared to that of complete transection. The BBB scores of mice with severe contusion injuries (0.8mm group) were not significantly different from those with complete transections at most time points. The average white matter sparing in the 0.8mm group was 2.5%, not different from zero sparing in the complete transection group. The results suggest that the degree of white matter sparing needed to substantially impact locomotor recovery

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may be greater in mice than in rats (Basso et al., 1996a). In addition, these results suggest that a 0.8mm displacement injury provides a good model of the most severe contusion, leading to nearly complete loss of function.

**Behavioral Outcome Measures**

Three behavioral tests were chosen to evaluate the effect of displacement amplitude on locomotor function. The BBB locomotor rating scale was chosen because it has become a standard across laboratories to evaluate locomotor recovery after spinal cord injuries in rats (Basso et al., 1995). The BBB scores revealed variations in the overall patterns of hindlimb recovery in mice across the full range of the injury groups. The correlation of the BBB scores with the peak force of impact and with the white matter sparing demonstrate that this rating scale is valid to assess locomotor recovery after spinal contusions in mice, as in rats (Basso et al., 1995; Basso et al., 1996a).

Use of the BBB scale for mice in our study also revealed some limitations. For example, following mild (0.3mm) contusion, the BBB scale distinguished the injured mice from laminectomy control animals only during the first week after injury. Similar results have been reported following mild contusion injuries (Basso et al., 1995), or excitotoxic injuries (Magnuson et al., 1999) at mid-thoracic levels in rats, in which a large percentage of ventral and lateral white matter has been preserved. The results suggest that adaptations may be necessary to improve the sensitivity of upper end of this scale in mice. Following a 0.5mm displacement injury, the mice exhibited an unambiguous pattern of recovery to a plateau of BBB scores ranging from 11-13. As described in Methods and Results, none of the mice sustaining a moderate injury...
demonstrated a smooth rhythmic gait consistently during the four minute test. Hence, they did not achieve consistent coordination. Because this criteria was not met, changes in paw rotation and toe clearance did not contribute to the BBB scores in this group. Therefore, scale modifications may help to differentiate locomotor changes that are not associated with coordination.

Despite these limitations, we feel that overall, the BBB scale has advantages, such as ease of use, objectivity, and inter-rater reliability, that some other behavioral tests do not have. It serves as a useful and effective measure to evaluate open field locomotion abilities in mice. Our results also support the current argument for using multiple behavioral tests to evaluate residual function and functional recovery after spinal cord injury in rodent models (Lardy and Wellman, 1953; Muir and Webb, 2000). In the present study, for example, the grid walk test and footprint analysis were also used.

The grid walking test is sensitive to small residual deficits following incomplete SCI in rats (Behrmann et al., 1992; Prakriya et al., 1993). Performance on this test may be related to the function of propriospinal pathways in the more medial white matter tracts (Bresnahan et al., 1987) or to the effects of corticospinal (Grill et al., 1997) or sensory fibers in the dorsal columns or rubrospinal fibers in the dorsolateral columns (Whishaw et al., 1992). In the present study, mice from the mild injury group exhibited long lasting deficits when tested on the grid. Therefore, a difficult task such as grid walking would be recommended as a secondary outcome measure for mice receiving discrete lesions or mild contusion injury.

Footprint analysis was first developed to evaluate functional recovery after sciatic nerve injury in rats (de Medinaceli et al., 1982) and later modified by other investigators.
to assess behavioral outcome after SCI (Behrmann et al., 1992; Kunkel-Bagden and Bregman, 1990; Kunkel-Bagden et al., 1992; Stokes and Reier, 1992). In this study, the recorded footprints provided a direct and objective assessment of toe clearance, base of support, and forelimb-hindlimb matching in the intermediate range of deficits. Consistent (>95%) toe clearance was confirmed in all mice that received a laminectomy only or 0.3 mm injury, while mice from the 0.5 mm injury group showed a range of deficits in toe clearance. After injury, mice that sustained 0.3 or 0.5 mm displacement showed a decreased base of support compared with pre-injury values. This finding illustrates the sensitivity of footprint analysis for detecting deficits following mild injury that were not evident with BBB scores alone. However, in contrast to results from rats (Behrmann et al., 1992), the base of support did not differ with injury severity in this species.

The footprint analysis revealed additional insights of mouse locomotor recovery with regard to coordination. The use of the forelimb-hindlimb “matching index” is a simplified method for evaluating the spatial register for forelimb-hindlimb coordination. In general, the matching index corresponded to the assignment of BBB scores in the open field. However, it is important to recognize that the two approaches differed with regard to the definition of coordination (a temporal rhythmic gait vs. discrete spatial forelimb-hindlimb matching), the number of passes required to obtain a score (as many as possible in four minutes vs. two trials), and locomotor environment (free to locomote in any direction vs. constrained, forward locomotion). Thus, there were some anticipated deviations in assessment between the two tests. However, an unexpected finding was the wide variability in forelimb-hindlimb matching ratios within groups of mice that
sustained the same level of injury (Fig. 4). The greatest variability was seen in the moderate (0.5 mm) injury group, suggesting that these animals employed the widest range of interlimb patterns of locomotion. A recent detailed analysis of forelimb-hindlimb coordination following mid-thoracic hemisections in Balb/C mice revealed a negative correlation between the accuracy and consistency of interlimb coordination and the area of residual white matter in the ventral funiculus (Basso et al., 2000). These findings suggest that the variability in coordination patterns may be common following mild-moderate injuries in the mouse.

Influence of Training on Hindlimb Function in Behavioral Tasks

Training and motivation play an important role in assessment of behavioral function. Previous studies have demonstrated that animals that are trained prior to injury will perform better on a similar task after injury than animals that are untrained (De Leon et al., 1999). We predicted that training prior to injury would improve the compensatory plasticity of the residual pathways in the spinal cord (De Leon et al., 1998). It might be more difficult to evaluate the relationship between tissue loss and functional recovery in animals that had mastered a skilled task prior to injury. Therefore, the mice in our studies were acclimated to the apparatus, but not trained prior to injury. Training began at six weeks post-injury when recovery of open field locomotion had reached a plateau. Three weeks of daily training had no effect on open field locomotion, grid walk, or footprint analysis. These results indicate that the deficits observed in these tasks are independent of plasticity associated with a simple 10-15 minute post-injury daily training paradigm started 6 weeks after injury. The correlation of performance with injury severity suggests
instead that functional recovery on these tasks is more closely related to the loss of ascending and/or descending function at the injury site (Magnuson et al., 1999). Future work will address if there is a critical period for initiating training either prior to or after injury that may improve functional outcome on these tasks.

**Chronic Histopathology**

Previous research has shown a high degree of correlation between white matter sparing and behavioral outcome after contusion or compression SCI in cats (Ford, 1983; Means et al., 1981), and rats (Basso et al., 1996a; Bresnahan et al., 1987; Noble and Wrathall, 1989). Treatments that preserve white matter also improve the degree of functional recovery after injury in the rat contusion model (Behrmann et al., 1993; Behrmann et al., 1994; Grossman et al., 1999). Results in the present studies demonstrate a similar correlation between the area of residual white matter at the lesion epicenter and the biomechanical parameters and behavioral outcome measures. The lesion length and size of the scar tissue defined by fibronectin deposition were also significantly different across injury groups and correlated inversely with injury severity.

There was no qualitative difference in the composition of the central scar region in the different injury groups, except the presence of moderate-sized cystic cavities in the 0.3mm injury group. One common finding after SCI in mice, following either contusion injury (Jakeman et al., 2000; Kuhn and Wrathall, 1998) or crush injury (Fujiki et al., 1996; Zhang et al., 1996), is the absence of cavitation at the lesion site. In the present study, we confirmed the absence of cavitation and accumulation of scar tissue following 0.5mm and 0.8mm injury. In the 0.3mm injury group, however, moderate cystic cavities...
were observed within the central lesion region. This finding suggests that cavitation and fibrosis represent different possible outcomes of the simultaneous proliferative and phagocytic processes that occur following injury. Molecular pathways that contribute to these and other processes of secondary injury can be examined in greater detail with specific genetic mutations using a consistent murine model of traumatic spinal contusion injury.

In conclusion, a spinal cord injury model has been developed in the C57Bl/6 mouse, using the ESCID injury device described previously (Jakeman et al., 2000). The present study demonstrates that the ESCID device produces graded contusion injuries with low variability, which results in distinct behavioral and histological outcomes between injury groups. Use of multiple behavioral outcome measures provides a thorough analysis on the chronic functional recovery process in the mouse after contusion injury. The availability of producing reliable contusion injuries with appropriate outcome measures represents great potential to use this mouse model to study the cellular mechanisms of secondary injury and repair after traumatic SCI.
FIGURE 1. Biomechanical measures and locomotor recovery following spinal cord injury with three levels of displacement. (A, B) Records of displacement (A) and force of impact (B) taken from a representative 0.3mm (c), 0.5mm (b), and 0.8mm (a) contusion injury. Graphs were plotted from recordings of the displacement and force transducers, collected every 0.125 msec. (C) Time course of locomotor recovery as measured by BBB locomotor rating scale for five groups. Each point represent the group mean ± SEM. Kruskal-Wallis nonparametric analysis of variance revealed differences between injury levels across time. Differences at each day post-injury were identified by Bonferroni's post-hoc test.
FIGURE 2

(A) Grid walking scores in the 0.3mm injury group across days. At each time point after injury, the group had more foot misses than pre-op level \((p < 0.01^{**}, 0.05^*)\). (B) Grid walking scores following laminectomy only, 0.3mm and 0.5mm displacement injury. After injury, the 0.3mm and 0.5mm groups had more deficits than pre-operative level \((p < 0.01^{**}, 0.05^*)\). At 42 days post injury, both injury groups had significant more foot misses than laminectomy control \((^p < 0.001)\). At 63 days post injury, the 0.5mm group but not the 0.3mm group had more foot misses than control \((^p < 0.001)\). Post-hoc test revealed significant differences between 0.3mm and 0.5mm group at both 42 and 63 days post injury \((^p < 0.001)\). All values are means ± SEM.
FIGURE 2

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FIGURE 3

Representative footprint records obtained from laminectomy control (A), 0.3mm injury (B), and 0.5mm injury (C) mice at 63 days post-operative. Forelimb prints are indicated in red. Hindlimb prints are black. The direction of mouse movement is indicated by the gray arrows. The base of support was measured as the distance between the heel spots of the two hindlimbs. A toe drag was counted for each hindpaw ink streak that extended beyond one paw length.
FIGURE 3

A  Laminectomy

B  0.3mm Injury

toe drag

C  0.5mm Injury

base of support

1 cm
FIGURE 4

Footprint analyses of the 0.3mm and 0.5mm injury groups. All values are means ± SEM.
(A) Base of support. Both injury groups showed a significant decrease in the base of support compared to laminectomy control (*p < 0.001). There were no differences between the two injury groups. (B) Toe drags. Toe drags were never observed in records from laminectomy controls, and rarely seen following the 0.3mm injury. In contrast, frequent toe drags were observed following the 0.5mm displacement injury. Significant differences in toe drags/cycle were found between the injury groups (*p < 0.01) at 42 and 63 days post-operative. (C) Forelimb-hindlimb matching ratio. This ratio was calculated by counting the number of forelimb step cycles that were in 1:1 correspondence with hindlimb prints. This value was then divided by the total number of forelimb step cycles in the full runway record. Each point represents an individual animal with the bar representing the group mean.
FIGURE 5

Photomicrographs of representative mouse spinal cord transverse sections taken at the level of the T9 vertebra from a laminectomy control animal (A), and from the lesion epicenter of subjects sustaining 0.3mm (B), 0.5mm (C), and 0.8mm (D) injury. All mice were sacrificed at 9 weeks post injury. The arrows in (A) illustrate the approximate distance of displacement. The Luxol fast blue (LFB) stained tissues revealed a central lesion area surrounded by a rim of spared white matter which decreased with increasing injury level. Dotted lines illustrate the rim of spared tissue that was quantified in histological analysis. Scale bars, 160μm.
FIGURE 6

Lesion morphometry from LFB stained sections. (A) Total cross-sectional (CS) area at the epicenter was decreased following 0.5mm and 0.8mm displacement vs. laminectomy (LA) control (*p < 0.001). Significant differences were also found between the 0.3mm and 0.5mm, and between the 0.3mm and 0.8mm injury groups (*p < 0.001). (B) The amount of spared white matter reported as the % of total CS area of the cord revealed significant differences between all three injury groups and laminectomy controls (*p < 0.001), and between each of the injury groups (*p < 0.001 vs. 0.3mm, *p < 0.001 vs. 0.5mm group). (C) Lesion length was significant greater when the injury level was higher (*p < 0.001 vs. 0.3mm group, *p < 0.001 vs. 0.5mm group).
FIGURE 6

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FIGURE 7

Photomicrographs of representative immunohistochemistry at the lesion center 9 weeks following 0.5mm contusion injury in mice. (A) Anti-neurofilament H-stained lesion site. (B-G) Lesioned area from adjacent sections shown at higher magnification. The central lesion area contained scattered axons (B) and macrophages (D), but was devoid of GFAP-positive astrocytes (C) except for small foci surrounding some blood vessels (*). The extracellular matrix stained most densely with antisera against fibronectin (F). Laminin immunoreactivity (E) was observed in association with vessels (arrowheads), but was also found in thickened cables (arrows). Collagen IV (G) antisera stained thin tendrils, many of which appeared to be oriented perpendicular to the plane of the section (arrowheads). Scale bars: A. 100μm. B-G. 20μm.
Photomicrographs of the immunohistochemistry studies showed different zones of the lesion area 9 weeks after 0.5mm injury. A discrepancy in spatial extent between LFB stained tissue (A and D) and adjacent GFAP stained tissue (B&E) revealed a transition region (outlined in C-I) of reactive gliosis at the edge of the lesion. While the central core area was occupied by dense fibronectic scar tissue (F), the transition area was characterized by a high density of demyelinated axons (D and G), reactive astrocytes (E), reactive microglia (H), and laminin (C). In contrast, fibronectin (F), and collagen IV (I) were less prominent in this region than in the nearby central core area. Scale bars: A-B. 100μm, C-I. 40μm.
FIGURE 9. Quantitative comparisons of fibronectin immunoreactivity at the lesion epicenter (0 position), and at selected intervals rostral (+) and caudal (-) to the epicenter for each of the contusion injury groups at 9 weeks after injury. One-way ANOVA revealed significant differences across groups at the epicenter. 0.5mm rostral and 0.5mm caudal to the epicenter ($p < 0.0001$). Comparisons at each site were made with Bonferroni's post-hoc test ($^* p < 0.001$ vs. 0.3mm group; $^* = vs. 0.5mm group, p <0.05*$. 0.01**, 0.001***).
TABLE 1. Comparison of injury parameters, and behavioral and histological outcomes following impact at three displacement levels.

<table>
<thead>
<tr>
<th>Group</th>
<th>Displacement (mm)</th>
<th>Peak Force (kdynes)</th>
<th>Impulse Momentim (kdyne-sec)</th>
<th>BBB Score At 63 dpo</th>
<th>Spared White Matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminectomy</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>21.0 ± 0.0^*#</td>
<td>69.3 ± 0.7^*</td>
</tr>
<tr>
<td>(n=7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3mm Injury</td>
<td>0.284 ± 0.001^*</td>
<td>51.5 ± 5.0^*</td>
<td>0.613 ± 0.069^*</td>
<td>21.0 ± 0.0^*#</td>
<td>51.2 ± 3.0^*</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5mm Injury</td>
<td>0.498 ± 0.002^*</td>
<td>102.4 ± 9.5^*</td>
<td>1.045 ± 0.158</td>
<td>12.4 ± 1.1^*#</td>
<td>17.3 ± 3.0^*</td>
</tr>
<tr>
<td>(n=8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.8mm Injury</td>
<td>0.792 ± 0.003^*</td>
<td>185.4 ± 13.9^*</td>
<td>1.634 ± 0.204^*</td>
<td>3.8 ± 1.0^*</td>
<td>2.5 ± 0.4^*</td>
</tr>
<tr>
<td>(n=8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transection</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.3 ± 0.4^*</td>
<td>-</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group Mean</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Significance</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

All values are means ± SEM. Group means were compared by one way ANOVA (++: p < 0.01, +++: p < 0.001) followed by Bonferroni's post-hoc test (^- vs. 0.3mm group, ^- vs. 0.5mm group, ^- vs. 0.8mm group, #- vs. complete transection group).
<table>
<thead>
<tr>
<th></th>
<th>Peak Force</th>
<th>Maximum I/M</th>
<th>BBB 63 dpo (rₚ)</th>
<th>Grid walk</th>
<th>White Matter Sparing</th>
<th>Lesion Length</th>
<th>Fibronectin Immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Displacement</td>
<td>0.873***</td>
<td>0.686**</td>
<td>-0.855***</td>
<td>0.798**</td>
<td>-0.898***</td>
<td>0.964***</td>
<td>0.781***</td>
</tr>
<tr>
<td>Peak Force</td>
<td>0.910***</td>
<td>-0.814***</td>
<td>0.431</td>
<td>-0.762***</td>
<td>0.873***</td>
<td>-0.645**</td>
<td></td>
</tr>
<tr>
<td>Maximum I/M</td>
<td></td>
<td>-0.709***</td>
<td>0.166</td>
<td>-0.594**</td>
<td>0.704**</td>
<td>0.519*</td>
<td></td>
</tr>
<tr>
<td>BBB 63 dpo (rₚ)</td>
<td></td>
<td></td>
<td>-0.855***</td>
<td>0.935***</td>
<td>-0.907***</td>
<td>-0.838***</td>
<td></td>
</tr>
<tr>
<td>Grid walk</td>
<td></td>
<td></td>
<td></td>
<td>-0.802**</td>
<td>0.774**</td>
<td>0.865**</td>
<td></td>
</tr>
<tr>
<td>White Matter Sparing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lesion Length</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.714***</td>
<td></td>
</tr>
</tbody>
</table>

The correlation between open field locomotion (BBB scores) and biomechanical parameters (displacement, peak force, and maximum impulse/momentum) or histological outcomes represent Spearman correlation coefficient (rₛ). All other values represent Pearson correlation coefficient (r). Significance is indicated where $p < 0.05*$, $0.01**$, $0.001***$. 
CHAPTER 2

MONOCYTE RECRUITMENT AND MYELIN REMOVAL ARE DELAYED FOLLOWING SPINAL CORD INJURY IN MICE WITH CCR2 CHEMOKINE RECEPTOR DELETION

Introduction

A primary mechanism for selective leukocyte infiltration after injury or disease is the differential expression of chemokines (chemoattractive cytokines) (Adams and Lloyd, 1997; Glabinski et al., 1995; Ransohoff, 1997; von Tschamer et al., 1986; Rollins, 1997; Murphy et al., 2000). Traumatic injury in the central nervous system (CNS) has been consistently associated with enhanced expression of the β-chemokine, CCL2, commonly referred to a monocyte chemoattractant protein-1 (MCP-1) (Ransohoff et al., 1993; Glabinski et al., 1996; Ransohoff, 1997; Hausmann et al., 1998; McTigue et al., 1998; Lee et al., 2000; Glabinski et al., 1995; Berman et al., 1996). MCP-1 mRNA expression in the CNS has been localized most frequently to reactive astrocytes (Muessel et al., 2000), but it may also be produced by microglia (Gourmala et al., 1997; Hesselgesser and Horuk, 1999) and neurons (Coughlan et al., 2000; Glabinski et al., 1996; Glabinski et al., 1995; Simpson et al., 1998). There are additional murine MCPs that exhibit high homology to MCP-1, with overlapping functional characteristics in vitro. However.
recent evidence from pharmacological and murine knock-out studies suggests that MCP-1 mediates important and non-redundant actions in CNS inflammation (Ghimikar et al., 1998; Huang et al., 2001).

In the mouse, the major monocyte receptor for MCP-1 is CCR2 (Boring et al., 1996; Charo, 1999). In addition to MCP-1, CCR2 also binds other β chemokines, including MCP-2, MCP-3, MCP-4, and MCP-5 (Ben-Baruch et al., 1995; Garcia-Zepeda et al., 1996; Gong et al., 1997; Sarafi et al., 1997). Thus, the CCR2 receptor represents an attractive target for examining the functional role of this closely related family of chemokines. Genetic deletion of CCR2 results in the impaired recruitment of macrophages in several models of injury and disease, including experimental peritoneal inflammation (Kurihara et al., 1997; Boring et al., 1997; Kuziel et al., 1997), apolipoprotein E-related atherosclerosis (Boring et al., 1998), and *Listeria monocytogenes* infection (Kurihara et al., 1997). CCR2-deficient mice also exhibit altered inflammatory responses in nervous tissue, including decreased susceptibility to experimental autoimmune encephalomyelitis (Fife et al., 2000; Izikson et al., 2000), and impaired recruitment of macrophages and removal of myelin debris following sciatic nerve injury (Siebert et al., 2000). Together, these results implicate CCR2 as a principal mediator of early macrophage recruitment in tissue inflammation. In the present study, we investigated the macrophage response to SCI in CCR2-deficient mice and wild type controls. The results demonstrate that chemokines acting through CCR2 play an important role in the early phase of monocyte recruitment and myelin phagocytosis in SCI.
Methods

Animals

The generation of CCR2 (-/-) mice by homologous recombination has been described previously (Boring et al., 1997). One line was maintained by crossing CCR2 (-/-) mice onto a C57Bl/6 background for 8 generations. A second line was produced on a hybrid C57Bl/6 x 129S4/SvJae background. Pairs of heterozygous breeders were obtained from Dr. Charo's laboratory and the colony maintained in pathogen-free housing at the Ohio State University vivarium for the course of these studies. The mice used for spinal cord injury were F2 progeny of heterozygous pairs from the C57Bl/6 line, the hybrid line, or heterozygous crosses from the two lines (Hesselgesser and Horuk, 1999; Steward et al., 1999). Littermate CCR2 (-/-) and CCR2 (+/+)) pairs were used when available, and mice from all three breeding lines were included in each experimental group to minimize any effects of background genes. Genotypes were determined by PCR analysis of tail samples collected at weaning and confirmed at the time of tissue harvest (Boring et al., 1997).

Spinal Cord Contusion Injury

Experimental spinal cord injuries were described in detail in the general methodology. The mice were sacrificed at 1 and 7 days post-injury. All analyses were done by an investigator who was blinded with regard to experimental groups. Tissues from animals in each group were mounted on the same slides and processed simultaneously.
**Behavioral Recovery**

The mice were tested at 1, 3, 7, and 14 dpi to evaluate the recovery of hindlimb function in spontaneous overground locomotion (Ma et al., 2001) using the Basso, Beattie, Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). Each mouse was scored over a 4-minute period by a team of two examiners who were blind to mouse genotype. The evaluators assigned an operationally defined score (0-21) for each hindlimb. The scores from the two hindlimbs were averaged at each time point.

**Histopathology**

*Lesion Epicenter and Tissue Sparing.* One complete set of evenly spaced sections (100µm apart) was stained with Luxol fast blue (LFB) to identify myelinated white matter regions. For each specimen, the lesion epicenter was defined as the tissue section with the smallest cross-sectional area of LFB staining in the peripheral rim (Behrmann et al., 1992; Jakeman et al., 2000; Ma et al., 2001). Borders were drawn on digitized, spatially calibrated images (SONY 970 color CCD), and the cross-sectional area of spared white matter and total tissue were determined using MCID. Lesion length was determined to the nearest 0.1 mm by examining the LFB stained sections rostrally and caudally from the epicenter until sections with a normal gray and white matter distribution and density of LFB staining were seen.

*Mac-1 Immunohistochemistry.* For immunohistochemical detection of activated microglia and macrophages, adjacent slide series were stained with a monoclonal rat anti-mouse antibody raised against complement receptor CR3 (Mac-1, Serotec Ltd., Oxford, England; 1:200). Biotinylated rabbit anti-rat antibody (Vector Labs, Burlingame, CA: 68
1:400) was applied overnight and then stained using Elite ABC (Vector Labs) and the chromagen, diaminobenzidine (Vector Labs). At 7 and 14 dpi, macrophages and microglia coalesce and cannot be distinguished and counted. Therefore, macrophage activation/infiltration was quantified using proportional area measurements, using a computer-assisted image analysis program as described previously (MCID: Imaging Research Inc., Ontario, Canada) (Popovich et al., 1997; Ma et al., 2001). The cross-sectional area of the spinal cord tissue section was drawn on digitized, spatially calibrated images (SONY 970 color CCD). Images from equally spaced sections at 100 μm intervals surrounding the epicenter were digitized, and the optical density detection threshold was adjusted to differentiate positive staining from background. Proportional area was computed as the fraction of the area of positive staining divided by the total cross-sectional area of the tissue section.

**Oil red O.** Myelin phagocytosis was analyzed from sections stained with Oil Red O (Fisher Scientific, Pittsburgh, PA) and counterstained with Mayer's hematoxylin (Zymed) to identify cell nuclei (Chester et al., 1971). Oil red O staining was measured using the MCID by determining the proportional area of an 0.18 mm² sampling region positioned in the center of the tissue sections that was occupied by the staining in the red range of the color spectrum (digital threshold settings set by hue and intensity of staining).
**RNA Preparation and RNase Protection Assay**

Mouse chemokine and chemokine receptor mRNA expression was measured by RNase protection assay (RPA) following *in vitro* transcription as described in general methodology.

**Statistical Analysis**

Histological outcome measures from CCR2 (+/+) and CCR2 (-/-) specimens were compared using the unpaired Student's *t* test. Two-way ANOVA was used to compare chemokine or chemokine receptor message across days and genotype.

**Results**

*Mac-1+ Immunoreactivity is Reduced at 7 dpi in CCR2 (-/-) Mice*

Mac-1 immunostaining revealed the distribution of reactive microglia, monocytes, and macrophages at 1, 7, and 14 dpi. Proportional area measures of stained tissues (stained area / total cross-section tissue area) were made in the section closest to the lesion epicenter as defined by staining with LFB. At 1 dpi, small, irregularly shaped Mac-1 positive cells were few in number and more prominent within the spinal gray matter regions than surrounding white matter (Figure 10A-C). There were no differences between CCR2 (+/+) and CCR2 (-/-) specimens at this time point. By 7 dpi, Mac-1 immunoreactivity was evident throughout the lesion epicenter of CCR2 (+/+) specimens. In contrast, the epicenter sections of CCR2 (-/-) mice at 7 dpi contained large regions that were devoid of Mac-1 staining, particularly in the region corresponding to the location of normal dorsomedial gray matter. Mac-1 distribution and cell morphology in the
peripheral rim of these specimens was similar in CCR2 (+/+ ) and CCR2 (-/- ) specimens. However, due to the marked reduction in Mac-1-positive cells in the center of the section, there was a significant decrease in the total Mac-1 immunoreactivity of the entire cross-sectional tissue area (Figure 10D-F). Although Mac-1 staining extended for over 1 mm in each direction, the decreased Mac-1 staining in CCR2 (-/- ) mice was highly localized to the lesion epicenter and 0.2 mm in the caudal direction (Figure 11).

By 14 dpi, the Mac-1 stained region area occupied the entire central area of all specimens (Figure 10G-I). Immunostaining was most prominent at the interface between the residual rim of white matter and the original gray matter region of the spinal cord. The cells within the peripheral tissue exhibited irregular morphology of reactive microglia. In the center of the lesion, the Mac-1 staining was associated with large round cells and within the surrounding extracellular matrix. There were no differences in the distribution or intensity of staining between the knock-out and wild-type animals at this time point.

**Reduced Mac-1 Immunoreactivity is Associated With Reduced Myelin Degradation**

Macrophages are important for removal of cellular and myelin debris after injury (Blight, 1985). At 7 dpi, LFB myelin staining was found within the peripheral tissue rim in both CCR2 (+/+ ) and CCR2 (-/- ) specimens. However, the CCR2 (-/- ) animals also exhibited regions within the lesion center that stained densely with LFB, while only small patches of similar staining were observed in the CCR2 (+/+ ) controls (Figure 12A,B). Close microscopic analysis of this LFB stained region within the gray matter revealed a different staining pattern (more densely stained) than the spared peripheral white matter.
suggestive of myelin debris that was not completely degraded. This region of dense LFB stain corresponded with those areas that were devoid of Mac-1 immunoreactivity (Figure 12C-H). The CCR2 (-/-) mice also showed significantly less Oil red-O staining in the lesion center than the CCR2 (+/++) mice at this time point. Oil red O stains myelin degradation products, indicating reduced phagocytic activity by macrophages at the lesion epicenter (Figure 12I-J; Figure 13). By 14 dpi, the center of the lesion was devoid of LFB, but exhibited Oil red O staining in both CCR2 (+/++) and CCR2 (-/-) mice.

Chemokine and Chemokine Receptor mRNA Expression in Contused Mouse Spinal Cord

The expression of selected α-chemokine (MIP-2, IP-10) and β-chemokine (MCP-1, MCP-3, MIP-1α, MIP-1β, and C10) mRNA was determined by RPA analysis of spinal cord tissue obtained at 1 and 7 dpi (Figure 14A). MCP-1, MCP-3, and MIP-2 were increased at 1 dpi compared with 7 dpi. Modest increases were observed for MIP-1α, IP-10, and C10. These data are similar to those reported following spinal contusion injury in rats (McTigue et al., 1998). There was no significant difference in the pattern or magnitude of chemokine message expression between CCR2 (+/++) and CCR2 (-/-) animals at either time point (Figure 15).

Chemokine receptor mRNA expression was also regulated after injury (Figure 14B). In wild-type mice, CCR1 and CCR2 mRNA levels were increased at 1 dpi relative to 7 dpi. CCR2 (-/-) mice had no detectable CCR2 mRNA. In addition, the CCR2 (-/-) mice had significantly reduced levels of CCR1 and CCR5 receptor mRNA at both 1 and 7 dpi compared with the CCR2 (+/++) mice (Figure 15).
**Lesion size and behavioral outcome**

Morphometric analyses of LFB-stained tissue revealed no significant differences between CCR2 (+/+) and CCR2 (-/-) mice with regard to lesion size or behavioral outcome. Surprisingly, these data instead revealed a trend toward decreased white matter sparing (t-test; p=0.07) and increased lesion length (t-test; p=0.08) in the CCR2 (-/-) mice at 7 dpi (Figure 16). The CCR2 (-/-) mice also exhibited a trend toward decreased behavioral recovery scores at 7 dpi (Mann Whitney U; p=0.07), but the behavioral scores were completely overlapping by 14 dpi (p=0.69). The reduced scores at 7 dpi were consistent with the trend toward decreased white matter sparing at the lesion epicenter at this time point. Thus, the delayed myelin removal in the CCR2 (-/-) was associated with trends toward increased tissue loss and delayed functional recovery.

**Discussion**

The interaction of MCP-1 with CCR2 is a critical signaling event in a number of models of inflammatory disease or trauma. In the present study, we investigated the role of CCR2 in the inflammatory response following spinal cord contusion injury in mice. CCR2 depletion impaired the recruitment of monocytes and the degradation of myelin at the impact site at 7 days following SCI. The results demonstrate an important role of this mouse chemokine receptor in the early phase of monocyte recruitment to the injured spinal cord.

Spinal cord contusion results in rapid activation of microglia and astrocytes and the recruitment of neutrophils to the site of impact within hours of the initial injury (Carlson
et al., 1998; Dusart and Schwab, 1994). This response is followed by the accumulation of monocytes and macrophages at the injury epicenter, beginning at about 24 hours post-injury and reaching a peak at approximately 7 days in the rat (Streit et al., 1998; Popovich et al., 1997). The time course of Mac-1 immunoreactivity in C57Bl/6 mice has shown for the first time the similar temporal sequence of macrophage accumulation following SCI. Microglia/macrophage staining reached maximal activation at 7 days post-injury and decreased in intensity by 14 dpi. Neutrophil and macrophage recruitment in this injury is a highly regulated process that is correlated with the expression of selective chemokines. Peak expression of mRNA for the α-chemokines, GRO-α and IP-10, precedes neutrophil invasion, while expression of the β-chemokines, MCP-1 and MCP-5, precedes the peak appearance of monocytes and macrophages (McTigue et al., 1998; Streit et al., 1998; Lee et al., 2000).

MCP-1 is a potent monocyte chemotactic mediator and is the predominant β-chemokine in models of CNS injury that result in a macrophage-rich inflammatory response. In addition to SCI, increased MCP-1 expression occurs in response to cortical stab wounds or cryolesions (Berman et al., 1996; Hausmann et al., 1998; Grzybicki et al., 1998), lysophosphatidylcholine injection leading to focal demyelination (Ousman and David, 2000), focal ischemia (Gong et al., 1997), or deafferentation (Muessel et al., 2000); review in (Ransohoff, 1997). Peripheral nerve injury also induces a selective expression of MCP-1 (Coughlan et al., 2000; Toews et al., 1998; Ransohoff, 1997). We have shown here that contusion injury in the mouse evokes a robust increase in MCP-1, MCP-3, and MIP-2 expression at 1 day post-injury relative to 7 days post-injury, and a more modest increase in MIP-1α at this time point. Thus, MCP-1 and MCP-3 represent
likely candidates for modulating monocyte/macrophage recruitment to the injured mouse spinal cord.

MCP-1 evokes chemotaxis of monocytes through CCR2 (Dzenko et al., 2001; Kuziel et al., 1997). The present results demonstrate that CCR2 is required for the initial phase of monocyte recruitment at the epicenter of a contusion lesion. The results are similar to those reported following sciatic nerve injury in CCR2 (-/-) mice, which demonstrated reduced macrophage recruitment and myelin phagocytosis during the first week post-injury (Siebert et al., 2000). As seen in the peripheral nerve injury model, the effect of CCR2 deletion after SCI was transient, and the reduction in macrophage accumulation and myelin phagocytosis was restored by 2 weeks post-injury. Thus, the effect of CCR2 deletion is a delayed recruitment of phagocytic cells to the lesion site. These results suggest that in the absence of CCR2, MCP-1 might act through other non-CCR2 receptors, such as CCR1 and CCR5 to accumulate macrophages to the lesion site with a slower time course. Alternatively, additional signals are able to recruit phagocytic macrophages to the center of the injury site and the surrounding tissues. Such signals might include other β-chemokines acting on macrophages or microglia, such as MCP-3, MIP-1α, and MIP-1β. These chemokines have also shown to be capable of binding to CCR1 and CCR5 (Murphy et al., 2000). However, the definitive role of these receptors in mediating inflammatory responses is not clear through receptor depletion studies. For example, CCR5 knockouts have been shown to exhibit reduced leukocyte recruitment into the CNS after Cryptococcus infection (Huffnagle et al., 1999), but were not responsible for macrophage accumulation after sciatic nerve axotomy (Siebert et al., 2000). CCR1 deficient mice showed exacerbated nephrotoxic nephritis with increased
accumulation of macrophages (Topham et al., 1999). Other possibilities may be that other cytokines and growth factors that are up-regulated after SCI, such as IL-1, TNF-α, or GM-CSF, can induce microglial and monocyte activation and inflammation (Bartholdi and Schwab, 1997; Streit et al., 1998). In the lysophosphatidylcholine model of demyelinating disease, for example, treatment at the injection site with specific antibodies to each of these cytokines resulted in partial suppression of macrophage recruitment, while robust suppression was observed following infusion of a cocktail of all four cytokines (Ousman and David, 2001).

The effects of CCR2 deletion on macrophage accumulation were restricted to the center of the impact site. The very localized effect is consistent with the known heterogeneous nature of CNS macrophages. These cells are derived from two distinct sources, including the endogenous tissue microglia and circulating hematogenous monocytes. The immunocytochemical marker, Mac-1, cannot distinguish these two populations of macrophages. However, a recent study has employed a bone marrow chimeric rat model to define the regional distribution of peripherally derived macrophages and central (microglial) macrophages after spinal cord contusion injury (Popovich and Hickey, 2001). The two cell types are distinguished in the chimeric rats by unique immunocytochemical cell surface markers derived from the different rat strains. Using this approach, the authors showed that by 1-3 days after a contusion, macrophage markers were associated primarily with host microglia and by 7 days post injury, blood monocytes were the predominant source of macrophages at the injury center, but virtually all of the macrophages in the surrounding white matter and rostral and caudal segments of the injured cord were derived from the microglia of the chimera.
hosts. Taken together with our current findings, these results strongly support the interpretation that CCR2 selectively mediates the recruitment of hematogenous monocytes to the injury site.

The role of monocytes and the prominent macrophage response in SCI remains enigmatic, in part because of the functional diversity of these cells. On one hand, macrophages play a beneficial role in axonal regeneration by facilitating the removal of cellular debris (Scheidt et al., 1986; Zeev-Brann et al., 1998; Avellino et al., 1995), and by secreting extracellular matrix molecules and anti-inflammatory cytokines (Nathan, 1987). The delayed infiltration of macrophages in the CNS relative to the periphery may contribute to the failure of regeneration after CNS injury (Perry et al., 1987; George and Griffin, 1994; Zeev-Brann et al., 1998; Rapalino et al., 1998). The findings in this study showing that reduced Mac-1 immunoreactivity at the epicenter at 7 days was associated with reduced Oil red O staining in CCR2 deficient mice suggest a primary role of monocyte derived macrophages in phagocytosis at this time.

However, macrophages within the CNS may also be deleterious to spared tissue following SCI. These cells produce neurotoxic molecules, including quinolinic acid (Blight et al., 1995; Popovich et al., 1994) and nitric oxide (Grzybicki et al., 1998; Yamanaka et al., 1998), which can contribute to secondary or bystander damage to tissues and cells that survive the initial wave of necrotic cell death (Blight, 1992). Systemic treatments that reduce the inflammatory response to SCI, including bolus infusions of the glucocorticoid, methylprednisolone, and the anti-inflammatory cytokine, IL-10, have been reported to result in improved tissue sparing and improved recovery in models of spinal contusion injury (Behrmann et al., 1994; Bethea et al., 1999). The
depletion of peripheral monocytes during the first week post-injury has been shown to enhance tissue sparing and promote functional recovery in guinea pig and rat models of SCI (Blight, 1994; Popovich et al., 1999). Thus, macrophages participate in a wide variety of functions in the injured CNS, which both facilitate and interfere with recovery after SCI. In the present study, we found no significant differences in tissue sparing as a result of CCR2 deletion. These data suggest that other mechanisms of macrophage recruitment are compensatory with regard to tissue sparing and functional recovery. However, recent studies suggest that cellular events associated with SCI may extend for several weeks after injury in rats and mice (Ghimikar et al., 2001; Sroga et al., 2001; Watanabe et al., 1999). For example, using MCP-1 antagonist with treatment to seven days delayed the migration of macrophages into the lesion but did not affect degeneration (Ghimikar et al., 2000); however, continuous treatment with the broad spectrum antagonist, vMIPII, up to 21 days was shown to promote tissue survival and provide a growth permissive environment (Ghimikar et al., 2001). Therefore, long term (>14 days) studies may reveal more striking effects of delayed monocyte recruitment on tissue sparing and functional outcome after injury.

The present study has shown for the first time the direct involvement of the β chemokine receptor, CCR2, in inflammation after spinal cord contusion. The results indicate that chemokines acting through CCR2 are necessary during the early phase of macrophage recruitment following SCI. However, the effects mediated by CCR2 following SCI are complex, reflecting the heterogeneity of cellular responses to chemokines and other intercellular signaling molecules following traumatic SCI.
FIGURE 10. Representative photomicrographs and quantitative analysis of Mac-1 distribution and proportional area measures at the lesion epicenter at 1 dpi (A-C), 7 dpi (D-F), and 14 dpi (G-I). Macrophage density was reduced at the injury epicenter in specimens from CCR2 (-/-) mice obtained at 7 dpi (t-test; *=p<0.05), but not at 1 or 14dpi. The effect was due to a reduction in Mac-1 immunoreactivity at the center of the lesion site (E). Scale = 200 μm.
FIGURE 11. Distribution of Mac-1 proportional area measures at intervals spanning the rostro-caudal extent of the lesion at 7 dpi. Differences in Mac-1 immunoreactivity were restricted to the center of the lesion and 0.2 mm caudal to the epicenter (t-test; * = p<0.05; ** = p<0.01). Solid bars correspond to CCR2 (+/+) specimens and open bars correspond to CCR2 (-/-) specimens.

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FIGURE 12
Digital micrographs the injury epicenter at 7 dpi. Representative sections from sections stained with LFB myelin stain (blue; A-D), Mac-1 immunoreactivity (brown; C-H) and Oil red O/hematoxylin stain (I, J). The dual staining in C and D was obtained by superposing images of LFB and Mac-1 stain using digital imaging. A, C, E. CCR2 (+/+) specimen contains Mac-1 immunoreactivity throughout the lesion epicenter, in areas devoid of LFB. B, D, F. Reduced Mac-1 immunoreactivity corresponds with greater residual LFB stain and absence of Oil red O reaction in the epicenter of a CCR2 (-/-) specimen. G, H. Higher magnification of area outlined in E and F, to illustrate paucity of Mac-1 staining in the central region. I, J. Oil red O from sections adjacent to G and H reveals active macrophages surrounding myelin debris in the CCR2 (+/+) specimen only. Scale A - F = 200 μm; Scale G - J = 50 μm.
FIGURE 12
FIGURE 13. Quantitative analysis of Oil red O staining in the center of the lesion. A. Representative photomicrograph illustrating the region selected for analysis from a CCR2 (+/+) specimen (see Figure 3 I for example). Staining in the red spectrum range was established as the target for analysis (black overlay in A). A fixed area of 0.18 mm² from the middle of the epicenter section was used for analysis as shown. B. Oil red O stain was reduced in CCR2 (-/-) specimens (n=9) compared with CCR2 (+/+) specimens (n=8); t-test, **p < 0.01.
FIGURE 14. Sample images from RPA using probe sets to identify chemokine (A) and chemokine receptor (B) mRNAs. Total RNA extracted from one spinal cord (20 μg) was loaded per lane.
Quantitative analysis of chemokine and chemokine receptor mRNA expression obtained from RPA. Bars represent mean ± SEM of the densitometric ratio of protected species, calculated relative to L32 density. Black bars represent CCR2 (+/+); n=7 at 1 dpi; n=5 at 7 dpi. Open bars are CCR2 (-/-); n=12 at 1 dpi; n=9 at 7 dpi. Two way ANOVA was used to determine the effect of dpi and genotype on group means (legend, upper right of each panel; int.= interaction effect). Asterisks above each pair of bars represent the results of t-tests to compare densitometric ratio for each chemokine at 1 or 7 dpi. * p<0.05; ** p<0.01; ***p<0.001.
FIGURE 15
FIGURE 16

LFB analysis of the peripheral white matter rim at the lesion epicenter at 7 dpi (A,B) and 14 dpi (C,D). A,C. Digital photomicrographs of LFB stained sections from CCR2 (-/-) and (-/-) specimens. Note the residual LFB stain in the center of the CCR2 (-/-) specimen at 7 dpi only. Residual or spared white matter in the peripheral rim was identified and manually outlined as indicated for quantitative analysis. Scale = 200 µm. B,D. White matter (LFB staining) in the peripheral rim was expressed as a percentage of the Cross-sectional Area (CS Area) at the lesion epicenter. CCR2 (-/-) mice showed a non-significant trend toward decreased tissue sparing at 7 and 14 days as compared with (+/+ controls. Values represent mean ± SEM: 7dpo (+/+. n=9; -/- n= 13; p=0.07); 14dpo (+/+ n= 3; -/- n=4; p=0.13).
FIGURE 16
CHAPTER 3

QUALITATIVE AND QUANTIATIVE IMMUNOHISTOCHEMICAL ANALYSES OF INFLAMMATORY RESPONSE AND WOUND HEALING AFTER SPINAL CORD CONTUSION INJURY IN TWO MOUSE STRAINS

Introduction

Various experimental models for SCI research in mice have revealed a very different histopathological sequelae than that have seen in rats and other species (Farooque, 2000; Fujiki et al., 1996; Kuhn and Wrathall, 1998; Jakeman et al., 2000; Ma et al., 2001; Zhang et al., 1996). Instead of cavitation, the lesion site in the mouse spinal cord was filled with macrophages and a continuous connective tissue matrix with minimal cavity development. Since progressive necrosis and cavitation prevent successful axon regeneration, consistent lack of central cavitation in the mice seems to solve this problem. However, our previous study described in Chapter 1 showed that axon growth was limited at the lesion epicenter after injury in C57Bl/6 mice despite the presence of a cellular terrain. Studies by Fitch and Silver (Fitch and Silver, 1997) demonstrate that increases in putative inhibitory molecules such as chondroitin sulfate proteoglycan (CSPG) correlate with the presence of activated macrophages and blood brain barrier compromise. The authors suggest that inflammatory response involving the interaction
between macrophages and astrocytes may be responsible for the failure of axons to regenerate successfully following traumatic SCI.

Cellular response to injury has been studied by Fujiki et al after a crush injury to the spinal cord of C57Bl/6 mice (Fujiki et al., 1996). In this study, the cellular response involving microglia/macrophages and astrocytes appeared comparable to what have been described in rats (Dusart and Schwab, 1994; Koshinaga and Whittemore, 1995; Popovich et al., 1997; Reier, 1986) and could not explain the unique wound healing response in the murine spinal cord (Zhang et al., 1996). Cellular processes after CNS injury have also been compared between several strains of mice and strain differences existed in some aspects of the response to injury. For example, a greater extent of secondary injury was found in some mouse strains that are vulnerable to kainic acid neurotoxicity (Inman et al., 1997; Zhang et al., 1997). However, these differences still could not explain the unique wound repair process in the mouse.

To understand the contribution of the inflammatory response in the post-injury pathological sequelae, it is essential that the time course of cellular activation, infiltration and scar formation be established. In the present study, we propose to characterize the time course of cellular response focusing on microglia activation and macrophage infiltration and astrocyte activation, and formation of selected extracellular matrix molecules following SCI using our mouse contusion model. The histopathological sequelae after injury is compared in two commonly used inbred mouse strains: C57Bl/6 and 129X1/SvJ. 129X1/SvJ mice, which are commonly used for embryonic stem cell research, have been recently reported to have a defect in inflammatory cell recruitment in a peripheral inflammation model (White et al., 2002). It is our hypothesis that the
inflammatory cellular reactions after contusion injury are different in these two inbred mouse strains. Parallel analyses of cellular responses with axon growth and extracellular matrix formation using immunohistochemical methods will provide baseline evidence that whether the unique wound healing response in the injured murine spinal cord could be explained by differences in the response of microglia/macrophage or astrocytes.

Methods

Spinal Cord Injury

Adult female C57Bl/6 mice (Taconic Farms, Germantown, NY) and 129X1/SvJ mice (The Jackson Laboratory, Bar Harbor, ME), 8-10 weeks old at the time of the injury, have been used for this study. Experimental spinal cord injuries were described in detail in the general methodology. Briefly, by using the ESCID, the mice were injured by a single, rapid displacement of the exposed dorsal surface of the spinal cord for a distance of 0.5 mm (10 msec peak displacement duration).

Histopathology

For histopathology and immunohistochemistry, the mice were sacrificed at 3 (C57Bl/6, n=3: 129X1/SvJ, n=4), 7 (n=6/strain), 14 (n=6/strain), 28 (C57Bl/6, n=5: 129X1/SvJ, n=6), and 63 (129X1/SvJ, n=10) days post-injury (dpi). 63 dpi tissues harvested from 129X1/SvJ mice were compared to tissues from C57Bl/6 mice that were used in the studies in Chapter 1 (0.5mm injury group). Tissue preparation was described in detail in the general methodology.
**Immunohistochemistry**

Adjacent slides of LFB staining from two strain of mice sacrificed at various time points were immunostained for the macrophage/microglia activation, astrocyte activation, axon profiles, and extracellular matrix formation by a panel of antibodies. The staining procedures for different antibodies have been described in the general methodology. Quantitative measurements of immunoreactivity of Mac-1, F4/80, GFAP, neurofilament, laminin, and fibronectin were made using the proportional area method that was described in the general methodology as a function of post-injury survival time.

**Statistical Analysis**

Two-way ANOVA was used to compare the histological outcome measures, immunohistochemistry across days post injury and strains. Within each strain, the immunoreactivity of each marker at different time points was compared using one way ANOVA followed by Bonferroni's post-hoc test.

**Results**

**Histopathology**

LFB stained transverse tissue sections were examined under light microscopy for quantitative measurements. At all time points examined from 3 days on, the lesion at the epicenter appeared to occupy a central position with nearly complete destruction of gray matter. At 3 dpi, tissue edema and swelling was obvious. The lesion site was filled with a cellular and extracellular matrix and no large cavities were observed, consistent with all other reports (Jakeman et al., 2000; Kuhn and Wrathall, 1998; Zhang et al., 1996).
spared white matter appeared as a peripheral rim of blue staining or was limited to a portion of ventral funiculus and sometimes a small portion of dorsal column. All the above changes were qualitatively similar in C57Bl/6 and 129X1/SvJ mice (Figure 17).

The total cross-sectional area of the cord at the epicenter was quantitatively analyzed as a function of post-operative interval (Figure 18A). Two way ANOVA analyses revealed differences in cross-sectional area of the cord over time ($p < 0.001$) and between two strains ($p < 0.01$). The cross-sectional tissue area diminished over time resulting in significantly smaller cord area by 28 and 63 days compared to 3 and 7 days post-injured tissue. When compared to 129X1/SvJ mice, the rate of tissue shrinkage was greater in C57Bl/6 mice. By 14 days, the tissue area was significantly smaller in C57Bl/6 mice than in 129X1/SvJ mice. By 28 days, the extent of shrinkage remained greater in C57Bl/6 mice. Although the cross-sectional area of the cord at the epicenter was comparable after 9 weeks post injury in two strains, the whole process of tissue shrinkage was significantly slower in 129X1/SvJ mice.

Measurement of the actual area of residual white matter at the epicenter revealed a progressive decrease in tissue sparing over time (Figure 18B). By 28 days, the spared white matter area was the least in both strains. There was no further decrease thereafter. Comparisons of total area of spared white matter in the two strains revealed significant strain difference ($p < 0.01$). A trend of more spared white matter in the 129X1/SvJ mice existed at all time points, but was only significantly different at 3 dpi ($p < 0.05$. Bonferroni post-tests). The residual white matter was also measured as a percentage of total cross-sectional area. Because of proportional decreases in cross-sectional cord area and total area of spared white matter over time, and same trend of greater cord area and
spared white matter in 129X1/Svj mice, two way ANOVA revealed no significant
differences in tissue sparing over time and between two strains when expressed as a ratio
(Figure 18C).

**Macrophage and Microglia Reactions Assessed by Mac-1 Immunoreactivity**

Mac-1 is a monoclonal rat anti-mouse antibody raised against complement type-3
receptor (CR3). The CR3 receptor was constitutively expressed on resting ramified
microglia throughout the spinal cord and its expression was increased after injury and
revealed by increased Mac-1 immunoreactivity. The time course of the changes in Mac-1
immunostaining was qualitatively and quantitatively described and compared in two
strains in the injury epicenter.

*Mac-1 immunostaining in C57Bl/6 mice.* At 1 dpi. small, irregularly shaped, Mac-
1+ cells were few in number and most prominent within the spinal gray matter regions
(Figure 19A-B). At 3 dpi. the number and size of Mac-1+ cells was greater. both within
the gray matter region and surrounding white matter (Figure 19C). Many of the stained
cells had an enlarged rounded phagocytic morphology. especially in the ventral gray
matter region and at the interface between the region of damaged and residual white
matter (Figure 19D). By 7 dpi. Mac-1 immunoreactivity was intense throughout the
lesion epicenter (Figure 19E). Large round macrophage profiles occupied most of the
cross-sectional area of the spinal cord (Figure 19F). Within the center region. staining
was heavily associated with the cytoplasm. Many large round cells had a lucent
cytoplasm containing lipid accumulations and indicating active phagocytosis of
degenerating myelin. By 14 dpi. the Mac-1 stained region area occupied the entire
central area of all specimens. Immunostaining was most prominent at the interface between the residual rim of white matter and the original gray matter region of the spinal cord (Figure 19G). In the center of the lesion, the intensity of Mac-1 immunoreactivity was less than that seen at 7 dpi in part because of less cytoplasm staining. Mac-1 staining was associated with large round cells with clear cytoplasm and distributed throughout the surrounding extracellular matrix (Figure 19H). The cells within the peripheral tissue rim exhibited irregular morphology characteristic of chronically reactive microglia (Figure 19I). By 28 dpi, the distribution of large round macrophages and reactive microglia was comparable to that of 14 dpi tissue, but the overall intensity of Mac-1 staining was much less than at 14 days. By 63 dpi, round Mac-1+ macrophages remained at the center of the lesion at the epicenter, while those in the surrounding spared white matter were typically reactive microglial cells.

Mac-1 immunostaining in 129X1/SvJ mice. The early microglia and macrophage reactions within the first week in 129X1/SvJ mice were similar to those in C57Bl/6 mice. At 3 dpi, Mac-1+ cells exhibited round phagocytic morphology and were most obvious in the ventral horns of the gray matter (Figure 20A). The density and staining intensity of Mac-1+ cells was significantly increased by 7 dpi. The distribution and morphology of Mac-1+ cells were comparable to that in C57Bl/6 mice (Figure 20B).

By 14 dpi, overall Mac-1 immunoreactivity appeared was decreased at the epicenter in comparison to 7 dpi (Figure 20C). Similar to C57Bl/6 mice, Mac-1 staining was most prominent in the transitional area between the core region and residual white matter. However, in the core lesion area at the epicenter, the pattern of Mac-1 staining in 129X1/SvJ mice was quite different than that seen in C57Bl/6 mice. Fewer large round
Mac-1+ macrophages were seen in this area. Instead of evenly distributed throughout the central area, these phagocytic macrophages were seen in small clusters and showed a scattered distribution in the lesion center (Figure 20D). The area in between clustered macrophages was mostly unstained for Mac-1. A few Mac-1+ cells with retracted processes representing activated microglia morphology could also be seen in this area (Figure 20D arrow). By 28 and 63 days, the intensity and distribution pattern of Mac-1 staining in the core region was similar to that observed at 14 days (Figure 20F&H). The transitional area still showed Mac-1 positive chronic reactive microglia, but the intensity was much less than that at 14 days post injury (Figure 20E&G).

Quantitative analyses of Mac-1 immunoreactivity at the core lesion area revealed no strain differences at 3 and 7 days post injury (Figure 22A). However, at 14 days post injury, Mac-1 staining in the core lesion area was significantly less in 129X1/SvJ than in C57Bl/6 mice (Figure 22A). The same difference was seen at 63 days post injury. At 28 days, although difference in Mac-1 staining pattern between the two strains was obvious (Figure 19H & 20H), there were no quantitative differences revealed by proportional area measurement. The decreased proportional area in C57Bl/6 mice was probably due to the decrease in Mac-1 immunoreactivity associated with these cells. The round phagocytic cells were still present but more lightly stained overall in C57Bl/6 mice at this time. Since proportional area measurement is based on optical density, decrease in staining intensity below certain threshold will make this measurement more difficult to interpret.
Macrophage and Microglia Reactions Assessed by F4/80 Immunoreactivity

Another microglia/macrophage cell marker F4/80 is a monoclonal rat anti-mouse antibody raised against Fc receptor (Perry and Gordon, 1988). Unlike the CR3 receptor, the Fc receptor is less constitutively expressed on resting microglia in the spinal cord, but its expression is greatly increased in activated microglia and macrophages. Thus, F4/80 is a sensitive marker for microglia/macrophage activation. In general, the intensity of F4/80 staining was stronger than Mac-1 staining. At all survival times examined (Figure 21), F4/80 staining revealed the same distribution and morphology of reactive microglia and phagocytic macrophages as described by Mac-1 staining. However, there were a few differences revealed by quantitative measurements of F4/80 immunoreactivity.

In C57Bl/6 mice, although a slight decrease in intensity compared to 7 days post injury, F4/80 immunoreactivity was still prominent in the core region at 14 days (Figure 21E). Unlike Mac-1 staining, F4/80 staining remained prominent from 28 to 63 days post injury (Figure 21G&I). Therefore, round phagocytic macrophages were clearly seen present at the core lesion site chronically. In 129X1/SvJ mice, the distinct distribution pattern of macrophages at the center lesion area in the epicenter from 14 to 63 days post injury revealed by Mac-1 staining was more obviously shown by F4/80 staining (Figure 21F, H, &J). In addition, quantitative analyses revealed a progressive decrease in F4/80 staining in 129X1/SvJ mice since 7 days, resulting in significant less F4/80 immunoreactivity in 129X1/SvJ mice than in C57Bl/6 mice at 14, 28, and 63 days post injury (Figure 22B). The decreased F4/80 proportional area in 129X1/SvJ mice was
more likely due to the decrease in F4/80 stained cells rather than the decrease in the F4/80 immunoreactivity.

The chronic macrophage response seen in 129X1/SvJ mice after contusion injury revealed by both Mac-1 and F4/80 staining was very characteristic and very different than that seen in C57Bl/6 mice. These results indicate that stronger and prolonged macrophage accumulation at the lesion epicenter occurred in the C57Bl/6 mice after contusion injury.

Astrocyte Reactions Assessed by GFAP Immunoreactivity

GFAP is an intermediate filament cytoskeletal protein expressed primarily by astrocytes (Bignami et al., 1972) and has been used as the marker for identifying astrocytes in the intact and lesioned CNS (Eng and Ghirmikar. 1994) (Bignami and Dahl. 1995). Increased GFAP expression is considered to be the main indicator of astroglial activation (Ridet et al., 1997).

**GFAP immunostaining in C57Bl/6 mice.** Figure 23 illustrates the pattern of GFAP staining at the lesion epicenter at 3, 7, 14, 28 dpi in C57Bl/6 mice. GFAP positive astrocytes were observed throughout the gray and white matter in normal spinal cord. Contusion injury to the spinal cord results in rapid response from resident astrocytes, manifested by increased GFAP immunoreactivity. At 3 dpi, GFAP staining revealed hypertrophied astrocytes, commonly referred to as reactive astrocytes (Figure 23A-B). At 7 dpi, GFAP+ reactive astrocytes were most prominent in the transition area between the central lesion and peripheral rim of spared white matter (Figure 23C). The central lesion area was mostly unstained for GFAP, except for minor staining associated with
cell debris or blood vessels (Figure 23D). By 14 dpi, strong and dense GFAP positive astrocytes were observed in the dorsal column, dorsolateral, and dorsoventral white matter tracts, whereas the central area of the lesion was entirely negative for GFAP staining (Figure 23E-F). The central area of the lesion remained GFAP-negative at 28 days and thereafter (Figure 23G-H). Interestingly, GFAP-negative area corresponded to the area occupied by round phagocytic macrophages.

**GFAP immunostaining in 129X1/SvJ mice.** Figure 24 illustrates the pattern of GFAP staining at the lesion epicenter at 3, 7, 14, 28, and 63 dpi in 129X1/SvJ mice. The early astrocyte response within the first week was similar to that described in C57Bl/6 mice. For example, by 7 dpi, GFAP+ astrocytes were almost cleared from the central lesion area, and became prominent in the surrounding area (Figure 24C-D). In striking contrast to C57Bl/6 mice, by 14 dpi, the central area that was unstained for GFAP at 7 days has begun to show increased GFAP-stained cells (Figure 24E-F). Thus, these GFAP+ astrocytes seemed to migrate into the regions where there were no phagocytic macrophages and circumscribe those regions containing nests of phagocytic macrophages. There were also GFAP-negative regions in the central scar that exactly corresponded to the area of tightly packed clusters of large round macrophages. The morphology of GFAP+ astrocytes in the central scar was different from those that were observed to wall-off the central lesion. These reactive astrocytes with thin and long processes formed interdigitating bridges extending into the central lesion area. These changes made the demarcation between central lesion and classical glial scar not as clear as seen in C57Bl/6 mice. The same pattern of GFAP staining was revealed at 28 or 63 dpi (Figure 24G-J).
Quantitative analysis of GFAP immunoreactivity in the central lesion area showed a consistent low GFAP immunoreactivity from 7 days after injury in C57Bl/6 mice. However, 129X1/SvJ mice had increased GFAP immunoreactivity from 14 days post injury (Figure 25A). Comparison between the two strains revealed that GFAP immunoreactivity was significantly higher in 129X1/SvJ mice at 14, 28, or 63 dpi (Figure 25A).

**Axonal Changes Assessed by Neurofilament (NF) Immunohistochemistry**

*NF immunostaining in C57Bl/6 mice.* This staining recognizes 200 Kd neurofilament proteins in axons that normally observed in gray and white matter of the spinal cord. At 3 dpi, NF immunoreactivity was increased and exhibited dense and darkly stained profiles in the gray matter (Figure 26A-B). The disorganized dark NF staining generally indicates axonal death and degeneration. At 7 dpi, NF stained axons were sparse at the central lesion area (Figure 26C-D). Increased NF staining was only noticed in the transition area. By 14 dpi, a few scattered axons appeared in the central region (Figure 26E). These axons exhibited either as small dark dots or elongated profiles indicating a different orientation. By 28 dpi, a few axons were still seen in the central lesion area (Figure 26F).

*NF immunostaining in 129X1/SvJ mice.* At 3 dpi, NF staining was similar to what was seen in C57Bl/6 mice (Figure 27A-B). Axonal clearance revealed by a few NF stained axons in the center region was also obvious by 7 dpi (Figure 27C-D). However, by 14 dpi, there were more axons in the core lesion area (Figure 27E). Interestingly, increased NF stained profiles seemed to be more distributed in the area of increases in
GFAP+ astrocytes. While in the regions of clusters of round macrophages, axons were rarely seen.

Proportional area measurements of NF immunoreactivity in the central lesion area in C57Bl/6 mice showed that NF-stained axon profiles remained unchanged since the first week (Figure 25B). In 129X1/SvJ mice, significantly more NF immunoreactivity at chronic time points (after 7 dpi) indicated that there was increased axonal regrowth into the central lesion area during that time. Therefore, NF immunoreactivity was significantly higher in 129X1/SvJ mice that in C57Bl/6 mice after 14 days post injury (Figure 25B).

**Extracellular Matrix Formation**

**Laminin immunostaining.** At 3 dpi, the laminin immunoreactivity was low in both strains. Laminin staining was usually associated with small blood vessels (Figure 28A-B; 29A-B). At 7 dpi, laminin immunoreactivity greatly increased and reached maximum density in both strains, while the densest laminin staining was more pronounced in 129X1/SvJ mice (Figure 28C-D; 29C-D).

In C57Bl/6 mice, from 14 days, the intensity of laminin staining within the central lesion area was decreased and its immunoreactivity was mainly found associated with blood vessels (Figure 28F). The intensity of laminin staining in the transition area between the core region and peripheral residual tissue was quite pronounced (Figure 28E). In 129X1/SvJ mice at 14 days post injury, however, the intensity of staining was still strong within the core region of the lesion (Figure 29E-F). In contrast to that was seen in C57Bl/6 mice, the laminin immunoreactivity was not only associated with blood
vessels, but also seemed to be more profusely deposited in the central lesion area. The laminin expression pattern remained the same within the core region, while the intensity of staining slightly decreased over time (Figure 29G-H). Proportional area measurement revealed significantly more laminin immunoreactivity in the core region of 129X1/SvJ mice than that was seen in C57Bl/6 mice after 7 days post injury (Figure 31A).

**Fibronectin immunostaining.** The overall pattern of fibronectin staining was generally similar in C57Bl/6 and 129X1/SvJ mice. Pronounced increases in fibronectin immunoreactivity were seen as early as 3 dpi (Figure 30A-B). Fibronectin staining remained intense within the central lesion area at 7 dpi (Figure 30C-D). Fibronectin immunoreactivity appeared to be closely associated with activated microglia and phagocytic macrophages. Difference between two strains in terms of fibronectin staining occurred at 14 and 28 dpi. The intensity of fibronectin staining in the core region was significantly higher in 129X1/SvJ mice than that seen in C57Bl/6 mice (Figure 31B).

**Discussion**

The present study provides detailed histological analyses of the inflammatory cellular reaction and wound healing response following spinal contusion injury in the mouse. We have examined the temporal relationship between the appearance of different cellular and extracellular components of scar formation after injury in the two mouse strains: C57Bl/6 and 129X1/SvJ. The results revealed distinct macrophage and astrocyte responses in the two strains. Differences in cellular responses between two mouse strains after contusion injury corresponded to a change in axon density and extracellular matrix.
composition. The current results provide important data about the complex cellular inflammatory response and repair seen after SCI.

Examination of the time course of inflammatory cellular reactions after mouse spinal contusion injuries revealed significant strain differences in the chronic macrophage response. In the first week, the macrophage response was very similar in two strains. The acute cellular response observed here was also consistent with previous reports in other SCI studies in rats (Popovich et al., 1997) or mice (Fujiki et al., 1996). However, beginning at 14 days post injury, the magnitude and distribution of macrophage response in the core region of the lesion epicenter appeared very different in the two strains. In C57Bl/6 mice, from 14-63 days post injury, round phagocytic macrophages remained in the center scar area and evenly distributed. In 129X1/SvJ mice, however, the F4/80 or Mac-1 immunoreactivity was significantly diminished by 14 dpi and thereafter. The decreased immunoreactivity could result from either downregulation of these cell surface markers or a decrease in F4/80 or Mac-1 stained macrophages. Qualitative analyses of immunostaining established which alternative was plausible. For example, the decreased Mac-1 immunoreactivity seen in C57Bl/6 mice at 28 dpi seemed to result from the downregulation of CR3 receptor on macrophages, while that seen in 129X1/SvJ mice was more likely due to fewer Mac-1 stained cells in the lesion site. In addition, it is suspected that reduced macrophages were those of monocytic origin (i.e. from vascular sources) recruited from the blood and what remained in the lesion site was derived from microglia. The present study therefore illustrated the first known defect in macrophage recruitment or function in the injured spinal cord of 129X1/SvJ mice. Similar defect in cell
recruitment in this strain of mice have been described in a peripheral inflammation model (White et al., 2002).

More interestingly, the strain differences in macrophage response could be established by the second post-operative week; the time period when profound wound healing responses began. Several characteristic observations in 129X1/SvJ mice are of particular importance: 1) the regions containing nests of round macrophages were devoid of GFAP+ astrocytes, while GFAP+ astrocytes were seen in the regions devoid of phagocytic macrophages; 2) an increased axon density at the core region of the lesion was noticed along the regions containing GFAP+ astrocytes but no round macrophages: and 3) laminin expression was extensive and not only associated with blood vessels as seen in C57Bl/6 mice. Taken together, these observations indicate that the defect in macrophage response in 129X1/SvJ mice contributes to the evolution of a permissive tissue environment and supports enhanced axon growth at the site of a spinal contusion injury.

The inflammatory response involving microglia/macrophages triggered by traumatic injury to the nervous system was known to participate in both tissue destruction and wound healing (Blight, 1994; Giulian and Robertson, 1990; Popovich et al., 1999). The temporal and spatial correlation between the regions of phagocytic macrophages and the area of absent astrocytes and limited axon growth suggest that early macrophage reaction is necessary for initiating the wound healing by supporting the clearance of myelin debris. Prolonged presence of phagocytic macrophages at the lesion site, however, is not beneficial for axonal growth.
Another important aspect of cellular response to injury is the activation of astrocytes. While this reaction is pronounced after an injury to the CNS, its function consequences in wound repair and axonal growth remain controversial (Eddleston and Mucke, 1993; Hatten et al., 1991; Manthorpe et al., 1986; Reier, 1986; Reier et al., 1989; Ridet et al., 1997). On one hand, it has been suggested that the astroglial scar can form a barrier for regeneration either physically or by the expression of inhibitory molecules (Reier et al., 1983; McKeon et al., 1991). On the other hand, reactive astrocytes have been implicated in positive aspects of tissue repair. For example, the glial scar can wall off areas of tissue damage and help to restore the structural integrity and play a role in axon guidance by the secretion of laminin (Frisen et al., 1995).

The role of reactive astrocytes is determined by their diverse molecular profiles (Eddleston and Mucke, 1993). Astrocytes upon activation may release a range of cytokines, neurotrophic factors and their receptors (Schwartz et al., 1994; Strauss et al., 1994), upregulate a variety of cell surface molecules, including cell adhesion molecules and extracellular matrix molecules such as laminin (Bernstein et al., 1985; Giftochristos and David, 1988; Liesi et al., 1984; Laywell et al., 1992). Our observation of increased GFAP immunoreactivity co-localized to the regions of more axon growth and laminin expression in 129X1/Svj mice indicates that reactive astrocytes are able to modify the tissue environment through the interaction with the ECM molecules. In addition, these astrocytes that appeared to support axon growth seemed to represent a different population of astrocytes. The reactive astrocytes in the peripheral glial scar exhibited dense GFAP-stained plexus encasing the central lesion area, commonly referred to as anisomorphic gliosis (Ridet et al., 1997). The astrocyte response at the epicenter in
C57Bl/6 mice was typically of anisomorphic gliosis, and is also seen in the rats after contusion injury (Popovich et al., 1997) or in the same strain of mice after crush injury (Fujiki et al., 1996). In 129X1/SvJ mice, however, there were also astrocyte responses in the core lesion. These GFAP+ astrocytes were characterized by hypertrophy and increased GFAP expression, but did not form a dense boundary. This would fit well with the description of another type of glial scar, isomorphic gliosis, which was more commonly seen in a more stable environment, such as distal region of the lesion (McGraw et al., 2001). Heterogeneity within the astrocyte population has been reported in many studies and its functional significance remains unclear (Bignami and Dahl, 1995; Malhotra et al., 1993). Although the factors responsible for the heterogeneity of reactive astrocytes seen in 129X1/SvJ mice were unknown, our current results suggest that it may relate to the astrocyte interaction with microglia and macrophages.

Interactions between reactive astrocytes and reactive microglia and macrophages following CNS injury have been mostly explored in vitro. These studies reveal that these cells interact with each other through either direct cell contact or secretion of factors. For example, cytokines and growth factors are important mediators of microglia-astroglial interactions (Furukawa et al., 1986; Kloss et al., 1997; Liu et al., 1994b; Tanaka and Maeda, 1996; Vincent et al., 1997; Vincent et al., 1998; Smith and Hale, 1997). We have shown here in the mouse contusion injury model that intricate functional interactions may exist between microglia/macrophage and astrocytes in vivo. Changes in the "permissiveness" of the extracellular matrix, i.e. ability to support axonal growth, seemed to be one potential consequence of the interaction between macrophages and reactive astrocytes. A reasonable scenario might then be deduced from the different cellular
processes seen in two mouse strains. The sustained presence of phagocytic macrophages in the lesion site may release cytotoxic products that prevent the migration of reactive astrocytes into the lesion site. This in turn results in less permissive extracellular matrix protein such as laminin, produced by reactive astrocytes, being deposited in the central lesion area. The coincidence of laminin in the region of more astrogliosis and less macrophages suggests that the interaction between these two cell types can significantly affect the wound healing processes by changing the nature of the tissue microenvironment.

The present study compared the reactive response of macrophages and astrocytes after SCI in two mouse strains by using immunohistochemical markers. The results revealed that 129X1/SvJ mice exhibited distinct macrophage and astrocyte responses from that seen in C57Bl/6 mice. Temporal and spatial analyses of these processes suggest that characteristic cellular response in 129X1/SvJ mice contribute to the evolution of a permissive extracellular matrix formation and enhanced axon growth at the injury site. The potential signals that trigger the different macrophage responses in the two mouse strains will be studied further in the next Chapter.
FIGURE 17

Representative photomicrographs of sections stained with Luxol fast blue (LFB) at the injury epicenter at 3 (A-B), 7 (C-D), 14 (E-F), and 28 (G-H) days post injury (dpi) in C57Bl/6 (A, C, E, G) and 129X1/SvJ (B, D, F, H) mice. Scale bars, 200 μm.
FIGURE 17

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FIGURE 18

Lesion morphometry from LFB stained section in C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice at 3, 7, 14, 28, and 63 dpi. (A) Total cross-sectional area at the epicenter was significantly decreased over time (***p < 0.001). The rate of tissue shrinkage was greater in C57Bl/6 mice than in 129X1/SvJ mice (**p < 0.01). (B) The total spared white matter was also decreased over time (***p < 0.001) and less was spared in C57Bl/6 mice (*p < 0.05). (C) The amount of spared white matter reported as the % of total cross-sectional area of the cord was not significantly changed over time and not different between two mouse strains.
Figure 18

A: Total CS Area

B: Total Spared White Matter

C: Spared White Matter as % of the Cross-sectional Cord Area

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Representative photomicrographs of Mac-1 immunoreactivity at the lesion epicenter of C57Bl/6 mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), and 28 days (G-H) after the 0.5mm contusion injury. A few round phagocytic profiles are found within the gray matter region and surrounding white matter at 3 dpi (A and B). By 7dpi, large round macrophages occupied most of the cross-sectional area of the spinal cord with intense Mac-1 immunoreactivity (D). By 14 dpi, the intensity of Mac-1 staining was less than that seen at 7 dpi, and was associated with large round cells in the center of the lesion (F). Reactive microglia profiles can be found within the peripheral tissue rim (E). By 28 dpi, large round cells were still present in the center of the lesion, but were very lightly stained by Mac-1 (H). Scale bars A, C, E, and G, 200 μm; B, D, F, and H, 50 μm.
FIGURE 20

Representative photomicrographs of Mac-1 immunoreactivity at the lesion epicenter of 129X1/SvJ mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), 28 days (G-H), and 63 days (I-J) after the 0.5mm contusion injury. The pattern and distribution of Mac-1 immunoreactivity at 3 and 7 dpi were similar as seen in the C57Bl/6 mice. By 14 dpi, there were less Mac-1 stained round cells in the center of the lesion (F), and the intensity of Mac-1 staining was less than that seen at 7 dpi. Reactive microglia profiles can also be found within the peripheral tissue rim with strong Mac-1 immunoreactivity (E). By 28 dpi, there were nests of round cells lightly stained with Mac-1 in the center of the lesion (H). Mac-1 immunoreactivity associated with peripheral reactive microglia decreased compared to that at 14 dpi (G). By 63 dpi, overall Mac-1 immunoreactivity was low, and revealed the same pattern of staining as seen at 28 dpi (I-J). Scale bars A, C, E, G, and I, 200 μm; B, D, F, H, and J = 50 μm.
Representative photomicrographs of F4/80 immunoreactivity at the center of the lesion at the epicenter of C57Bl/6 (A, C, E, G, I) and 129X1/SvJ (B, D, F, H, J) mice at 3, 7, 14, 28, and 63 days after the 0.5mm contusion injury. The pattern and distribution of F4/80 immunoreactivity at 3 (A-B) and 7 (C-D) dpi were similar as revealed by Mac-1 immunostaining and between two strains of mice. By 14 dpi, F4/80 immunoreactivity remained robust and was associated with large packed round cells in the lesion center in C57Bl/6 mice (E). While in 129X1/SvJ mice, there were less F4/80 stained round cells in the center of the lesion (F). By 28 dpi in C57B1/6 mice (G), those large round cells remained in the lesion center and were still stained strongly with F4/80, while in 129X1/SvJ mice, there was much less F4/80 stained cells (H) and the intensity of F4/80 staining appeared less than that seen in C57Bl/6 mice. The differences in F4/80 staining pattern between C57Bl/6 (I) and 129X1/SvJ (J) mice were also clearly seen at 63 dpi. Scale bars, 50 μm.
Quantitative comparisons of Mac-1 (A) and F4/80 (B) immunoreactivity at the center of the lesion of the injury epicenter in C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice at different time points post injury. Two way ANOVA revealed significant strain differences in both staining. At 14 and 63 dpi, Mac-1 and F4/80 stained macrophages were significantly less in 129X1/SvJ mice (***p < 0.001 vs C57Bl/6 mice). At 28 dpi, F4/80 stained macrophages were also significantly less in 129X1/SvJ mice (***p < 0.001 vs C57Bl/6 mice).
A

**MAC1 Immunoreactivity**

![Bar graph showing MAC1 Immunoreactivity over time](image)

B

**F4/80 Immunoreactivity**

![Bar graph showing F4/80 Immunoreactivity over time](image)

**FIGURE 22**

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FIGURE 23

Representative photomicrographs of GFAP immunoreactivity at the lesion epicenter of C57Bl/6 mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), and 28 days (G-H) after the 0.5mm contusion injury. At 3dpi, GFAP stained astrocytes showed hypertrophied morphology in both gray and white matter. By 7 dpi, GFAP+ reactive astrocytes were most prominent in the peripheral rim. In the lesion center, GFAP staining was greatly reduced and only seen associated with some blood vessels (D). From 14 dpi and thereafter, the center of the lesion remained unstained by GFAP (E-H). Scale bars A, C, E, and G, 200 μm; B, D, F, and H, 50 μm.
FIGURE 24

Representative photomicrographs of GFAP immunoreactivity at the lesion epicenter of 129X1/SvJ mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), 28 (G-H), and 63 (I-J) days after the 0.5mm contusion injury. At 3 and 7 dpi, GFAP staining was similar as seen in C57Bl/6 mice. By 14 dpi, unlike in C57Bl/6 mice, there were increased GFAP stained astrocytes seen in the center of the lesion. These GFAP stained astrocytes remained in the lesion center by 28 and 63 dpi. Scale bars A, C, E, G, and I. 200 μm; B, D, F, H, and J. 50 μm.
FIGURE 25

Quantitative comparisons of GFAP (A) and neurofilament (B) immunoreactivity at the center of the lesion of the injury epicenter in C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice. *p < 0.05. **p < 0.01. ***p < 0.001 vs C57Bl/6 mice at same time interval.

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FIGURE 25

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**FIGURE 26**

Representative photomicrographs of neurofilament immunoreactivity at the lesion epicenter of C57Bl/6 mice at 3 day (A-B), 7 days (C-D), 14 days (E), and 28 days (F) after the 0.5mm contusion injury. Note a few axons in the center of the lesion since 7 dpi. Scale bars A and C. 200 μm; B, D, E, and F, 50 μm.
FIGURE 27

Representative photomicrographs of neurofilament immunoreactivity at the lesion epicenter of 129X/SvJ mice at 3 day (A-B), 7 days (C-D), 14 days (E), and 28 days (F) after the 0.5mm contusion injury. Note few axons were left in the center of the lesion at 7 dpi (D), but obvious more axon profiles by 14 (E) and 28 (F) days after injury. Scale bars A and C. 200 μm; B, D, E, and F. 50 μm.
FIGURE 27
FIGURE 28

Representative photomicrographs of laminin immunoreactivity at the lesion epicenter of C57Bl/6 mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), and 28 days (G-H) after the 0.5mm contusion injury. At 3dpi, laminin immunoreactivity was mainly associated with small vessels. By 7 dpi, there was intense laminin staining in the center of the lesion (C-D). By 14 dpi, laminin immunoreactivity was most prominent at the transitional area between the core region and peripheral residual tissue (E), while in the lesion center, the intensity of laminin staining decreased and laminin stained profiles were restricted to vessels (F). The same pattern was also seen at 28 dpi (G-H). Scale bars A, C, E, and G. 200 μm; B, D, F, and H, 50 μm.
FIGURE 29

Representative photomicrographs of laminin immunoreactivity at the lesion epicenter of 129X1/SvJ mice at 3 day (A-B), 7 days (C-D), 14 days (E-F), and 28 days (G-H) after the 0.5mm contusion injury. Note that the laminin immunoreactivity in the center of the lesion remained intense even by 14 (F) and 28 (H) dpi. Scale bars A, C, E and G, 200 μm; B, D, F, and H, 50 μm.
FIGURE 30

Representative photomicrographs of fibronectin immunoreactivity at the center of the lesion at the epicenter of C57Bl/6 (A, C, E, G) and 129X1/SvJ (B, D, F, H) mice at 3, 7, 14, and 28 days after the 0.5mm contusion injury. The pattern of fibronectin immunostaining was similar between two strains of mice. Scale bars. 50 µm.
FIGURE 31

Quantitative comparisons of laminin (A) and fibronectin (B) immunoreactivity at the center of the lesion of the injury epicenter in C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice. *p < 0.05. **p < 0.01. ***p < 0.001 vs C57Bl/6 mice at same time interval.
A  
Laminin Immunoreactivity

Proportional Area

3d  7d  14d  28d  63d

B  
Fibronectin Immunoreactivity

Proportional Area

3d  7d  14d  28d  63d

FIGURE 31
CHAPTER 4

CHEMOKINE EXPRESSION AFTER SPINAL CONTUSION INJURY IN TWO
MOUSE STRAINS

Introduction

In the CNS, chemokines are known to be essential for normal development and homeostasis and play a key role in diverse CNS pathological states (Asensio and Campbell, 1999; Mennicken et al., 1999; Bacon and Harrison, 2000; Ransohoff, 1997; de Groot and Woodroofe, 2001; Huang et al., 2000). For example, extensive studies on the role of chemokines in MS and EAE demonstrate that chemokines are uniquely important for mediating leukocyte entry into CNS tissue during immune-mediated inflammation (Ransohoff et al., 1996). The important role of chemokines in post-traumatic inflammation is also implicated by studies of chemokine mRNA expression in different CNS injury models (Glabinski et al., 1996; Grzybicki et al., 1998; Kim et al., 1995; Muessel et al., 2000; McTigue et al., 1998; Gourmala et al., 1997). Our previous expression study in the rat spinal cord after contusion injury has shown that selective and temporal patterns of chemokine expression were integral to the appropriate inflammatory response as a mechanism of leukocyte recruitment (McTigue et al., 1998).

Genetic approaches that involve the use of mouse provide new ways for defining the molecular cascades that regulate the histopathological sequelae after injury (Steward et
al., 1999). One useful approach is to take advantage of naturally occurring genetic variability, i.e. to use genetic differences between inbred strains of mice (Steward et al., 1999). In Chapter 3, we observed strain differences in inflammatory cellular responses following contusion injury. Specifically, a defect in macrophage recruitment was noticed in the inbred mouse strain, 129X1/SvJ. Since chemokine expression is an important mediator for the recruitment of inflammatory cells to the lesion area, we hypothesized that the differences in macrophage response can be attributed to different chemokine (especially β chemokines) expression in these two strains. To test this hypothesis, in this chapter, we compared chemokine mRNA expression profiles in the two strains of mice that were found to differ in macrophage recruitment. The chemokines that have been examined include α chemokines MIP-2 and IP-10, and β chemokines MCP-1, MCP-3, MIP-1α, MIP-1β, RANTES, and C10.

**Methods**

**Spinal Cord Injury and Tissue Preparation**

Adult female C57Bl/6 mice (Taconic Farms, Germantown, NY) and 129X1/SvJ mice (The Jackson Laboratory, Bar Harbor, ME), 8-10 weeks old at the time of the injury, have been used for this study. Experimental spinal cord injuries were described in detail in the general methodology and methods in Chapter 3.

**RNA Extraction and RPA**

The mice were sacrificed by decapitation under ketamine/xylazine anesthesia at 6hr, 12hr, 24hr, 72 hr, 7, and 14 days post-injury (n=3/strain/survival time). Control mice
including naïve mice (n=3/strain) and laminectomy only (n=4/strain) were sacrificed at 12 hr post-operative. The procedures of RNA extraction and RPA for quantification of chemokine mRNA expression in spinal cord tissues were described in the general methodology.

**Statistical Analysis**

Two-way ANOVA was used to compare the chemokine mRNA expression across days and strains. Strain differences were further compared at each time point using Bonferroni post-hoc test. Within each strain, each chemokine expression was evaluated with one-way ANOVA. Post-hoc analyses were performed using a Dunnett test to compare values at each time point with control.

**Results**

The expression of selected α and β chemokine mRNA was determined by RPA analysis of spinal cord tissue obtained at various time points (Figure 32). For all the chemokines that were examined in both strains of mice, low levels of chemokine expression were measured in laminectomized non-injured spinal cords. Detected levels were not significantly different from naïve animals. Expression of most chemokines decreased to levels that were comparable to that in matched control animals after day 7, and there were no differences between the two strains.
**Chemokine expression**

*MIP-2 (Figure 33A).* In C57Bl/6 mice, MIP-2 expression began to elevate by 6 hr post-injury, and reached a peak level by 12 hr post-injury \( p < 0.01 \) with a 97-fold increase over naïve control values. In 129X1/SvJ mice, MIP-2 mRNA levels was significantly elevated by 6 hr post-injury \( p < 0.05 \), and peaked by 12 hr post-injury. The peak level at 12 hr post-injury was about 43-fold higher than naïve controls \( p < 0.01 \). The magnitude of MIP-2 increase at this time was significantly higher in C57Bl/6 mice than in 129X1/SvJ mice (Bonferroni post test, \( p < 0.01 \)). MIP-2 expression began to decrease toward control levels by 24 hr in both strains, but slightly slower in C57Bl/6 mice. The pattern of MIP-2 expression was significantly different between the two mouse strains (two way ANOVA, \( p < 0.05 \)).

*IP-10 (Figure 33B).* Expression of IP-10 was significantly elevated and peaked at 6 hr with a 11-fold increase in both C57Bl/6 mice and 129X1/SvJ mice over their matched naive controls \( p < 0.01 \). Significantly elevated IP-10 expression plateaued between 12 hr and 24 hr post-injury in C57Bl/6 mice \( p < 0.01 \), and decreased to control levels by 72 hr post-injury. In 129X1/SvJ mice, IP-10 mRNA level remained significantly elevated at 12 hr post-injury \( p < 0.01 \), and began to decrease by 24 hr and returned to control level thereafter. IP-10 levels at 24 hr post-injury in C57Bl/6 mice were significantly higher than that in 129X1/SvJ mice (Bonferroni post test, \( p < 0.01 \)). Two way ANOVA revealed no strain differences.
**Chemokine expression**

**MCP-1 (Figure 34A).** Expression of MCP-1 was significantly elevated and reached a peak level by 6 hr post-injury and remained significantly elevated by 12 hr post-injury ($p < 0.01$). At 6 hr post-injury, the MCP-1 levels were 43-fold higher in C57Bl/6 mice, and 39-fold higher in 129X1/SvJ mice than naive controls. By 24 hr post-injury, MCP-1 expression began to decrease to control levels in both strains. Two way ANOVA revealed no strain difference in the pattern of MCP-1 expression.

**MCP-3 (Figure 34B).** Expression of MCP-3 was significantly elevated by 6 hr and peaked between 6 and 12 hr post-injury in both strains, albeit the magnitude of increase was higher in C57Bl/6 than in 129X1/SvJ mice. At 6-12 hr post-injury, MCP-3 mRNA levels were 34-fold greater than naive control values in C57Bl/6 mice, and were 22-fold greater in 129X1/SvJ mice. By 24 hr post-injury, MCP-3 expression in both strains returned to laminectomy control levels. Two way ANOVA revealed significant differences in the pattern of MCP-3 expression between two strains ($p < 0.05$).

**MIP-1 α (Figure 35A).** MIP-1α expression was significantly elevated by 6 hr post-injury and remained a peak level between 6-24 hr post-injury in both strains. There are low constitutive expressions of MIP-1α in normal mice and therefore the peak mRNA levels only reached 3- to 4-fold higher than control values. The increases in MIP-1α expression were consistently greater in C57Bl/6 than in 129X1/SvJ mice at 12-24 hr post-injury (Bonferroni post test, $p < 0.05$) and resulted in significantly strain differences ($p < 0.01$). Thereafter, MIP-1α expression approached to laminectomy control levels.

**MIP-1 β (Figure 35B).** In C57Bl/6 mice, MIP-1β expression was significantly elevated and peaked by 6-12 hr post-injury ($p < 0.01$) with a 19-fold increase over control
values. In 129X1/SvJ mice, MIP-1β expression was also significantly elevated by 6-12 hr post-injury \((p < 0.01)\), but only with a 12-fold increase. The peak level of MIP-1β in C57Bl/6 mice between 6-12 hr was significantly higher than that of seen in 129X1/SvJ mice \((p < 0.05)\). The MIP-1β mRNA levels began to decrease by 24 hr post-injury in both strains, but slightly slower in 129/SvJ mice so that the levels in this strain did not return to the control level until 72 hr post-injury. The pattern of MIP-1β expression was significantly different between the two mouse strains \((p < 0.001)\).

**RANTES (Figure 36A).** Expression of RANTES showed a similar time course and magnitude of peak elevation between 6-12 hr post-injury as MIP-1α expression, with a 4-fold increase compared to controls in both strains. By 24 hr post-injury, the mRNA levels began to decrease in both strains, but slower in C57Bl/6 mice. Thus, the levels were still significantly higher in C57Bl/6 mice at 24 hr post-injury and returned to laminectomy control level by 72 hr post-injury. While in 129X1/SvJ mice, the levels decayed to control values by 24 hr post-injury. Overall, there were no strain differences in RANTES expression.

**C10 (Figure 36B).** C10 expression was not elevated until 12 hr post-injury and reached a peak at that time in both strains \((p < 0.01)\). In C57Bl/6 mice, the peak level showed a 15-fold increase over controls and the level remained significantly elevated by 24 hr post-injury \((p < 0.05)\). In 129X1/SvJ mice, the peak level was 12-fold higher than naïve controls. By 24 hr post-injury, C10 levels decreased to the level that was not different from laminectomy controls. However, C10 level was significantly higher than naïve control values by 72 hr post-injury \((p < 0.05)\). Thereafter, C10 expression
approached laminectomy control values. There were no strain differences in expression of C10.

**Discussion**

The present study reports expression of a wide variety of both α and β chemokines in the contused spinal cords of two strains of mice. Using sensitive RPA analyses, we observed significant increases in chemokine expression after injury in both strains. In C57Bl/6 mice, MCP-1 and MCP-3 showed the most dramatic increases as early as 6 hr post-injury; MIP-2 elevations were observed at 12 hr post-injury. Marked increases were also observed for MIP-1β. and IP-10 that peaked between 6 and 12 hr post-injury, and for C10 that displayed delayed increases at 12 hr post-injury. MIP-1α and RANTES displayed modest but prolonged increases in expression between 6-24 hr post-injury. In 129X1/SvJ mice, in general, the peak times of all chemokine elevation was similar to those seen in C57Bl/6 mice. Nevertheless, striking differences exist between the two strains in terms of the magnitude or kinetics of selected chemokine mRNA expression. For example, although elevated to the peak level at the same time as in C57Bl/6 mice, the magnitudes of MCP-3. MIP-1α. MIP-1β. and MIP-2 increases were significantly lower in 129X1/SvJ mice. Furthermore, these elevated chemokine levels declined more rapidly in 129X1/SvJ mice so that mostly by 24 hr post-injury, the levels were not different from control values. These results strongly suggest that the pattern of chemokine expression is distinct between the two mouse strains and this difference may account for the different macrophage responses after contusion injury observed in the same two strains (described in Chapter 3).
The dramatic increase in MCP-1 mRNA expression in the contused spinal cords of both strains of mice is consistent with data from other CNS injury models (Berman et al., 1996; Glabinski et al., 1996; Gourmala et al., 1997; Hausmann et al., 1998; McTigue et al., 1998; Muessel et al., 2000). Expression of MCP-1 that peaked between 6 and 12 hr post-injury preceded microglia activation and monocyte infiltration. Comparable pattern and magnitude of MCP-1 expression shown in the two strains corresponded to the similar early macrophage responses revealed by immunohistochemical studies within the first week after injury in the two strains. Our current findings add further evidence to suggest that MCP-1 may be a primary and potent mediator for the early inflammatory process following SCI.

The temporal profile of MCP-1 mRNA expression following spinal contusion injury exhibited an earlier upregulation of MCP-1 (6-12 hr post-injury) in the mice than that in the rat in which MCP-1 peak expression was seen between 12-24 hr post-injury (McTigue et al., 1998). However, the time course of macrophage accumulation during the first week was similar between mice and rats (Popovich et al., 1997). This phenomenon was also observed in other CNS injury models. For example, in response to traumatic brain injury, increased MCP-1 expression was observed to peak at 24 hr post-injury in the rat (Gourmala et al., 1997) but at 6 to 12 hr post-injury in the mice (Hausmann et al., 1998; Glabinski et al., 1996). There is no clear explanation for this difference. It is possible that translation efficiency of MCP-1 mRNA differs between mice and rats. For instance, the effect of protein produced through translation of mRNA in mice may continue longer than in the rat.
Although MCP-1 expression revealed by RPA analysis in the present study did not address its cellular source, the early peak elevation between 6 and 12 hr post-injury indicated that it was probably expressed by cells endogenous to the spinal cord. For example, MCP-1 expression has been localized to astrocytes following a stab wound to the mouse brain (Glabinski et al., 1996) or ischemic injury to the rat brain (Gourmala et al., 1997). Other studies have found that MCP-1 expression in response to traumatic CNS injury was associated with endothelial cells or microglia cells (Berman et al., 1996; Kim et al., 1995).

Despite the clear indication of MCP-1 involvement in the early inflammatory cascade after SCI, the similarity of the expression pattern in the two mouse strains excludes MCP-1 from being primarily responsible for the strain differences in macrophage response seen subacutely and chronically in the injury site. Our results suggest that differences in the pattern of other β-chemokine expression, such as MCP-3, MIP-1β, and MIP-1α, could contribute to different macrophage responses in the two mouse strains.

MCP-3, another member of β-chemokine subfamily MCPs, also show chemotactic preference for monocytes, lymphocytes, eosinophils and basophils as MCP-1 (Rollins, 1997). MCP-3 interacts with CCR1, CCR2, and CCR3. In terms of its chemoattractant activities for monocytes, MCP-3 has been shown to be less potent and efficacious than MCP-1 (Proost et al., 1996). However, the role of MCP-3 in the CNS has not been characterized as extensively as MCP-1. Wang et al. has shown that the upregulation of MCP-3 mRNA occurs following ischemic injury to the rat brain which suggests a role for MCP-3 in inflammatory cell recruitment into ischemic tissue (Wang et al., 1998). The
early and marked increase in MCP-3 mRNA expression shown in the present study provides the evidence, for the first time, for the possible involvement of MCP-3 signaling in spinal contusion injury. Similar to MCP-1, MCP-3 were also significantly upregulated, peaking between 6 to 12 hr post-injury. In addition, MCP-3 also displayed prolonged elevation until 24 hr post-injury in C57Bl/6 mice. Prolonged upregulation of MCP-3 corresponded with sustained phagocytic macrophage accumulation in the lesion site of C57Bl/6 mice. In 129X1/SvJ mice, MCP-3 expression not only showed a smaller peak level between 6 and 12 hr post-injury, but also downregulated more rapidly. In parallel, after the peak macrophage response at 7 days post-injury, significant less macrophages stayed in the lesion epicenter chronically. These results suggest a possible role of MCP-3 in extending and supplementing the effects of MCP-1 in monocyte recruitment into the damaged tissue.

Same implications may apply to MIP-1α and MIP-1β, the other two β chemokines since their mRNA expression also revealed significant strain differences. Similar to MCP-3, the upregulation of these chemokines were also less in magnitude and shorter in 129X1/SvJ mice, suggesting that these chemokines may have a role in regulating inflammatory response in the injured spinal cords. In both strains of mice, the peak level of expression was higher for MIP-1β than for MIP-1α, and the levels of both chemokines were lower than MCP-1. This was in agreement with the report using a spinal cord hemisection model (Bartholdi and Schwab, 1997). Previous study in our lab of chemokine expression in the rat contused spinal cord also revealed modest upregulation of MIP-1α (McTigue et al., 1998). These results indicate MCP-1α and MCP-1β were less efficient than MCP-1 in recruiting monocytes. However, their upregulation
following injury to the spinal cord does suggest their contribution to the inflammatory cascade, maybe by the involvement in other aspects, such as inducing activation of microglial cells (Bartholdi and Schwab, 1997) or modulating macrophage function (Fahey et al., 1992).

Other β chemokines displayed either very modest (RANTES, about 4-fold increase over controls) or delayed increases (C10, onset of increase at 12 hr post-injury) in expression. RANTES has been shown to be able to attract T lymphocytes, eosinophils, basophils, NK cells, and dendritic cells (Rollins, 1997). In vitro, RANTES is also a potent chemoattractant for monocytes (Schall et al., 1990). In those in vivo CNS injury models where RANTES expression has been examined, the level was only elevated modestly (Hausmann et al., 1998), as was seen in the present study. The role of RANTES expressed in the damaged CNS tissue remains speculative. C10 is a member of MIP-1 family of unknown function (Orlofsky et al., 1991). Recently, C10 has been identified as a novel chemokine expressed in EAE and a potentially significant factor for the migration of macrophages to sites of tissue inflammation (Asensio et al., 1999). Our study showed that following contusion in the mouse, C10 expression was elevated about 12-15 fold over controls in a delayed fashion. This may suggest an important role of C10 in participating the inflammatory process after SCI.

The α chemokine IP-10 expression were also examined following contusion injury. IP-10, a non-ELR α chemokine, is a chemoattractant for monocytes and activated lymphocytes (Katz et al., 1993). Elevated level of IP-10 mRNA has been demonstrated in ischemic rat brain and is suggested to be responsible for prolonged leukocyte migration elicited by ischemia (Hausmann et al., 1998). Expression of IP-10 was also
observed following cortical injury (Hausmann et al., 1998) or EAE (Ransohoff et al., 1993). In the present study, IP-10 also significantly increased rapidly, peaking between 6 to 12 hr post-injury. Although no strain differences revealed in terms of upregulation magnitude and peaking time, there was a difference seen at 24 hr post-injury. At this time, IP-10 mRNA expression in C57Bl/6 mice remained at a high level, while that in 129X1/SvJ mice was already back to control level. The results suggests that prolonged IP-10 expression may also contribute to sustaining the macrophage accumulation seen in C57Bl/6 mice, a similar role as described in MCP-3, MIP-1α, and MIP-1β above.

Finally, MIP-2 was the other α chemokine that was examined in this study. MIP-2 is a murine analogue of IL-8, a prototypic ELR positive α chemokine which has been shown to be a potent chemoattractant and activating factor for neutrophils (Rollins, 1997). In the CNS, a marked recruitment of neutrophils to the mouse brain was evoked by the direct injection of MIP-2 into the brain parenchyma (Anthony et al., 1998). Following brain trauma, MIP-2 was upregulated rapidly and the peak levels seen between 4 to 6 hr post-injury were followed by neutrophil infiltration which was maximal at about 24 hr post-injury (Hausmann et al., 1998; Zhang et al., 2001). These studies have demonstrated the important role of MIP-2 in neutrophil recruitment in response to CNS insults. However, our results of MIP-2 expression pattern following SCI argue against this notion. First, the peak elevation of MIP-2 was observed at 12 hr post-injury, a time when large numbers of neutrophils already infiltrated into the injured tissue (Dusart and Schwab, 1994). This suggests that MCP-2 may not be the major signal for neutrophil recruitment. An in situ hybridization study by Bartholdi and Schwab (Bartholdi and Schwab, 1997) of chemokine expression upon spinal hemisection in mouse also failed to
detect MIP-2 positive cells until 24 hr. Second, contusion injury to the mouse spinal cord evoked a dramatic MIP-2 expression with a 97-fold increase over controls in C57Bl/6 mice. MIP-2 expression in 129X1/SvJ mice was also significantly elevated but only as half as much in C57Bl/6 mice. This pattern strongly indicates that MIP-2 exerts additional actions apart from being a chemoattractant. It might, for example, be involved in the regulation of macrophage responses, analogous to those β chemokines, such as MCP-3, MIP-1α, or MIP-1β.
FIGURE 32. Sample images from RPA using probe sets to identify chemokine mRNAs in the spinal cords of C57Bl/6 (B) and 129X1/SvJ (W) mice.
FIGURE 33

Time course of MIP-2 (A) and IP-10 (B) mRNA expression in the injured spinal cords of C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice after the 0.5mm contusion injury. Bars represent mean ± SEM of the densitometric ratio of protected species, calculated relative to L32 density. Two way ANOVA was used to determine the effect of dpi and genotype on group means (legend, upper right of each panel). Asterisks above each bar represent the results of Dunnett post-hoc test to compare values at each time point with matched control (* p < 0.05; ** p < 0.01). Strain differences at each time point were revealed by Bonferroni’s post-hoc test (^^ p < 0.01)
MIP-2

![Graph A](image)

**FIGURE 33**

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FIGURE 34

Time course of MCP-1 (A) and MCP-3 (B) mRNA expression in the injured spinal cords of C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice after the 0.5mm contusion injury. Bars represent mean ± SEM of the densitometric ratio of protected species, calculated relative to L32 density. Two way ANOVA was used to determine the effect of dpi and genotype on group means (legend, upper right of each panel). Asterisks above each bar represent the results of Dunnett post-hoc test to compare values at each time point with matched control (* p < 0.05; ** p < 0.01).
FIGURE 34

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FIGURE 35

Time course of MIP-1α (A) and MIP-1β (B) mRNA expression in the injured spinal cords of C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice after the 0.5mm contusion injury. Bars represent mean ± SEM of the densitometric ratio of protected species, calculated relative to L32 density. Two way ANOVA was used to determine the effect of dpi and genotype on group means (legend, upper right of each panel). Asterisks above each bar represent the results of Dunnett post-hoc test to compare values at each time point with matched control (\(^*\) p < 0.05; \(**\) p < 0.01). Strain differences at each time point were revealed by Bonferroni’s post-hoc test (\(^\wedge\) p < 0.05).
A MIP-1α

Densitometric Ratio

naive LA 6h 12h 24h 72h 7d 14d

B MIP-1β

Densitometric Ratio

naive LA 6h 12h 24h 72h 7d 14d

FIGURE 35

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FIGURE 36

Time course of RANTES (A) and C10 (B) mRNA expression in the injured spinal cords of C57Bl/6 (solid bars) and 129X1/SvJ (open bars) mice after the 0.5mm contusion injury. Bars represent mean ± SEM of the densitometric ratio of protected species, calculated relative to L32 density. Two way ANOVA was used to determine the effect of dpi and genotype on group means (legend, upper right of each panel). Asterisks above each bar represent the results of Dunnett post-hoc test to compare values at each time point with matched control (* p < 0.05; ** p < 0.01).
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GENERAL DISCUSSION

Summary

Extensive experimental studies of SCI support the long-held belief that much of the damage to cells and axons after SCI occurs as a consequence of secondary events that begin after the initial trauma (Balentine, 1978; Tator and Fehlings, 1991; Young, 1993). The inflammatory response as one of these secondary events is proposed to have a major impact on the pathophysiological sequelae of SCI (Blight, 1992; Dusart and Schwab, 1994; Zhang et al., 1997). One approach to defining how inflammation affects the outcome of traumatic insults is to identify the signals that mediate the recruitment and activation of inflammatory cells to the injury site. Evidence that chemokines selectively recruit target leukocytes focuses attention on the possibility that selective chemokine expression could account in part for the characteristic CNS inflammation that is primarily mononuclear in nature. Our hypothesis is formulated from the findings that chemokine expression, especially MCP-1, a potent monocyte attractant molecule, has been associated with a number of CNS injury models, and correlated with macrophage infiltration to the injured tissues (McTigue et al., 1998; Ransohoff, 1997). The data presented in this dissertation serves to establish the precise roles that selected chemokines might play in contributing to further injury or repair following traumatic spinal injury using a clinically relevant model of SCI.
Specifically, a three-step approach has been used to test our hypothesis, in which initially a mouse model of graded spinal contusion injury was characterized using biomechanical, behavioral and histological outcome measures. We then applied this model in transgenic mice with depletion of CCR2, a principal receptor for MCP-1 to determine the specific role of binding of MCP-1 to this receptor in the inflammatory process and functional outcome. Finally, we used this mouse model to further characterize the general patterns of chemokine expression, cellular reactions, and scar formation in two commonly used inbred mouse strains. In light of the present data, we provided insights into the pivotal roles for β chemokines and MCP-1 in particular and their target cells e.g. macrophages and microglia in wound healing response of SCI in the mouse.

In order to clearly elucidate the role of individual chemokines in SCI using transgenic approaches, the injury model and the phenotypic chemokine response to the specific injury stimuli must first be well understood (Jakeman et al., 2001). Therefore, the purpose of Chapter 1 was to characterize the functional outcomes of a contusion injury model that has been developed in the C57BI/6 mouse, using the ESCID injury device described previously (Jakeman et al., 2000). The results demonstrate that the ESCID device produces graded contusion injuries with low variability, which results in distinct behavioral and histological outcomes between injury groups. Use of multiple behavioral outcome measures provides a thorough analysis on the chronic functional recovery process in the mouse after contusion injury. The availability of producing reliable contusion injuries with appropriate outcome measures enables us to directly
assess the effects of the genetic manipulations on functional recovery and histopathological evolution.

In Chapter 2, evaluation of the role of CCR2, a primary MCP-1 receptor in inflammation toward wound healing after contusion injury revealed that mice with a deletion of CCR2 resulted in significantly reduced macrophage accumulation at the lesion epicenter at 7 days post-injury. The regions of reduced macrophage accumulation corresponded to areas of reduced myelin degradation at this time. The results suggest that chemokines acting through CCR2 contribute to the early phase of macrophage recruitment following SCI. On the other hand, since only a delayed monocyte recruitment in response to injury was seen upon deletion of CCR2, this reflects the potential complex interactions between chemokines and their target cell recruitment. In addition, since the effects of CCR2 deletion on macrophage accumulation were restricted to the injury epicenter, our work reflects the complicated local heterogeneity of cellular reactions to chemokines after SCI.

The study presented in Chapter 2 has shown for the first time the direct involvement of the β-chemokine receptor CCR2 in inflammation after spinal cord contusion by using gene deletion technology. Recent studies that also use CCR2 deficient mice have demonstrated the involvement of CCR2 in macrophage recruitment to the injured peripheral nervous system (Siebert et al., 2000) and in the induction of EAE (Fife et al., 2000). These results suggest that application of transgenic approaches could provide important information about the role of specific signaling molecules in the relevant models. However, these experiments involving transgenic mice also raise some concerns about data interpretation. For example, given that macrophage-rich inflammatory
response after SCI has a great impact on the functional outcomes and MCP-1 represents an important signaling molecule to govern macrophage infiltration. We would expect that genetic deletion of its primary receptor CCR2 would reduce the recruitment of monocytes into the injured spinal cord resulting in ultimately altered histopathology or even functional recovery. Although we did see limited changes on macrophage infiltration, the subsequent changes were not as clear as was initially predicted. There are several potential explanations. One possible explanation for the absence of effects of CCR2 deletion on histopathology is the redundancy in the chemokine network. This is highly relevant for complex processes such as those that occur after SCI, in which dozens of different molecular signals participate in the sequence of events leading to damage and repair. As a result, it may be difficult to evaluate the impact of individual chemokine in wound repair after SCI in the context of numerous compensatory and functionally redundant processes by using only knockout or transgenic mice. Second, as discussed in Chapter 2, the cellular events associated with SCI may extend for several weeks after injury. Therefore, unless long-term (> 14 days) studies were conducted, we may not see any significant effects of delayed monocyte recruitment on tissue survival. In addition, lack of appropriate and sensitive outcome measures could also account for the failure of detection of any functional changes.

In the present study, the effects of CCR2 deletion were suspected to be on recruited monocytes since reduced macrophages were found restricted in the core region of the lesion epicenter. The number and immunoreactivity of chronic reactive microglia located in the surrounding transitional zone did not seem to be affected. Thus, any corresponding changes in these cellular phenotypes in that core region should be further studied. For
example, differences in myelin degradation by macrophages were shown by Oil Red O staining in these studies. Future analyses could also include an evaluation of neuronal death, axonal sparing, and astrogliosis by using sensitive immunohistochemical markers.

Another concern raised from the study in Chapter 2 and other studies using genetically modified animals for neurotrauma research relates to the strains of animals used in the studies. The development of gene knockout mice involves the use of homologous recombination method and embryonic stem cells to produce mice with a deletion of the gene of interest and crossing the gene knockout onto various inbred backgrounds (Galli-Taliadoros et al., 1995). The phenotype of a mutant mouse is not only the result of the targeted gene, but directly reflects the influence from the background genes. There are known differences in behavioral traits and cellular processes in response to neural trauma and neurogenesis between such inbred mouse strains (Crawley, 1996; Schauwecker and Steward, 1997; Steward et al., 1999; Kempermann et al., 1997). Therefore, the genetic background of the inbred mouse strains must be carefully considered in the interpretation of results from knockout mice (White et al., 2002). Substrains of 129 mice are commonly used for embryonic stem cells and C57Bl/6 is a strain commonly used for breeding, and as the background strain for spontaneous mutations. For example, the CCR2 (-/-) mice used in the present study was originally produced on a hybrid of C57Bl/6 X 129S4/SvJae background (Boring et al., 1997). One approach to reduce the influence from background genes is by sufficient backcrossing to standard inbred mice as we did in maintaining the CCR2 (-/-) mice (described in Chapter 2 methods). Another important step that also forms an integral part of this dissertation to test the hypothesis is to evaluate the phenotypic inflammatory...
response and chemokine expression in the two parental strains, and in their F1 hybrids if possible.

In Chapter 3, we characterized the time course of cellular and extracellular processes following spinal contusion injury in two mouse strains: C57Bl/6 and 129X1/SvJ. One reason we chose 129X1/SvJ strain is that the 129S4/SvJae strain that was used to produce CCR2 knockout mice is no longer commercially available (Simpson et al., 1997), while the 129X1/SvJ mouse strain appears to be another common substrain of 129s that are used for embryonic stem cells (Schwartzberg et al., 1985). A recent study on the role of vitamin D-binding protein (DBP) on innate immune function in a peripheral inflammation model found a profound defect in macrophage recruitment in the DBP knockout mice. Further analysis revealed that this defect was not linked to the DBP-locus, but instead correlated with the 129X1/SvJ genetic background of the mice being studied (White et al., 2002). Interestingly, our qualitative and quantitative studies in comparison of macrophage response to SCI between C57Bl/6 and 129X1/SvJ mice also revealed a significant defect in macrophage response in 129X1/SvJ mice. These results suggest a genetic control of the inflammatory response. In the study by White et al (White et al., 2002), the authors considered the genetic basis for the genetic defect in the 129X1/SvJ mice by examining the F1 progeny of the 129X1/SvJ and C57Bl/6 intercross. They concluded that the inflammatory response is polygenic which means the trait is controlled by more than one gene locus.

In the study described in Chapter 2, macrophage recruitment was delayed and did not reach a peak accumulation at 7 days post-injury in the CCR2 (-/-) mice. The reduced macrophage infiltration resulted in reduced myelin degradation. In the study described in
Chapter 3. after the peak accumulation at 7 days, macrophage accumulation was markedly decreased in 129X1/SvJ mice at 14 days post injury. Smaller numbers of phagocytic macrophages in the central lesion area chronically corresponded to a more permissive tissue environment where axon growth was enhanced. Taken together, these observations indicate that the timing of the appearance of macrophages is a key issue to determine their functional roles in preventing or promoting the wound healing processes after injury. Our results support the view that the uncoordinated post-traumatic inflammatory response contributes to the unsuccessful wound healing in the CNS (Zhang et al., 1997).

Further studies on chemokine expression after injury in the same two mouse strains used in the histological study demonstrate that the inflammatory cellular responses following spinal contusion injury are precisely regulated by the expression of specific chemokines. There are two conclusions drawn from the studies in Chapter 2 and 4. First, MCP-1 and its related chemokines that act through CCR2 receptor play a primary role in early macrophage recruitment. Second, other β chemokines such as MCP-3, MIP-1α, or MIP-1β may play a supplemental role of macrophage recruitment, especially in the late phase of inflammatory response. Therefore, the regulation of inflammatory response by chemokines is complex and probably requires coordinated actions of selected chemokines.

**Future Directions**

The present data provide important evidence of the involvement of β chemokines in the histopathological sequelae of SCI through the regulation of inflammatory response.
The role of MCP-1 has been confirmed in our mouse model using both transgenic strategies (CCR2 knockout mice) and expression analyses. To further clarify its direct role in this setting, MCP-1 knockout or double knockout mice to delete both MCP-1 and its receptor CCR2 could be used. Although MCP-1 is asserted to be the primary chemokine responsible for early inflammatory response, it does not appear to be the only possible candidate to alter the histopathological outcome after spinal injury. Ghirnikar et al have evaluated the effectiveness of a chemokine receptor antagonist MCP-1 (9-76) that specifically inhibits the interaction of MCP-1 with its receptor in promoting tissue survival after SCI (Ghirnikar et al., 2001). Infusion of MCP-1 (9-76) in rat contused spinal cord only resulted in modest reduction in cellular infiltration without significant tissue survival. Instead, some other chemokines have been identified to be such potential candidates. Further studies by blocking the action of these chemokines pharmacologically or genetically will be needed to clarify their roles in the outcome of the inflammatory response after SCI.

In the present study, differential chemokine expression follows a time course that correspond to selective inflammatory cell recruitment suggesting that chemokine expression is also a strictly regulated process. For example, chemokine expression is known to be regulated by inflammatory cytokines such as TNF-α, IL-1β, interferon-γ, and TGF-β (Hurwitz et al., 1995; Majumder et al., 1996; Zhou et al., 1998). Future studies aimed at examining the expression of these cytokines will provide hints about how chemokine expression is being regulated. In situ hybridization methods could also be used to define the cellular source of these cytokines and chemokines.
The mouse strain 129X1/SvJ has been shown in our study to exhibit a characteristic inflammatory response after SCI. The significant feature of this mouse strain is that the reduced macrophage infiltration in the late phase of inflammation seems to influence the environment for axonal regeneration. It will be necessary to further examine the detailed interrelationship between macrophage response and wound repair to take advantage of this mouse model. For example, it would be interesting to see if reduced macrophage infiltration is associated with less production of cytotoxic substances. One type of neurotoxin, quinolinic acid, was known to become elevated in the injured spinal cord concomitant with a dense macrophage infiltration and microglia activation (Popovich et al., 1994). In these experiments, analysis of quinolinic acid by gas chromatography/mass spectroscopy could be conducted in 129X1/SvJ and C57Bl/6 mice following contusion injury.

We have found that one of the consequences of the interaction between macrophages and astrocytes was to change the components of extracellular matrix. For example, less macrophage infiltration but more astrogliosis resulted in more laminin deposition in the lesion site in 129X1/SvJ mice. A more detailed examination of potential extracellular matrix proteins that of inhibitory or permissive nature should be done in the 129X1/SvJ and C57Bl/6 mouse.

Conclusions

The data presented in this dissertation have utilized a mouse model of spinal cord contusion injury to examine the roles of selective β chemokines in inflammatory response and pathophysiological sequelae after SCI. Detailed characterization of this
mouse model has proved that it represents a reliable and suitable model to study the molecular and cellular mechanisms of secondary injury and repair after traumatic SCI. Genetic approaches including the use of transgenic mice with targeted gene deletion (CCR2 knock out mice) and two different inbred strains of mice in the present studies have greatly advanced our understanding of the important roles of MCP-1 and other β chemokines in this setting. These work represent an important step to elucidate the complex cellular and molecular mechanisms following SCI and will have important implications for developing strategies to improve the pathophysiological outcome after injury.
LIST OF REFERENCES


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