ORIGINAL ARTICLE

Soluble complement receptor type 1 inhibits complement system activation and improves motor function in acute spinal cord injury

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Study design: Spinal cord injured rat model, treated with soluble complement receptor type 1 (sCR1).

Setting: Experimental Animal Department of China Medical University, Shenyang, China.

Objectives: Soluble CR1 is a powerful inhibitor of complement activation. In this study, we investigate the effectiveness of sCR1 on spinal cord injury (SCI) in rats.

Methods: Spinal cord injury was induced in Sprague–Dawley rats. Three experimental groups were examined; the sCR1 group was administered sCR1 at 1 h after the SCI, whereas the control group was administered saline at 1 h after SCI and the sham group underwent a sham operation without SCI or administration. The expressions of C9 and CD59 in the injured spinal cords were evaluated by immunohistochemistry, and numbers of positive cells counted. Furthermore, myeloperoxidase (MPO) activity and motor function were evaluated in each group.

Results: At all postoperative time points, the numbers of C9- and CD59-positive cells in the sCR1 group were reduced compared with the control group and MPO activity was significantly decreased compared with both other groups. Moreover, the Basso, Beattie and Bresnahan score for the sCR1 group was significantly improved as compared with that of the control group after 7 days postoperatively.

Conclusion: Soluble CR1 decreases inflammation reactions by inhibiting activation of the complement system and improves motor function after acute SCI.

Spinal Cord (2010) **48**, 105–111; doi:10.1038/sc.2009.104; published online 8 September 2009

Keywords: spinal cord injury; complement; soluble complement receptor type 1; regeneration medicine; myeloperoxidase

Introduction

In the last decade, several studies have shown that the complement system has an important function after spinal cord injury (SCI) and represents a therapeutic target for improving functional recovery by inhibiting complement activation and inflammation.^{1–3} In earlier studies, we revealed the expression of C9 and CD59 in a rat model of SCI and showed that inhibiting complement activation improved functional recovery after SCI.⁴ C9 is the most important inherent component needed to compose membrane attack complex (MAC). Therefore, the level of C9 thoroughly reflects MAC expression. In contrast, CD59 is a homogeneous restriction factor in the complement system end phase and an important regulatory factor, whose main biological activities are to inhibit the formation of MAC and

prevent cytolysis.⁵ In combination, C9 and CD59 expression provide valuable insight into the mechanism of action of potential therapeutic agents that target the complement system.

Complement receptor type 1 (CR1) (CD35, receptor of C3b/C4b) is a transmembrane protein of hematopoietic cells and soluble CR1 (sCR1) is a potent inhibitor of the complement system. Several studies have revealed that the complement system is important not only for protection against microbial infection, but also contributes to the pathophysiology of non-infectious diseases.⁶ Therefore, a better understanding of this system in various disorders may yield new strategies for anti-inflammatory therapy.⁷ sCR1 has significant therapeutic effects in animal experiments and clinical cases associated with such complement immunoreactions as secondary brain injury, ischemic–reperfusion injury and autoimmune disease.⁸ However, the effects of sCR1 on acute SCI have not been reported.

Spinal cord injury triggers an acute inflammatory reaction that leads to secondary damage at the cord lesion site.⁹

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Received 25 March 2008; revised 10 July 2009; accepted 17 July 2009; published online 8 September 2009

Neutrophil is the main inflammatory cell in acute inflammatory reactions. Myeloperoxidase (MPO) activity, associated with the infiltration and activity of neutrophil, is the specific and sensitive marker of inflammation in the injured tissue.¹⁰ In this study, we investigated the function of sCR1 with regard to the expression of C9 and CD59, MPO activity and the recovery of motor function of acute SCI.

Materials and methods

Animal and spinal injury model

One-hundred fifty adult Sprague–Dawley rats (Experimental Animal Department of China Medical University, Shenyang, China) weighing 200–250 g were used in this study. Animals were maintained and handled according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996, USA). Induction of SCI was carried out following a modified weight-drop method with the NYU impacting equipment as described previously.¹¹ Briefly, the rat was anesthetized by intraperitoneal injection of pentobarbital (50 mg kg⁻¹). A laminectomy was carried out at T9 and T10 to expose the cord under a microscope. The rat was placed in a stereotactic device, and the spinous processes of T8 and T11 were clamped to stabilize the spine. In addition, an impounder (2 mm in diameter) was placed gently on the exposed dorsal surface of the exposed cord at the T10. SCI was induced by directly dropping a 10 g weight from a height of 5 cm onto the impounder. The muscles, subcutaneous tissue and incision were sutured.

Operation and experimental groups

One-hundred rats that received a spinal injury were randomly administrated either sCR1 (sCR1 group, $n=50$) or saline (control group, $n=50$). In the sCR1 group, recombinant sCR1 (6 mg kg⁻¹) (TP10, AVANT Immunotherapeutics Inc., Needham, MA, USA) was administered into the tail vein at 1 h and everyday post-injury, whereas saline was administered in the same manner to the control group. In addition, rats in the sham operated group (sham group, $n=50$) underwent a laminectomy at the T9 and T10 without SCI, and no administration of sCR1 or saline. Eight rats in each group were killed for tissue samples at 12 h, and 1, 3, 7 and 14 days after SCI.

Hematoxylin and eosin, and immunohistochemical staining

For staining of specimens, 15 rats (each group, $n=5$) were anesthetized at each time point after SCI, and then transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The injured spinal cord was removed, and post-fixed in 4% paraformaldehyde at 4 °C, then transferred to 30% sucrose solution. Each specimen was cut into 12 μ m axial sections with a freezing microtome. Hematoxylin–eosin staining, C9 and CD59 immunohistochemical staining were performed.

For the C9 and CD59 immunohistochemical staining, two primary antibodies (rabbit anti-rat C9, 1:1000 and mouse anti-rat CD59, 1:1000) were obtained from Dr Morgan, Wales

University in England. Slide-mounted sections were placed in an oven at 37 °C for 1 h. After incubating under constant agitation in 3% hydrogen peroxide (H₂O₂) in methanol for 30 min, the sections were blocked for 1 h in 5% normal goat serum with 0.1 M phosphate-buffered saline and 0.1% Triton X-100. Next, the primary antibody was applied and the preparations were left for 24 h at 4 °C. The sections were incubated for 20 min with a biotinylated secondary antibody (goat anti-rabbit and goat anti-mouse, 1:200, Sigma, St Louis, MO, USA), followed by a 20-min incubation at 37 °C incubation with avidin–biotin complex reagent (ABC Kit, Sigma). In the control sections, the primary antibody was substituted with 1% normal goat serum. The reaction product was revealed by incubation for 3–5 min with 0.02% diaminobenzidine tetrahydrochloride and 0.5% H₂O₂ in 0.05 M Tris-HCl (pH 7.6). Nine sections of C9 or CD59 staining were selected from the same region of the injury site at each time point. Positively stained cells was counted from 10 fields, which were randomly selected in each section (five fields were from gray matter and the others were from white matter). The positive cells were counted using a Meta Morph automatic color image analyzer by a blinded observer at magnification of $\times 400$, with cell numbers shown as an inverse ratio.

Assay of myeloperoxidase activity

Fifteen rats were used in this assay at each time after SCI (each group, $n=5$). After anesthesia, 1 cm of cord encompassing the entire lesion was excised, then frozen and stored at -20 °C before myeloperoxidase (MPO) enzyme activity evaluation. MPO activity level was measured using the method described by Taoka *et al.*¹² Briefly, 10% (w/v) of the spinal cord tissue specimen was homogenized and suspended in 20 mmol l⁻¹ of phosphate buffer (pH 6.0) containing 0.5% cetrimonium bromide, then the mixture was sonicated and incubated for 20 min. After centrifugation (12 500 g at 4 °C for 30 min), 0.1 ml of the supernatant was added to 0.6 ml of 0.1 mol l⁻¹ of phosphate buffer (pH 6.0) with 0.05% H₂O₂ containing 1.25 mg ml⁻¹ of o-dianisidine. Absorbance at 460 nm after 2 min was measured using a spectrophotometer, and MPO activity was calculated using a standard curve prepared with purified MPO. The results are expressed as units (U) g⁻¹ of wet tissue.

Motor functional analysis

The motor function of 30 rats (each group, $n=10$) was evaluated using Basso, Beattie and Bresnahan (BBB) scores. Scores ranged from 0 to 21, with 21 points considered normal and 0 points correlating to no movement of the hindlimbs.¹³ Motor function was evaluated by two independent and blinded observers and the average of three trials was recorded at each time point after SCI.

Statistical analysis

Statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA) for Windows, with Student's *t*-test used to compare the results between two groups. Data are expressed as the mean $\pm$ s.d., with $P < 0.05$ considered being significant.

Results

Hematoxylin–eosin staining

In the sham group, the spinal cord was normal at all periods after injury (data not shown). As for the control group, there were some spotty hemorrhage areas in the gray matter and neutrophils in the injured tissue at 12 h (Figures 1a and b). On the first day, neurons were beginning to swell with dissymmetrical nuclei, while they were obviously swollen and had become round, with nuclear concentration and fragmentation detected, and a large amount of neutrophil infiltration and large spotty hemorrhaging in the gray matter on the third day (Figure 2a). On the seventh day, the remnant neurons were decreased in number, although some neutrophil infiltration still existed in the gray matter. On the fourteenth day, fewer remnant neurons remained, while a small amount of neutrophil infiltration was seen in the gray matter and porosis had occurred.

In the sCR1 group, less vacuolization of cells in the white matter and more polygon-shaped neurons in the gray matter

were revealed as compared with the control group. At each time point, neuron swelling, degeneration, necrosis and neutrophil infiltration were all at lower levels as compared with the control group (Figure 2b).

Immunohistochemical staining

In the sham group, no expression of C9 or CD59 was observed in the spinal cord specimens at any time point (data not shown). In sCR1 and the control group, C9 and CD59 expressions were observed in some parts of the neuron cytomembrane and cytoplasm in the anterior horn regions at 12 h as well as in the neuropile on day 1. On the third day, C9 and CD59 expressions in the gray matter could be seen in the remnant neuron cytomembrane and cytoplasm, and expressions in the neuropile were also greatly increased (Figures 3a, b and 4a, b, respectively). In the white matter, C9 (Figures 5a and b) and CD59 expressions (data not shown) were also detected, indicating that the positive cells were

Figure 1 Hematoxylin–eosin (HE) staining of injured spinal cord specimens on the 12 h postoperatively. (a) HE-stained specimens in injured level of spinal cord in control group. (b) Several spotty hemorrhage areas were detected in the gray matter and neutrophils (arrow). The rectangle in panel a is magnified in panel b (magnification; (a): $\times 40$, (b): $\times 80$).

Figure 2 Hematoxylin–eosin (HE) staining in gray matter of injured spinal cord specimens on the third day postoperatively. (a) Control group: round swollen neurons (white arrow), nuclear concentration, nuclear fragmentation and a great deal of neutrophil infiltration (white arrowhead) were seen. (b) Soluble complement receptor type 1 group. A little neutrophil infiltration in reparative tissue was seen, whereas a lot of polygon-shaped neurons (arrow) were observed (HE, magnification: $\times 200$).

Figure 3 Immunohistochemical staining specific for C9 in gray matter of injured spinal cords on the third day postoperatively. The sections are stained with antibody against C9. C9 is stained in the remnant neuron cytomembrane, cytoplasm (white arrow) and the neuropile (white arrowhead) in panels a and b. (a and b) Shows the control and soluble complement receptor type 1 groups, respectively (magnification: $\times 400$).

oligodendrocytes. C9 and CD59 were gradually decreased by day 7. On day 14, C9- and CD59-positive neurons remained in the gray matter, with only slight C9 and CD59 staining in the neuropile.

Enumeration of C9- and CD59-positive cells

The number of C9- and CD59-positive cells differed between the sCR1 and control groups at each time point (Table 1). C9-positive cells were significantly reduced in the sCR1 group compared with the control group at all time points ($P < 0.01$). In addition, the number of CD59-positive cells in the sCR1 group was significantly lower than in the control group at 12 h, and days 1, 3 and 7 postoperatively ($P < 0.01$, $P < 0.05$), whereas there was no significant difference on the fourteenth day. The expression of C9 and CD59 in the injured spinal cord in both groups increased slowly after the 12-h time point and reached a peak on day 3, and then decreased gradually until day 14.

Figure 4 Immunohistochemical staining specific for CD59 in gray matter of injured spinal cord on the third day postoperatively. The sections are stained with antibody against CD59. CD59 is also stained in the remnant neuron cytomembrane, cytoplasm (white arrow) and the neuropile (white arrowhead). (a) and (b) Shows the control and soluble complement receptor type 1 group, respectively (magnification: $\times 400$).

Myeloperoxidase activity

The activities of MPO in the sCR1 and control group showed the same trends (Figure 6), although the sCR1 group was significantly less at all time points ($P < 0.01$). Interestingly, peak activity occurred on the third day after SCI in both groups, and then decreased in the same manner as C9- and CD59-positive cells (Table 1). MPO activity levels did not change in the sham group (data not shown). There were significant differences in MPO activity levels between the sCR1 and sham groups, and control and sham groups ($P < 0.01$).

The motor function

The BBB scores of both the control and sCR1 groups showed gradual improvements over the 6-day period after the procedure (Figure 7). However, the BBB scores of only the

Figure 5 Immunohistochemical staining specific for C9 in white matter of injured spinal cords on the third day postoperatively. The sections are stained with antibody against C9. C9 is widely stained in white matter of injured spinal cord (white arrow). The positive cells are oligodendrocytes. (a and b) Shows the control and soluble complement receptor type 1 groups, respectively (magnification: $\times 400$).

Table 1 Enumeration of C9- and CD59-positive cells in the sCR1 and control groups with a Meta Morph automatic colored image analyzer at magnification of $\times 400$, with cell numbers shown as an inverse ratio

Postoperative time	C9		CD59	
	Control	sCR1	Control	sCR1
12h	82.17 $\pm$ 4.18	88.53 $\pm$ 5.04*	95.34 $\pm$ 4.82	121.74 $\pm$ 9.52*
1 day	73.48 $\pm$ 3.34	81.98 $\pm$ 2.23*	87.55 $\pm$ 6.29	98.38 $\pm$ 8.01*
3 days	61.62 $\pm$ 2.33	72.01 $\pm$ 2.40*	70.12 $\pm$ 6.04	81.13 $\pm$ 5.45*
7 days	70.88 $\pm$ 5.34	81.56 $\pm$ 5.27*	80.35 $\pm$ 5.02	83.56 $\pm$ 6.09#
14 days	85.72 $\pm$ 5.31	95.87 $\pm$ 3.02*	97.59 $\pm$ 6.96	97.31 $\pm$ 7.88

Values were calculated by inverse ratio (mean score $\pm$ s.d.).

Asterisks (*) indicate significant difference vs control group ($P < 0.01$) and sharp (#) indicates significant difference vs control group ($P < 0.05$).

sCR1 group improved on day 7, suggesting that the effect of sCR1 administration became apparent after 1 week ($P < 0.05$). Subsequently, the motor function scores increased as

Figure 6 Histograms showing the myeloperoxidase (MPO) activity. MPO activity was assessed in the injured spinal cords of rats in the soluble complement receptor type 1 (sCR1), control and sham groups. There were significant differences between the sCR1 and control groups. Asterisk (*) indicates a significant difference vs the sham group ($P < 0.01$), Pound (#) indicates a significant difference vs the sCR1 group ($P < 0.01$) (U g^{-1} watery weight, $n = 5$, mean $\pm$ s.d.).

Figure 7 Histograms showing motor function graded with Basso, Beattie and Bresnahan (BBB) score. There was no significant difference between the soluble complement receptor type 1 (sCR1) and control groups during the first 6 days. Thereafter, scores for the sCR1 group scores increased significantly as compared with the control group. Asterisk (*) and sharp (#) indicate significant difference vs the control group $P < 0.01$ and $P < 0.05$, respectively ($n = 10$, mean $\pm$ s.d.).

compared with the control group from day 8 ($P < 0.01$). In the sham group, the BBB score was 21 at all time points.

Discussion

The mechanisms of repair after SCI have not been well defined, but it has been clearly shown that the inflammatory process has a significant role in functional recovery. Immune-related inflammation cannot only trigger a series

of reactions in the injured tissue at the cellular and molecular levels, but also leads to local glial cell cicatrization and hampers regeneration of the injured spinal cord.¹⁴

Neutrophils are the main inflammatory cells in acute immune inflammatory reactions. MPO is a specific enzyme present in large quantities in azurophilic granules of neutrophils, contributing to its oxygen-dependent bactericidal activity.¹⁵ In this study, sCR1 administration decreased inflammation reactions, as shown by decreased MPO activity and improved motor function. There was little improvement of motor score in the control group between 7 and 14 days, despite the concurrent decrease in MPO activity. We believe that it is the natural course of recovery from acute SCI of this model without any intervention. In contrast, there was a remarkable improvement of motor function in sCR1 group between 7 and 14 days, suggesting the impressive effect of sCR1 on SCI.

The complement system is an important component of the immune protection mechanism and significant with regard to immune system-related inflammation following pathological injury. In complement activation, MAC mediates neuron death, that is, cytolytic necrosis and apoptosis. MAC also provokes the cell into releasing leukotriene, prostaglandin and active oxygen. Numerous complement components, namely C3a and C5a as well as others, upregulate the expression or enhance the stimulation of multiple cytokines and chemokines and aggravate inflammatory reactions. Hence, inhibition of complement system activation is a viable therapeutic target for the suppression of the negative effects of inflammation after SCI.¹⁶

Soluble CR1 shows a potent inhibition to the complement system by inactivating the two critical enzymes (C3 and C5) in classical, alternative and lectin complement activation signaling pathways (Figure 8). In this study, C9 and CD59 staining were found in injured spinal tissues from both the sCR1 and control groups, and decreased by administrating of sCR1. The C9 expression in the sCR1 group was lower than that of control group, indicating that the complement cascade reaction is activated to the end phase following acute SCI and clearly suppressed by sCR1. The expression of CD59 in the sCR1 group was also lower as compared with the control group.

In several studies, recombinant sCR1 has been applied to animal experiments and clinical studies of severe trauma, ischemic-reperfusion injury and autoimmune diseases. In a study of traumatic brain injury model in rat, recombinant sCR1 reduced tissue neutrophil infiltration and relieved local immune inflammation by inhibiting activation of the complement system.¹⁷ Dobschuetz *et al.*¹⁸ have shown that sCR1 effectively improved the pancreatic ischemia-reperfusion-induced microcirculation in rats and they also described the possibility of treatment after post-ischemic pancreatitis. Li *et al.*¹⁹ showed that recombinant sCR1 effectively inhibited complement activation during cardiopulmonary bypass in humans, and referred the tolerance and improvement of vascular functions in infant's case.

This study shows a novel use of recombinant sCR1 in rats with an acute SCI. Our findings suggested that sCR1 can reduce inflammation and is effective in protecting the rat

Figure 8 Soluble complement receptor type 1 (sCR1) and complement activation signaling pathways. sCR1 inhibits the complement activation cascade at the two levels by inactivating the C3 and C5 in signaling pathways. At the terminal stage, C9 is needed to compose the MAC/C5b-9, and CD59 inhibits the formation of MAC/C5b-9.

spinal cord from secondary injury by inhibiting activation of the complement system. We believe that our results provide a theoretical basis for anticomplementary treatment for acute SCI.

Acknowledgements

We gratefully acknowledge AVANT Immunotherapeutics Inc. for their gift of recombinant sCR1. We also acknowledge Prof Morgan of Wales University in England for his devoted assistance and Dr Mimura of Shiga university of Medical science in Japan for his assistance. This work was supported by a grant-in-aid the 'Young Foundation Project Funds of the Educational Department of Liaoning Province, China' (05L531).

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