The role of complement in spinal cord injury and represents a therapeutic target for improving recovery following trauma

PhD Thesis

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INTRODUCTION

Spinal cord injury (SCI) is characterized by an initial traumatic injury phase, followed closely by secondary events that result in edema, ischemia, excitotoxicity, and inflammation. The mechanisms of secondary injury are not well defined, but it is clear that inflammatory processes play a significant role in functional recovery. While the initial traumatic injury is difficult to guard against, the subsequent inflammatory cascade represents a therapeutic target for SCI. The only clinical therapy accepted currently for acute SCI is methylprednisolone, a therapy that has yielded disappointing results, with the data from clinical trails being contradictory and inconclusive.

The complement system is known to perform a wide range of functions in the human body. It forms an essential component of the host immune system and is associated with the clearance of molecules of foreign origin as well as the elimination of invading pathogens from the body. It plays an important role in adaptive immunity. However, it is nonspecific in action and unable to distinguish between self and non-self. Under normal conditions, it is strictly regulated by complement regulatory molecules. However, in neuroinflammatory disorders, the complement regulatory molecules fail to control the activated complement components. These activated complement components then act as a double-edged sword and are responsible for the degeneration of neurons. The devastating roles of the complement components in neurodegenerative disorders are well documented. Spinal cord injury (SCI), traumatic brain injury (TBI), Alzheimer's disease (AD), multiple sclerosis (MS), myasthenia gravis and Parkinson's disease (PD) are examples of a few disorders in which activated complement components play an important role. Not only do these disorders arise out of dysfunction in the normal metabolic machinery, but also neuroinflammatory disorders associated with microbial infections show involvement of complement components.

We have shown that complement deficient mice and normal mice treated with a complement inhibitor are protected from neuronal injury following SCI, and that they have significantly improved functional scores over time compared to control mice.

These data indicate an important role for complement in secondary injury, and indicate complement inhibition will reduce inflammation and provide neuroprotection following spinal cord injury. However, complement-dependent mechanisms involved in secondary injury following SCI are not known, and there remain concerns regarding the clinical application of the currently available systemic (body-wide) complement inhibitors with regard to their safety and efficacy. Complement activation products are important for host defense and immune maintenance mechanisms, and systemic complement inhibition can compromise the protective and beneficial roles of complement. This will not be optimal in patients at risk of infection, and urinary tract infection is a frequent complication during initial and ongoing medical rehabilitation after SCI.

We propose to develop a neuroprotective strategy based on attenuating complement-dependent secondary damage after SCI using a novel and validated approach. The strategy involves the targeting of complement inhibitors to sites of complement activation and injury, and we have shown the approach to be highly effective in vitro and in mouse models of SCI and other inflammatory disease conditions. The targeting strategy enhanced the activity and protective effect of complement inhibitors by 10-20 fold compared to untargeted counterparts. Furthermore, untargeted, but not targeted complement inhibitors systemically inhibited complement and increased susceptibility to infection in a mouse model.

We propose to fully characterize our targeted complement inhibitors in a mouse model of SCI. In addition to therapeutic endpoints, we will use different types of complement inhibitor to investigate complement-dependent disease mechanisms. We will determine relationships between the generation of different complement activation products and other molecules associated with inflammation in order to gain a better understanding of the mechanisms of secondary tissue injury following SCI. These preclinical studies will establish the guidelines necessary for the translation of this therapy to the clinic using recombinant human proteins.

OBJECTIVES

The objectives of this thesis are as follows:

1. To investigate the dynamics of complement activation and its role in the development of SCI in mice.
2. To determine in vivo relationships between the generation of different complement activation products with cytokine production, adhesion molecule expression, leukocyte infiltration and activation, and injury.
3. To investigate the neuroprotective effect of a novel targeted complement inhibitor and develop a neuroprotective strategy based on attenuating complement-dependent secondary damage after SCI.

METHODS AND MATERIALS

1. Animals

Female wild-type C57BL/6 and C57Bl/6 C3-deficient (C3-/-) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Breeding pairs of fB-deficient (fB-/-) mice on C57BL/6 background were generated as described and provided by Dr. J. Thurman (University of Colorado Health Sciences Center, Denver, CO) and a breeding colony established. In mice, there are two genes encoding CD59; CD59a is widely expressed and is the primary regulator of the MAC in mice, whereas CD59b expression is limited to the testis and, at very low levels, bone marrow. In this study, CD59a-deficient mice on C57BL/6 background were used and were generated as described. fB and CD59 deficiency was confirmed by genotyping. Mice weighing between 18–22 g (6–8 weeks old) were used in experiments. All mice were fed on standard laboratory food and given tap water ad libitum with a light–dark cycle of 12 hours.

2. Complement Inhibitor-CR2-Crry

The fusion protein CR2-Crry was produced and purified as described previously. In brief, a cDNA construct of the recombinant fusion protein was prepared by joining the mouse CR2 sequence encoding the four N-terminal short consensus repeat (SCR) units (residues $1-257$ of mature protein, National Center for Biotechnology Information Gen-Bank, accession number M35684) to sequences encoding extracellular regions of mouse Crry. The Crry sequence used encoded residues 1–319 of the mature protein (National Center for Biotechnology Information GenBank, accession number NM013499). To join CR2 to Crry, linking sequences encoding $(GGGGS)_2$ were used. The recombinant protein was expressed in NSO cells and purified by anti-Crry affinity chromatography as described. CR2-Crry has a circulatory half-life in C57BL/6 mice of ~ 8 hours.

3. Anti-fB anti-body

The isolation and characterization of anti-fB mAb 1379 used in these studies was described previously, and the mAb effectively inhibits the mouse alternative pathway[72]. The mAb was generously provided by Drs. V. M. Holers, J. M. Thurman (University of Colorado Health Sciences Center, Denver, CO), and G. S. Gilkeson (Medical University of South Carolina).

4. Spinal cord injury surgery and antibody treatment

Wild-type (wt) mice were randomized into sham (laminectomy, no SCI damage), vehicle control (phosphatebuffered saline [PBS]), and CR2-Crry treatment and anti-fB mAb treatment groups. Other groups consisted of C3-deficient (C3-/-), fB-deficient (fB-/-) and CD59a-deficient (CD59-/-) mice. For CR2-Crry treatment were administered a single dose of 0.25 mg of CR2-Crry by tail vein injection. All other

animals received intravenous injections of phosphate buffered saline. For anti-fB mAb treatment, wt mice were randomized into four groups, with mice in each group receiving an intravenous injection of 100 µl PBS vehicle control or 2 mg anti-fB mAb in 100µl PBS at 1 and 12 hours after surgery, 12 and 24 hours after surgery, or 24 and 36 hours after surgery. The 2mg dose used was based on previous studies characterizing the therapeutic effect of this Ab in mouse models of inflammation.

5. Surgery and care

Mice subjected to laminectomy at the level of the 12th thoracic vertebra (T12), followed by contusion-induced SCI using the NYU weight drop impactor as described previously. After injury, the muscles and the subcutaneous tissue were closed in layers, the skin was closed with metal wound clips (World precision instruments), and mice were given 1 ml of saline per day for 3 days to compensate for loss of blood and dehydration. Bladders were expressed before testing, and each mouse was evaluated for 4–5 minutes on the day before surgery, immediately after surgery, and then manual bladder expression was performed twice daily until full bladder function was observed. Sham mice received a dorsal laminectomy without impact injury. There was less than 5% surgical mortality, and zero mortality of mice that recovered from surgery. Groups of mice were sacrificed at 1, 3, 7 and 21 days after injury, and spinal cords were isolated for analysis. The care of mice and surgical procedures were approved by the Medical University of South Carolina's committee for animal research, in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

6. Locomotor Function Analysis

Open-field observations of locomotor function recovery were independently scored by two observers blinded to experimental groups using the Basso, Beattie and Bresnahan (BBB) rating scale developed for rats, but later adapted by others for mice or Basso Mouse Scale (BMS)

7. Histopathological Analysis

Spinal cords were removed at 1, 3, 7 and 21 days postinjury for histological analysis. Immediately after sacrifice, mice were perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. Spinal cords were then removed, placed into 4% paraformaldehyde/PBS, and either cryoprotected in 30% sucrose for 48 hours before storing at -80°C or placed in formalin and processed to paraffin for histological analysis. For histological assessment, sections of spinal cord were stained with hematoxylin and eosin (H&E) or luxol fast blue (LFB) as previously described (Clark G: Staining Procedures. Baltimore, MD: Williams and Wilkins, 1981, pp 111–129) and Histopathological damage was assessed quantitatively by an independent reviewer blinded to the experimental groups. H&E and LFB sections were scored from 0 to 3 for the presence and intensity of inflammatory cell infiltration, neuronal vacuolation, and hemorrhage (where 0, is no evidence and 3 severe). Scores were then expressed as a cumulative score of 0–9. When assessing recovery at 21 days after injury, the extent of demyelination, graded between 0–3 (with 0 being no evidence of demyelination) was added for a cumulative score of 0–12. To further quantify spinal cord injury, morphometric analyses was conducted to determine the degree of tissue sparing after injury. Transverse sections of spinal cord were stained with H&E, and the cross-sectioned area of spinal cord was measured at 150 μm increments extending 2mm either side of the injury epicenter using Zeiss Axiovision image analysis software (Carl Zeiss, Oberkochen, Germany). Measurements were averaged for animals in each group at each time point as previously described. All assessments were performed in a blinded fashion.

8. Neutrophil and Macrophage Infiltration

The presence of infiltrating neutrophils and macrophages was assessed using immunohistochemistry on frozen spinal cord sections. Standard immunohistochemical methods were used as previously described. Neutrophils and macrophages were identified by anti-mouse Gr-1 and Mac-3 (BD Biosciences), respectively. Neutrophils and macrophages were quantified at the spinal cord injury epicenter, defined as the section exhibiting maximal tissue damage. The total number of neutrophils and macrophages were quantified using computerized image analysis methods, as previously described. Results are expressed as the number of neutrophils/macrophages per mm^2. Specificity of immunostaining was confirmed by both the use of isotype control antibody and by the omission of primary antibody.

9. Complement Deposition

Spinal cord cryosections were fixed in cold acetone for 5 minutes and then washed in running water followed by PBS. Sections were then incubated for 1 hour at room temperature with either anti-mouse C3 fluorescein isothiocyanate (FITC) (Dako, Ely, UK), mouse anti-mouse fB mAb 1379 (see above), rabbit anti rat C9 that is cross reactive with mouse C9, or rat anti-mouse CD59 mAb 7A6. For C9 and CD59 visualization, donkey anti-rabbit FITC and donkey anti-rat FITC antibodies were used. For fB visualization, we used a mouse on mouse staining kit and protocol from Vector laboratories (Burlingame, CA). Sections were then counterstained with DAPI (Thermo Scientific, Rockford, IL) for nuclear detail. Sections were then coverslipped and analyzed for fluorescence intensity using a Zeiss LSM5 Confocal microscope. Fluorescence intensity for each complement component was scored on a scale of 0–3, where 0 is no staining, 1, mild, 2, moderate, and 3, intense. All observations were made by an observer blinded to group identities.

10. Statistical Analysis

All data are presented as Mean±SEM or mean±SD as indicated. Analyses were performed using Statview Analysis Software (version 5; SAS Institute Inc., Cary, NC) or SPSS 13.0 for Windows. Statistical significance between groups was determined by two-way analysis of variance with Bonferroni/ Dunn's corrected post hoc t-tests. For Locomotor functional analysis, repeated measures of analysis of variance was used to determine differences between the groups. $P < 0.05$ was considered statistically significant.

RESULTS

1. Effect of C3 Deficiency and of Complement Inhibition on Locomotor Recovery following SCI

To investigate the role of complement in SCI, we induced contusion injury to the spinal cord in wt mice and in mice deficient in C3, a central protein of the complement system and common for all pathways of activation. Following injury, locomotor recovery was assessed using the modification of the BBB rating scale. All animals had a BBB score of 21 pre-injury and a score of 0 immediately after injury, with bilateral hindlimb paralysis (Fig. 3). Two days after injury, and every day thereafter through the termination of the study at day 21, the C3-deficient mice had a significantly improved BBB score compared to the wild-type controls ($P < 0.001$) (Fig. 3). By day 21 after injury, the C3-deficient mice showed a near normal BBB score of 19.6 ± 1.2 ($P< 0.001$), whereas the BBB score for wild-type mice was only 11.5 ± 2.14 which was significantly lower than that of C3-deficient mice ($P< 0.001$). These data indicate that C3 plays an important role in the posttraumatic events that affect functional recovery. Next, we determined whether C3 blockade, using an intravenously administered inhibitor previously shown to target to sites of complement activation, is a feasible posttraumatic therapeutic approach for improving functional recovery. Using the same spinal cord paradigm, a group of mice were

treated with a single intravenous injection of 0.25 mg CR2-Crry at 1 hour after SCI. As with the C3-deficient mice, the CR2-Crry-treated mice had a significantly improved BBB score compared to shamoperated controls at all time points from day 2 following traumatic injury ($P<0.001$) (Figure 3). The C3-deficient mice appeared to have a better outcome than the CR2-Crry-treated mice, but the difference was not significant.

2. Effect of fB and CD59 Deficiency and of alternative complement pathway inhibition on Locomotor Recovery following SCI

Contusion injury to the spinal cord was induced in mice deficient in fB or CD59, in wt untreated mice, and in wt mice subsequently treated with anti-fB mAb or PBS (vehicle). Locomotor function was assessed by the BMS scale, and all mice subjected to contusion injury exhibited a BMS score of 0 immediately after injury. Over the course of 21 days, locomotor function significantly improved in fB-/- mice and wt mice treated with anti-fB mAb at 1 and 12 hours after SCI compared to wt and PBS treated controls. There were no significant differences in BMS scores between fB-/- mice and antifB–treated (1/12 hours) mice over the course of the experiment. When anti-fB mAb was administered at 12 and 24 hours after SCI, there was a trend toward improved locomotor function when compared to controls, but the difference did not reach significance. We also administered anti-fB mAb at 24 and 36 hours post-SCI, but there was no difference in functional recovery compared to PBS treated mice (data not shown). Unless otherwise stated, all data shown below for anti-fB treated mice was obtained using a 1 and 12 hour post-SCI treatment schedule. In contrast to the improvement seen in fB-/-mice and anti-fB (1/12 hours) treated mice, recovery of locomotor function was significantly impaired in CD59-/-mice compared to wt and vehicle treated controls. There was no difference in functional recovery between PBS (vehicle) treated mice and untreated wt mice after SCI.

3.Effect of Complement Deficiency and Complement Inhibition on Tissue Damage And Demyelination after SCI

Spinal cord contusion results in a primary hemorrhage, inflammation, and loss/damage of neurons. We determined the effect of fB deficiency, fB inhibition (1/12 hours), or CD59 deficiency on spinal cord tissue injury by macroscopic examination of spinal cords and by assessment of histological changes within the spinal cords post injury. At 72 hours after injury, macroscopic examination of control spinal cords demonstrated marked indentation at the injury site with evidence of hemorrhage. These features were markedly reduced in fB-/- and anti-fB mAb treated animals, but appeared exacerbated in spinal cords from CD59-/- mice.

4. Effect of C3 Deficiency and of Complement Inhibition on the Extent of Tissue Destruction following SCI

To determine whether C3 deficiency or complement inhibition with CR2-Crry attenuated overall spinal cord tissue damage, we determined the cross-sectioned area of spinal cords at 100 μm increments extending 2 mm either side of the initial injury impact site. Measurements were made using spinal cords isolated from C3-deficient mice and mice treated with CR2-Crry or vehicle control (PBS). At 24 hours after injury, the profile of tissue damage was similar in both C3-deficient and CR2-Crrytreated groups. In the control group, there was a clear trend toward increased injury compared to the C3-deficient/inhibited groups, but by 24 hours after SCI the difference did not reach statistical significance at the injury site or on either side of the injury site. Comparable relative profiles were obtained for the three groups of animals at 72 hours after SCI. Seven days after injury, however, there was significantly more tissue sparing at and around the injury site in C3-deficient mice and in mice treated with CR2-Crry compared to vehicle control mice. There was no

difference in tissue sparing between C3-deficient and CR2-Crrytreated mice at 7 days after SCI.

5. Effect of C3 deficiency and of complement inhibition on the extent of tissue demyelination following SCI

We also analyzed the extent of necrosis and demyelination in cords isolated from the different groups of animals 7 days and 21 days after SCI. In the vehicle control group, H&E staining of cord sections (centered around the injury site) revealed marked areas of necrosis with vacuolization of cells at day 7, with necrosis being somewhat less evident at day 21. In contrast, the white matter beneath the injury site in cords isolated from C3-deficient mice appeared grossly intact at days 7 and 21. Cords from CR2-Crry-treated mice also exhibited significant attenuation of injury when compared with vehicle controls, although there appeared to be more vacuolization in the cells within the white matter compared to the C3-deficient animals.

6. Histopathology of spinal cord sections after SCI

We next analyzed spinal cords microscopically at the injury epicenter using H&E stain to assess the extent of inflammatory cell infiltrate, neuronal vacuolation and hemorrhage. Sections were prepared from cords isolated at 24 and 72 hours postinjury, and the sections were graded for a total cumulative score of 0–9 (see Materials and Methods). Injury to spinal cords was evident in all groups at both 24 and 72 hours.

No significant differences were noted at 24 hours postinjury, but at 72 hours there was significantly less damage seen in fB-/- mice and anti-fB mAb - treated mice compared to control mice. In contrast, damage was significantly exacerbated in CD59-/- mice compared to controls 72 hours after injury.

7. Quantitative assessment of histopathological inflammation and injury

Representative images of spinal cord sections postinjury are shown in Fig. 8, with quantification of data shown in. Histologically, control and CD59-/- spinal cord sections demonstrated evidence of hemorrhage, pronounced inflammation, neuronal cell vacuolation, and demyelination, and while these features existed in fB-/-mice and anti-fB mAb - treated mice, they were markedly reduced.

To further quantify the impact of complement deficiency or inhibition on SCI, we analyzed tissue destruction by determining the cross-sectional area of spinal cords at 150μm increments extending 2 mm either side of the initial injury impact site. In accord with our subjective histological assessments, there was significantly increased tissue sparing in fB-deficient and fB-inhibited mice, and significantly less tissue sparing in CD59-/- mice, when compared to control mice.

8. Expression of Neutrophils and Macrophage Infiltration after Spinal Cord Injury

After SCI, the infiltration and activation of neutrophils and macrophages is considered to play an important role in the propagation of inflammation and tissue damage. Neutrophils are typically the first leukocytes to arrive at the injury site, while macrophages/microglia appear later but, unlike neutrophils, persist in the spinal cord. Complement activation products provide chemotactic and activating signals for neutrophils and macrophages, as well as induce the expression of inflammatory cytokines/chemokines and adhesion molecules. Using complement deficient and inhibited mice, we investigated the influence of the alternative and terminal complement pathways in neutrophil and macrophage infiltration after SCI. Inflammatory cell influx was evaluated by immunohistochemical analysis using Gr-1, a marker antibody for neutrophils, and Mac-3, a marker antibody for microglia/ macrophages. Neutrophils and macrophages were quantified at the site of injury in mice from all groups at 1 and 3 or 7 days post-SCI.

In this study, the total number of neutrophils and macrophages present on each section was counted. No distinction was made between white and gray matter, due to the extensive damage noted at 24 and 72 hours postinjury in some groups and because sections were analyzed using immunohistochemistry, which did not permit morphological evaluation.

9. Targeting and Biodistribution of Therapeutically Administered CR2-Crry

We recently demonstrated that intravenously administered CR2-Crry targets sites of complement activation in a mouse model of complement-dependent intestine ischemia and reperfusion injury. The microenvironment of the spinal cord is different, and access of macromolecules and inflammatory cells is restricted by the bloodspinal cord barrier. On damage, however, the bloodspinal cord barrier becomes temporarily more permeable, and this may account for the access of intravenously administered CR2-Crry to its targeting ligand (C3) within the spinal cord. To support the concept that CR2-Crry functions by targeting specifically to the spinal cord following injury, we assessed the tissue distribution of ^{125}I-labeled CR2-Crry in normal control mice and in mice subjected to SCI. ^{125}I-labeled CR2-Crry was injected intravenously 1 hour after SCI, as per therapeutic protocol, and biodistribution determined 12 hours later. In control mice (no SCI), CR2-Crry was distributed primarily in the blood, with tissue localization restricted to the liver and kidney and, to a lesser extent, the heart. The location of CR2-Crry within the kidney and liver is likely associated with the nonspecific clearance of the protein. Of note, no ^{125}I-labeled CR2-Crry was detected in the CNS tissues of the spinal cord or brain, indicating that under normal physiological conditions CR2-Crry cannot pass the blood-brain/spinal cord barrier. In contrast, there was significant localization of ^{125}I-labeled CR2-Crry within the spinal cord of injured mice. In the spinal cord, levels of CR2-Crry was highest at the site of injury but was also detected at sites rostral and caudal to the site of injury. The absence of ^{125}I-labeled CR2-Crry in brain tissue of injured mice is also supportive of

the specific targeting of CR2-Crry. To further investigate the specific targeting of CR2-Crry to the injured spinal cord, we performed immunofluorescent staining with an anti- CR2 antibody. CR2 is expressed conservatively and found primarily on B cells and dendritic cells. Therefore, the presence of CR2 immunoreactivity within the spinal cord was deemed to be indicative of localization of recombinant CR2-Crry protein, and indeed no immunoreactivity for CR2 antibody could be detected in sham controls or SCI control (untreated) animals (not shown). CR2 immunoreactivity was, however, seen in CR2-Crrytreated animals when sections of spinal cord were assessed 12 hours after SCI. Staining was seen primarily within the white matter around the injury site and morphologically appeared to stain oligodendrocytes, fiber tracts, and vascular structures. This distribution of CR2 corresponds to the distribution of deposited C3 in injured spinal cords reported above, and is a further indication of the C3 targeting specificity of CR2- Crry. Staining was also noted, to a lesser degree, in the dorsal horns of the gray matter with immunolocalization seen in cells with neuronal morphology.

10. Deposition of Complement Activation

To correlate post-traumatic inflammation and injury with complement activation, we examined spinal cord sections for deposited C3, fB, and C9 (MAC) by immunofluorescence microscopy.

11. Time Course of Complement Activation 1, 24 and 72 hours after SCI

The presence of C3, deposition of which marks a site of complement activation by any pathway, was assessed in mice that had undergone SCI and in sham laminectomy controls. No staining for C3 was observed in sham operated- animals in any compartments of the spinal cord. In contrast, C3 deposition was evident following SCI in spinal cords harvested at 1 hour, 2 hours, 4 hours, 12 hours, and 24 hours after injury. At 1 hour, 2 hours, and 4 hours post-SCI, C3 deposition was centered to the

white matter of the injury site and within the ventral horns of the gray matter. At later time points of 12 hours and 24 hours, C3 staining was evident in surviving white matter, with staining also present throughout the gray matter and extending into the ventral and dorsal horns. By day 3 after injury, complement deposition was almost undetectable, with no C3 staining evident at the injury site. This result is different from that reported for complement deposition in the rat spinal cord following injury, in which complement deposition was evident for up to 42 days after injury. An additional apparent difference in the mouse model was that, at all time points, spinal cord sections 10 mm rostral and caudal to the injury site showed a much reduced C3 staining pattern compared to sections taken from the injury site. Complement deposition was seen up to 20 mm from the injury site in rats, with no apparent decrease in immunoreactivity and with increasing distance from the site of injury. However, this apparent difference is likely a consequence of animal size and differences in size of impact injury required to produce an equivalent condition.

12. Complement deposition at epicenter of injury 24 h post-SCI

Representative immunofluorescence images showing complement deposition, as well as CD59 expression, at 24 hours after injury are shown in Fig. 16. There was no evidence of C3, fB, or C9 staining in spinal cords from sham operated mice, and expression of CD59 was similar in wt control treated and fB-/- mice and absent in CD59-/- mice (Fig. 16).

13. Quantitative assessment of complement deposition post-SCI

In agreement with previous data obtained using mice (for C3 deposition) and rats (for fB and C9 deposition), there was pronounced deposition of all three complement proteins in the white and gray matter of control mice at 24 hours postinjury. C3 and C9 deposition was significantly lower in fB-deficient and fB inhibited mice compared to control mice. As expected, fB was undetectable in fB-deficient mice and was detected at only very low levels in fB-inhibited mice. Deficiency of CD59 resulted in

significantly increased levels of C9 deposition compared to all groups (including controls) but did not effect levels of C3 or fB deposition.

Similar relative profiles for C3, fB, and C9 deposition were seen at 72 hours post-SCI in all groups, although C3 and fB levels were lower than at 24 hours after SCI. C9 levels were similar at 24 and 72 hours after SCI. In this study, we detected C3 deposition at 72 hours post-SCI, although at low levels. This is in contrast to our previous report that C3 was undetectable in mouse spinal cords 72 hours after injury, and we attribute this difference to the use of a different detection antibody (see Materials and Methods).

14. Histological Analysis of Recovery

Data presented above show that after SCI, fB-deficient mice and fB-inhibited mice (when treated at 1 and 12 hours after SCI) have a significantly improved outcome compared with controls in terms of inflammation, tissue injury, and functional locomotor recovery. To determine the extent of histological recovery, we analyzed spinal cords 21 days after the initial impact injury. H&E and LFB staining of cord sections from wt and PBS treated controls revealed demylineation in the central core of the white matter beneath the impact site and evidence of vacuolation with some necrosis with inflammatory cells still present (Fig. 18 A and B.). By comparison, at 21 days after SCI, there was markedly less demyelination and inflammation and no evidence of necrosis in cords from fB deficient mice and mice treated with anti-fB mAb at 1 and 12 hours after SCI. There was no apparent difference in the extent of demyelination between the fB-deficient and fB-inhibited (1/12 hours) groups. In contrast, CD59-/- mice exhibited a serious lack of structural organization, abundant inflammatory cell infiltration, scaring, vacuolation, and no obvious myelin structure. Also, in agreement with locomotor recovery data, when anti-fB mAb was administered 12 and 24 hours after SCI (or 24 and 36 hours, not shown), there was no

significant improvement in histological evidence of recovery when compared to wt and PBS treated controls.

Conclusions

An impact to the spinal cord results in a primary injury, but the impact also triggers a series of downstream events that lead to secondary injury of tissue and the progressive degeneration of the spinal cord. Therapeutic interventions that minimize secondary injury will improve functional recovery after traumatic injury. Inflammation plays a key role in secondary injury following spinal cord injury (SCI). Inflammation is a rapid immune response that can be initiated by infection or tissue damage. An important component of an inflammatory response is the complement system, a collection of blood proteins that form part of the immune system and that can be activated by injured cells and tissues. Activation of complement amplifies the inflammatory response and produces molecules that can be directly toxic or that can recruit and activate cells of the immune system to produce toxic molecules. Inhibiting the complement system has been shown to be an effective therapy for inflammatory disease in various animal models, and some complement inhibitors are in clinical trials.

We have shown that complement deficient mice and normal mice treated with a complement inhibitor are protected from neuronal injury following SCI, and that they have significantly improved functional scores over time compared to control mice. These data indicate an important role for complement in secondary injury, and indicate complement inhibition will reduce inflammation and provide neuroprotection following spinal cord injury. However, complement-dependent mechanisms involved in secondary injury following SCI are not known, and there remain concerns regarding the clinical application of the currently available systemic (body-wide) complement inhibitors with regard to their safety and efficacy. Complement activation products are important for host defense and immune maintenance

mechanisms, and systemic complement inhibition can compromise the protective and beneficial roles of complement. This will not be optimal in patients at risk of infection, and urinary tract infection is a frequent complication during initial and ongoing medical rehabilitation after SCI.

We propose to develop a neuroprotective strategy based on attenuating complement-dependent secondary damage after SCI using a novel and validated approach. The strategy involves the targeting of complement inhibitors to sites of complement activation and injury, and we have shown the approach to be highly effective in vitro and in mouse models of SCI and other inflammatory disease conditions. The targeting strategy enhanced the activity and protective effect of complement inhibitors by 10-20 fold compared to untargeted counterparts. Furthermore, untargeted, but not targeted complement inhibitors systemically inhibited complement and increased susceptibility to infection in a mouse model.

We propose to fully characterize our targeted complement inhibitors in a mouse model of SCI. In addition to therapeutic endpoints, we will use different types of complement inhibitor to investigate complement-dependent disease mechanisms. We will determine relationships between the generation of different complement activation products and other molecules associated with inflammation in order to gain a better understanding of the mechanisms of secondary tissue injury following SCI. These preclinical studies will establish the guidelines necessary for the translation of this therapy to the clinic using recombinant human proteins.

PUBLICATIONS

1. List of publication related to the thesis

1) Carl Atkinson, Hongbin Song, Bo Lu, **Fei Qiao**, Tara A. Burns,V. Michael Holers, George C. Tsokos, and Stephen Tomlinson. Targeted complement inhibition by C3d recognition ameliorates tissue injury without apparent increase in susceptibility to infection. J Clin Invest. 2005 September 1; 115(9): 2444–2453.

2) Fei Qiao, Carl Atkinson,Hongbin Song, Ravinder Pannu, Inderjit Singh and Stephen Tomlinson. Complement Plays an Important Role in Spinal Cord Injury and Represents a Therapeutic Target for Improving Recovery following Trauma. The American Journal of Pathology. September 2006; Vol. 169(3)

3) Yuxiang Huang, **Fei Qiao(Co-1st Author),** Carl Atkinson, V. Michael Holers and Stephen Tomlinson A novel targeted inhibitor of the alternative pathway of complement and its therapeutic application in ischemia/reperfusion injury. The Journal of Immunology, 2008 Dec 1; 181(11):8068-76.

4) Fei Qiao, Carl Atkinson, Mark Kindy, Anandakumar Shunmugavel, B. Paul Morgan, Hongbin Song and Stephen Tomlinson. The Alternative and Terminal Pathways of Complement Mediate Post-traumatic Spinal cord Inflammation and Injury. Am J Pathol. December 2010, Vol. 177, No. 6.

5) Songqing He, Carl Atkinson, **Fei Qiao,** Katherine Cianflone, Xiaoping Chen and Stephen Tomlinson. A complement-dependent balance between hepatic ischemia/reperfusion injury and liver regeneration in mice. J Clin Invest. 2009 August 3; 119(8): 2304–2316.

2. Other publications

1) Ishibashi Y, Takahashi M, Isomatsu Y, **Qiao F**, Iijima Y, Shiraishi H, Simsic JM, Baicu CF, Robbins J, Zile MR, and Cooper G 4th. Role of microtubules versus myosin heavy chain isoforms in contractile dysfunction of hypertrophied murine cardiocytes. Am J Physiol Heart Circ Physiol. 2003 Sep; 285(3): H 1270- 85 .

2) Cheng GM, **Qiao F**, Thomas N Gallien, Dhandapani Kuppuswamy and George Cooper,IV. Inhibition of Beta-Adrenergic Receptor Trafficking in Adult Cardiocytes by MAP4 Decoration of Microtubules. Am J Physiol Heart Circ Physiol. 2005 Mar; 288(3):H1193-202. Epub 2004 Nov 4.

3) Yuxiang Huang, **Fei Qiao,** Ruben Abagyan, Starr Hazard and Stephen Tomlinson. Defining the CD59-C9 Binding Interaction. The Journal of Biological Chemistry Vol. 281, No. 37, 27398–27404, September 15, 2006

4) Carl Atkinson, Hong Zhu, **Fei Qiao,** Juan Carlos Varela, Jin Yu, Hongbin Song, Mark S. Kindy and Stephen Tomlinson. Complement-Dependent P-Selectin Expression and Injury following Ischemic Stroke. The Journal of Immunology, 2006 Nov 15; 177(10): 7266-7274.

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