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Delayed post-injury administration of C5a improves regeneration and functional recovery after spinal cord injury in mice

Q. Guo,*[1] J. Cheng,‡[1] J. Zhang,* B. Su,§ C. Bian,* S. Lin§ and C. Zhong†
*Department of Neurobiology, Chongqing Key Laboratory of Neurobiology, Third Military Medical University, †Department of Otorhinolaryngology, Southwest Hospital, Third Military Medical University, ‡Chongqing Normal University, Chongqing, and §Development and Regeneration Key Laboratory of Sichuan Province, The Key Medical Subject of Burn Injury in Sichuan Province, Department of Histology and Embryology and Neurobiology, Chengdu Medical College, Chengdu, China

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Correspondence: C. Zhong, Department of Otorhinolaryngology, Southwest Hospital, Third Military Medical University, Chongqing 400038, China.
E-mail: 13883065477@163.com

[1]These authors contributed equally to this work.

Summary

The activation of a complement system can aggravate the secondary injury after spinal cord injury (SCI). However, it was reported recently that the activation of a complement could have both a secondary injury and a neuroprotective effect, in which C5a is the most important factor, but there is no direct evidence for this dual effect of C5a after SCI. In order to investigate the potential neuroprotective effect of C5a after SCI, in this study ectogenic C5a was injected intraperitoneally before/after SCI *in vivo*, or administrated to mechanically injured neurones *in vitro*; following this, neurone apoptosis, neurite outgrowth, axonal regeneration and functional recovery were investigated. The *in-vivo* experiments indicated that, following treatment with C5a 24 h before or immediately after injury, locomotor function was impaired significantly. However, when treatment with C5a took place 24 h after injury, locomotor function improved significantly. *In-vitro* experiments indicated that a certain concentration of C5a (50–100 nM) could inhibit caspase-3-mediated neurone apoptosis by binding to its receptor CD88, and that it could even promote the neurite outgrowth of uninjured neurones. In conclusion, delayed post-injury administration of C5a within a certain concentration could exert its neuroprotective effect through inhibiting caspase-3-mediated neurone apoptosis and promoting neurite outgrowth of uninjured neurones as well. These data suggest that C5a may have opposite functions in a time- and concentration-dependent manner after SCI. The dual roles of C5a have to be taken into account when measures are taken to inhibit complement activation in order to promote regeneration after SCI.

Keywords: apoptosis, complement, neurite outgrowth, neuroprotection, spinal cord injury

Introduction

The role of the complement system after spinal cord injury (SCI) has received considerable attention. Inhibition of complement activation can reduce the expression of inflammatory factors, improve neuroprotection and attenuate the secondary injury following SCI [1–4]. Our previous work indicates that the blocking of complement activation by C3 deficiency has a neuroprotective effect and can improve regeneration and functional recovery after SCI [5], indicating that inhibiting complement activation may be a promising therapy for SCI.

However, it was reported recently that complement activation had another neuroprotective role [6–9] in addition to that involving the secondary injury [10,11], and C5a was thought to be the most important factor in this respect. Although C5a can induce the chemotactic response and mediate the early phase of inflammation that is harmful to cells at the lesion site [12,13], it can exert its neuroprotective effect by inhibiting neurone apoptosis [6,7,14] and promoting microglial phagocytosis [15,16], which is beneficial in alleviating the secondary injury. However, in terms of SCI, existing research is focused on the secondary injury caused by C5a, and it is not known whether it exerts a neuroprotective effect after SCI. If it does, the favourable and unfavourable roles of C5a have to be balanced following SCI in order to achieve greater efficiency in neuroneal regeneration.

Therefore, in the present study the neuroprotective effect of C5a after SCI was investigated. Our data indicated that although exogenous C5a administrated before or immediately after injury exacerbated inflammation after SCI, delayed post-injury C5a administration could attenuate the secondary injury and improve regeneration after SCI.

Materials and methods

Spinal cord injury and C5a injection

The study was performed in accordance with protocols approved by Third Military Medical University. All surgical procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Every effort was made to minimize the number of animals used and their suffering. Female wild-type C57/BL6 mice (weighing 18–20 g, 6–8 weeks old) were purchased from the Experimental Animal Center of the Third Military Medical University. In order to minimize the influence of the oestrogen level, a vaginal smear examination was performed and only animals in dioestrus were used. A forceps crush injury was performed using the procedure described in a previous report [17] after a laminectomy at T12. Manual bladder expression was performed twice a day after surgery.

The C5a protein (Abcam, Cambridge, MA, USA) was solubilized in 0·01 M phosphate-buffered saline (PBS). An effective concentration of C5a (100 nM) was injected intraperitoneally (10 μl/g body weight) 24 h before injury (−24 h), immediately after injury (0 h) or 24 h after injury (24 h), respectively. As for the control, PBS replaced C5a protein for injection at different time-points.

Locomotor function analysis

Functional recovery after SCI was scored in an open field using the Basso, Beattie and Bresnahan (BBB) rating scale. Animals were assessed daily before and after surgery by an observer blinded to animal treatment.

Histology analysis

Mice were perfused with 4% paraformaldehyde 9 weeks post-injury. Tissue segments extending from 3 mm rostral to 3 mm caudal to the lesion site were sectioned in the longitudinal plane (30 μm) on a cryostat. Immunohistochemistry was performed for growth-associated protein-43 (rabbit anti-GAP43, 1:500; Abcam) according to the protocol described in a previous report [18], and visualized with a species-specific rhodamine (tetramethylrhodamine isothiocyanate: TRITC)-conjugated antibody (1:500, Serotec, Raleigh, NC, USA). The tissue segments were also used to perform haematoxylin and eosin (H&E) staining.

Primary neurone culture and mechanical injury

Newborn mice (0 day) were used for the neurone culture. After being digested in 0·125% trypsin for 15 min, cells were collected and resuspended in neurobasal medium A (Sigma, St Louis, MO, USA) with 10% B27, 2 mM glutamine, 100 IU/ml penicillin/streptomycin and 10 ng/ml nerve growth factor (NGF) and plated at a density of 1×10^6/ml.

The neurone mechanical injury was performed according to the procedure described in a previous report [19]. In brief, the medium was refreshed 1 day before it was used. Then '#'-like scuffing was performed on neurones plated on a coverslip. The interval between scratch lines was 5 mm.

Neurone apoptosis analysis

C5a treatment was performed with different concentrations (50 nM, 100 nM, 200 nM) 24 h after the mechanical injury to the 5-day-old cultures. For the control, 0·01 M PBS was added into the culture media instead of C5a. To further confirm the effect of C5a, the C5a receptor antagonist AcF-[OP(D-Cha)WR] (PMX53; Shenzhen Jingmei, Shenzhen, China) was added at the same concentration as that of C5a in each group. Immunofluorescence staining was performed 3 days after injury. The proportion of caspase-3 positive cells (red) to 4′,6-diamidino-2-phenylindole (DAPI)-positive nuclei (blue) was calculated by counting, respectively, from 10 random visual fields under a microscope. The average proportion represented the ratio of apoptosis neurones to survival neurones.

Neurite assay

When neurone adherence was completed, different concentrations of C5a (50 nM, 100 nM, 200 nM) were added into the culture media for 2 days or 6 days until the neurite assay was performed. For the control, 0·01 M PBS was added into the culture media instead of C5a. Three different visual fields were selected randomly from each concentration group, and the area occupied by neurites was quantified using Image Pro. version 5.0 according to the method described by Bilsland *et al.* [20]. The average value of each group was used to represent the neurite outgrowth.

Statistical analysis

Statistical significance between groups was determined by analysis of variance with repeated measures using Scheffé's test for *post-hoc* comparisons; $P < 0{\cdot}05$ was considered statistically significant.

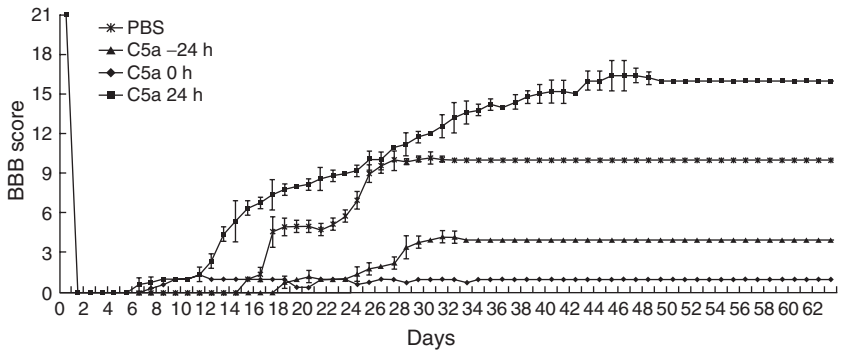

Fig. 1. Locomotor function assessed by the Basso, Beattie and Bresnahan (BBB) scale for 9 weeks after spinal cord injury (SCI). When pretreated with C5a 24 h before injury (C5a, –24 h), the locomotor function was impaired significantly in comparison with phosphate-buffered saline (PBS)-treated mice. Mice treated with C5a immediately after injury (C5a, 0 h) even showed a remarkable lower score throughout the observation period. However, locomotor function improved significantly only when mice were treated with C5a 24 h after injury (C5a, 24 h); $n = 5$ per group.

Results

Recovery of locomotor function after SCI

Locomotor function was assessed using the BBB scale for 9 weeks after SCI (Fig. 1). The BBB scores of all mice were 0 immediately after SCI, and there was no difference in functional recovery among each of the PBS-treated groups. When mice were pretreated with C5a 24 h before injury, locomotor function was impaired significantly in comparison with PBS-treated mice. Mice treated with C5a immediately after injury even showed a remarkable lower score throughout the observation period. However, locomotor function improved significantly only when mice were treated with C5a 24 h after injury.

Analysis of histological recovery

To detect the level of histological recovery, H&E staining was performed 9 weeks after injury. In mice treated with PBS, the normal tissue of the spinal cord was damaged and constricted, and several irregular cavities were formed in the injured region (Fig. 2a). In mice pretreated with C5a 24 h before injury (Fig. 2b), especially those treated immediately after injury (Fig. 2c), the extent of the damage was exacerbated, and the areas where there were cavities were enlarged. However, in mice treated with C5a 24 h after injury (Fig. 2d) the integrity of the spinal cord was improved, and the areas where there were cavities were clearly smaller than those of the other three groups.

Axonal regrowth after SCI

Although several GAP43-positive fibres were observed in the injured region in mice treated with PBS (Fig. 3a,a′), fewer GAP43-positive fibres were detected in mice pretreated with C5a 24 h before injury (Fig. 3b,b′, $P < 0{\cdot}01$ *versus* the PBS group). In mice treated with C5a immediately after injury, very few GAP43-positive fibres could be detected (Fig. 3c,c′, $P < 0{\cdot}01$ *versus* the PBS group). However, in mice treated with C5a 24 h after injury (Fig. 3d,d′), more GAP43-positive fibres were observed

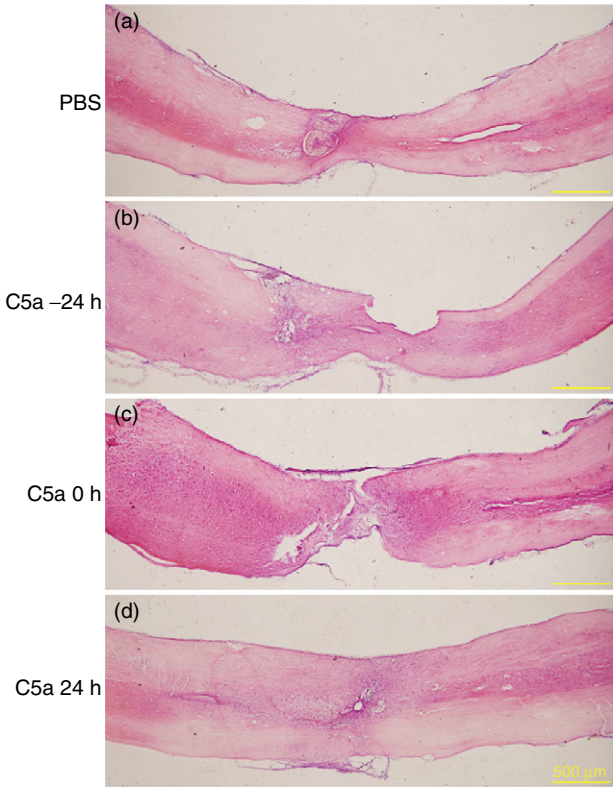

Fig. 2. Histology of spinal cord sections after spinal cord injury (SCI). Mice were treated with phosphate-buffered saline (PBS) (a), 100 nM C5a 24 h before injury (b), immediately after injury (c) or 24 h after injury (d), respectively. Longitudinal sections from the epicentre of the injury were stained with haematoxylin and eosin (H&E) 9 weeks after SCI; $n = 5$ per group. Scale bar: 500 μm.

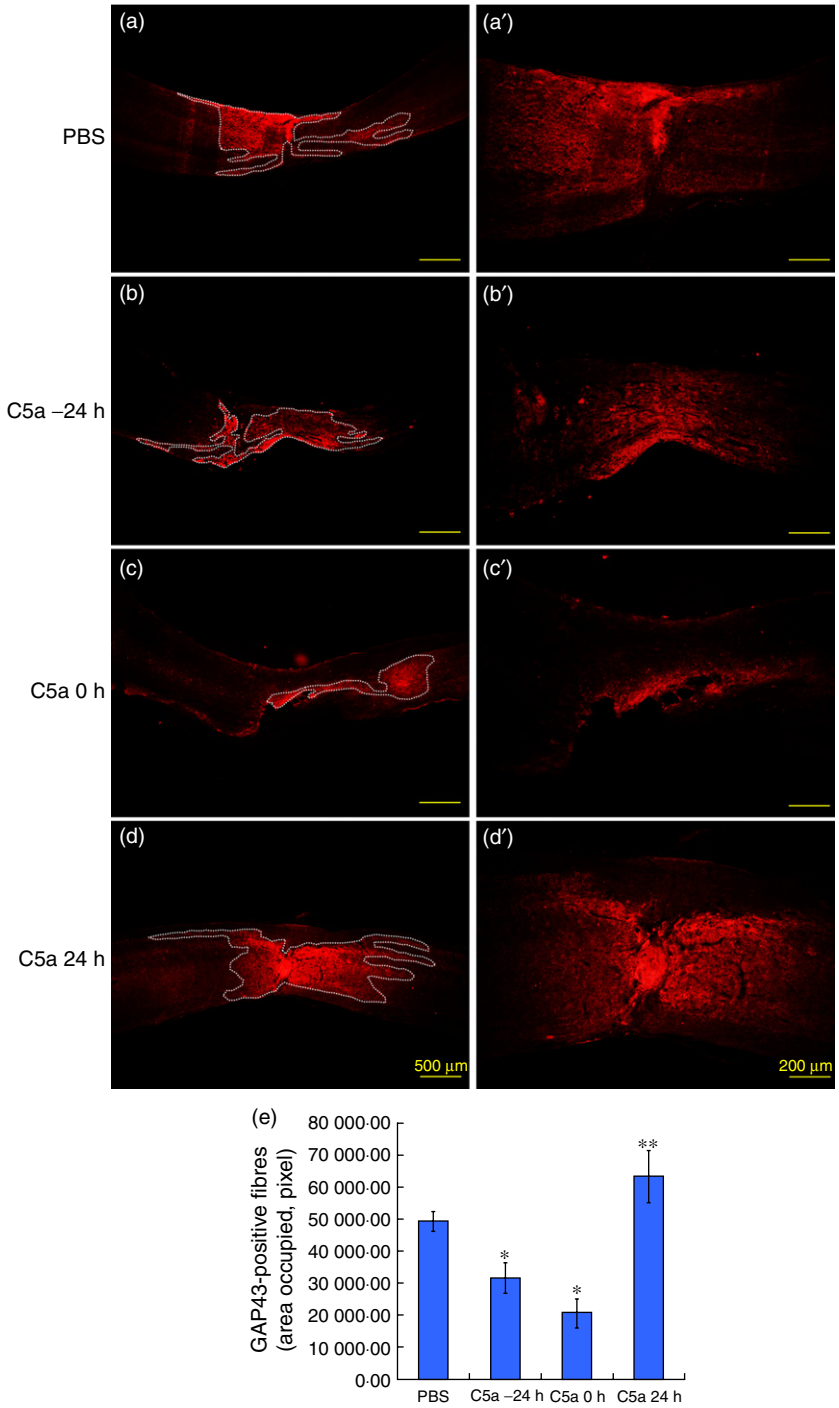

Fig. 3. The delayed post-injury administration of C5a improves regeneration and functional recovery after spinal cord injury (SCI). Longitudinal sections from the epicentre of the injury showing GAP43 immunostaining in spinal cords 9 weeks after SCI in mice treated with phosphate-buffered saline (PBS) (a, a′), 100 nM C5a 24 h before injury (b, b′), immediately after injury (c, c′) and 24 h after injury (d, d′), respectively; (a′–d′) are higher magnifications of (a–d). (e) The areas that GAP43-positive fibres occupied in (a–d) were marked with broken white lines and measured; $n = 5$ per group. Scale bars: (a–d) 500 μm, (a′–d′) 200 μm. *$P < 0{\cdot}01$ *versus* PBS group; **$P < 0{\cdot}05$ *versus* PBS group.

than was the case with the PBS-treated mice ($P < 0{\cdot}05$), suggesting that delayed post-injury administration of C5a can promote axonal regrowth in the injured spinal cord.

Neurone apoptosis

At lower concentrations of C5a (50 nM, 100 nM), neurone apoptosis was inhibited significantly by 38% ($P < 0{\cdot}05$) and 45% ($P < 0{\cdot}01$), respectively (Fig. 4b,b′,c,c′,i). However, at a higher concentration (200 nM), the apoptosis was no longer inhibited by C5a (Fig. 4d,d′,i). The apoptosis cells increased significantly compared with those in the 50 nM and 100 nM groups, and even exceeded the level of the control group ($P < 0{\cdot}01$) (Fig. 4a,a′,i). This inhibition effect could be reversed by PMX53 administration (Fig. 4e–h,e′–h′,i).

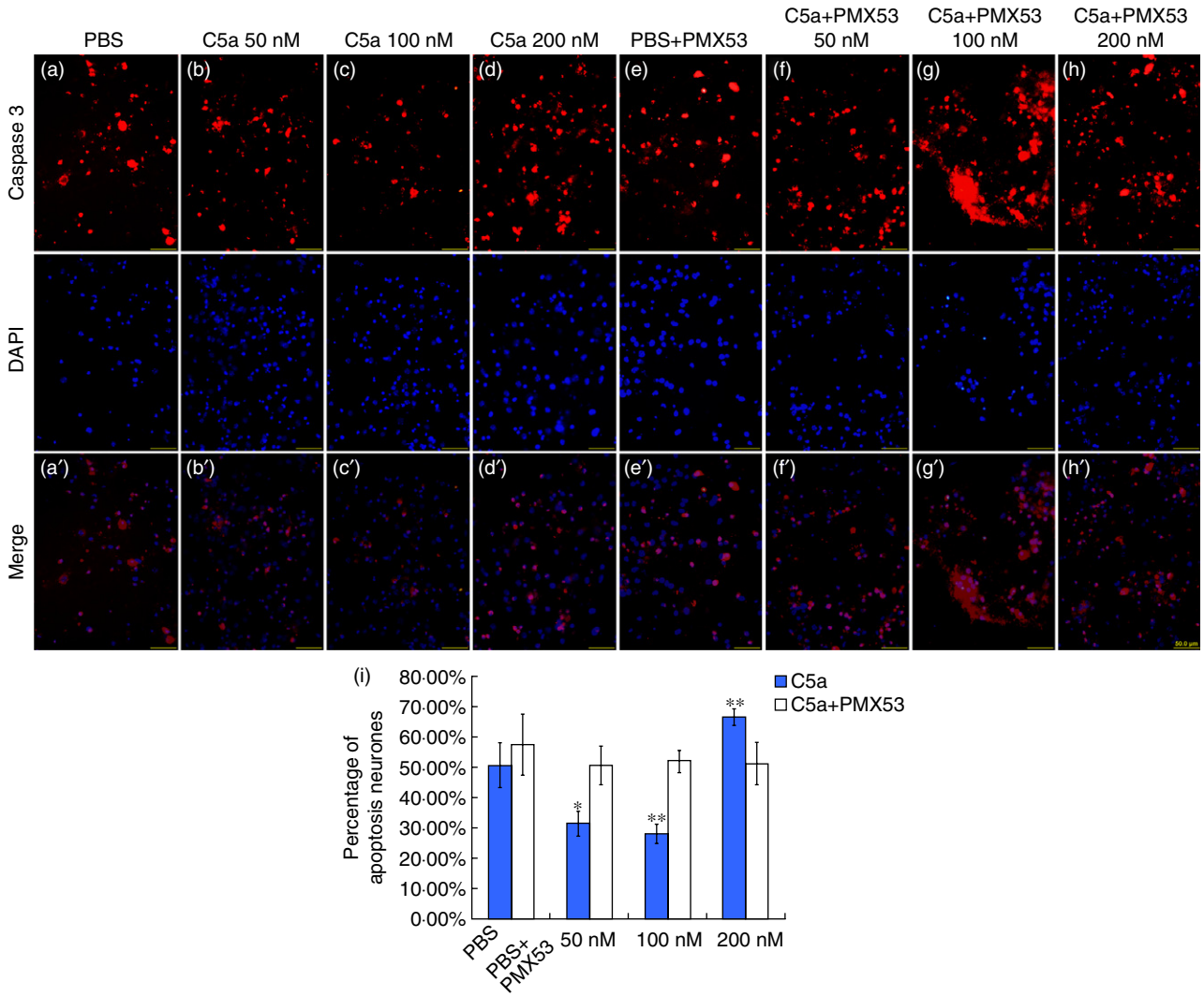

Fig. 4. Neurone apoptosis analysed 3 days after the mechanical injury to neurones treated with different concentrations of C5a (50 nM, 100 nM, 200 nM) 24 h after injury. (a–d) Caspase-3 immunofluorescence staining of neurones treated with phosphate-buffered saline (PBS) (a), 50 nM C5a (b), 100 nM C5a (c) and 200 nM C5a (d). (e–h) Caspase-3 immunofluorescence staining of neurones treated with PBS + PMX53 (e), 50 nM C5a + 50 nM PMX53 (f), 100 nM C5a + 100 nM PMX53 (g) and 200 nM C5a + 200 n PMX53 (h). 4′,6-Diamidino-2-phenylindole (DAPI) was used to identify all neuronal nuclei within the field. (a′–h′) A merging of caspase-3 and DAPI staining. (i) The percentage of apoptosis neurones in different groups; $n = 10$ per group. Scale bars: 50 μm. *$P < 0{\cdot}05$ *versus* PBS group; **$P < 0{\cdot}01$ *versus* PBS group.

Neurite outgrowth

Neurite outgrowth could be observed in each group at 2 days, with no significant difference in neurite areas being observed between the control (Fig. 5a,i), 50 nM (Fig. 5b,i) and 100 nM (Fig. 5c,i) groups. However, the neurite area of the 200 nM group (Fig. 5d,i) was significantly lower than that of the other three groups ($P < 0{\cdot}05$). A large quantity of interlacing neurites could be observed in each group at 6 days, with significantly larger neurite areas in the 50 nM (Fig. 5f,i) and 100 nM (Fig. 5g,i) groups than in the control ($P < 0{\cdot}05$) (Fig. 5e,i). For the 200 nM group (Fig. 5h,i), its neurite area was slightly smaller than that of the control, but the difference was not significant.

Discussion

Among complement components, C5a is thought to be the most effective inflammatory factor. Following central nervous system (CNS) lesions, C5a exerts its role by binding to its receptor, and CD88 is considered to be its main functional receptor [21,22]. C5a can induce neutrophil chemotaxis, up-regulate the expression of CD11b, interleukin (IL-2) IL-6, IL-8 and tumour necrosis factor (TNF)-α and mediate the secondary injury after SCI [12,13,23,24]. Meanwhile, there is positive feedback between the expression of TNF-α and that of CD88 [25]. Administration of C5a before or immediately after SCI will promote this feedback and increase the expression of endogenous C5a, CD88

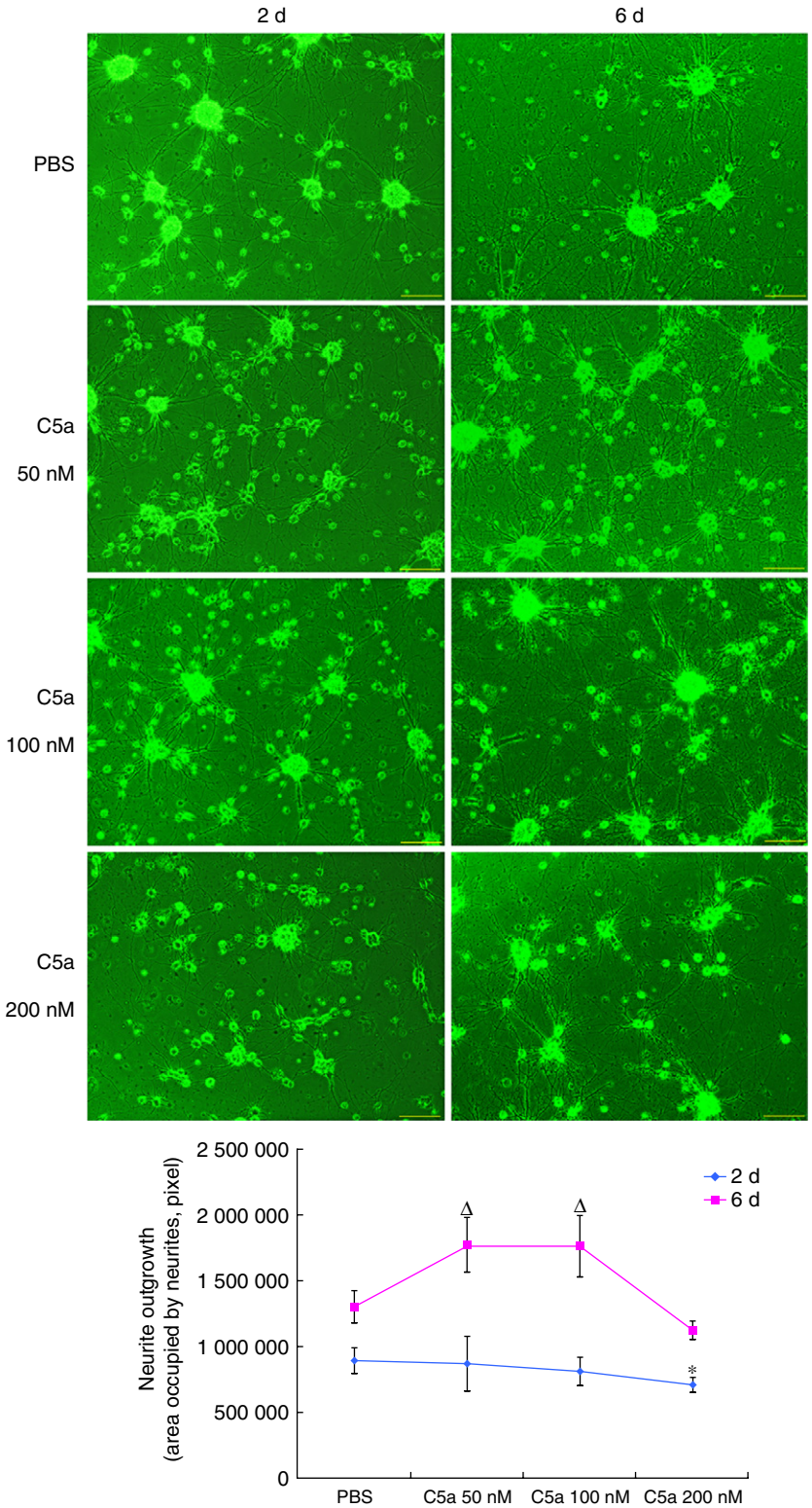

Fig. 5. Neurite outgrowth when treated with different concentrations of C5a. There was no significant difference between groups treated with phosphate-buffered saline (PBS) (a), 50 nM C5a (b) and 100 nM C5a (c) at 2 days. However, the neurite area of the 200 nM C5a-treated group (d) was significantly lower than that of the other three groups. At 6 days, significantly larger neurite areas in the 50 nM (f) and 100 nM (g) groups, but not in the 200 nM group (h), could be observed, compared with the PBS group (e). (i) The area occupied by neurites in the different groups at 2 days and 6 days, respectively; $n = 3$ per group. Scale bars: 100 μm. *$P < 0{\cdot}05$ *versus* PBS group at 2 days; Δ: $P < 0{\cdot}05$ *versus* PBS group at 6 days.

and TNF-α, and ultimately aggravate the secondary injury after SCI. The differing degrees of injury observed between the −24 h and 0 h groups may be relevant in terms of the metabolism of C5a. C5a *in vivo* is quickly metabolized by carboxypeptidases to form C5adesArg [26]. It was thought previously that both C5a and C5adesArg could be cleared from the body with a half-life of 2–3 min, mediated partly by internalization by CD88 and C5L2 [27,28]. However, a recent study has indicated that the C5a internalized by CD88 is not retained and degraded. Instead, it is released

into the extracellular medium in an intact state [28], so this part of C5a is not really cleared and could still participate in the secondary injury. Therefore, C5a administered 24 h before SCI might be only partially cleared, and that still remaining might participate in the secondary injury. When C5a was administered immediately after SCI, all of it participated in the secondary injury before it had time to be cleared. This could probably explain why the degree of injury in the −24 h group was more severe than that in the control, but better than in the 0 h group.

It is worth noting that C5a administered 24 h after SCI attenuated the secondary injury and enhanced the functional recovery. Our previous work showed that the expression of both C5a and CD88 increased, and peaked at 24 h after SCI [25]. Instead of exacerbating the secondary injury, C5a administration at this time-point reduced it significantly, suggesting an alternative role. Vidal *et al.* [29] reported that the inhibition of peripheral TNF-α, starting 14 days after injury, had no effect on functional recovery after SCI. Beck *et al.* [30] reported that late inflammation 14 days post-injury could promote functional recovery and myelination after SCI, and the macrophage/microglia response might prevent the further loss of function during the early chronic phase (14–28 days after injury) of inflammation. These studies indicated that the acute phase of inflammation might exacerbate the secondary injury after SCI, while the chronic phase could have a protective effect. Even C5a could probably have an opposite role during this second phase, as Beck *et al.* [30] predicted that the blocking of subacute to chronic inflammation by the C5a receptor antagonist would reduce functional recovery after SCI, which was confirmed in $CD88^{-/-}$ mice by Brennan *et al.* [9]. Perhaps for this reason, in our study the functional recovery of control mice started from 15 days after injury. When mice were treated with C5a 24 h after SCI the probable neuroprotective effect of C5a, rather than its proinflammatory effect, resulted in enhanced functional recovery. However, the precise time window during which C5a exerts this protective effect needs to be studied further.

To investigate further the neuroprotective role of C5a, we observed its effect on neural survival and neurite outgrowth and found that C5a within a certain concentration range (50–100 nM) could reduce the apoptosis of injured neurones and promote the neurite outgrowth of uninjured neurones, which indicated that the effect of C5a on neurones was related to the concentration range as well as to the time window. The inflammatory effect of C5a could convert to a neuroprotective effect as its concentration changed. It was reported that the expression of the C5a receptor in neurones remained weak under normal physiological conditions but increased significantly when stimulated by inflammation [25], which could be a self-protection response. For example, Mukherjee and Pasinetti [6] found that the neurones in the hippocampus that were pretreated with C5a showed high tolerance to glutamate, and Bénard *et al.* [7] reported that the C5a receptor antagonist could promote neural survival. Our data also showed that a certain concentration of C5a could reduce the caspase-3 mediated apoptosis of injured neurones, and that this effect could be reversed by PMX53 administration. Therefore, C5a could exert its neuroprotective effect by binding to its receptor on the neurone surface, inhibiting the expression of caspase-3 and ultimately reducing neurone apoptosis. To our surprise, in the case of uninjured neurones C5a could promote outgrowth of their neurites, which was a further illustration of its promotion of neural regeneration *in vivo*, although the mechanism is as yet unknown.

Finally, it should be noted that the localized concentration of C5a in the spinal cord after injection of 100 nM C5a into the peritoneum was much lower than the *in-vitro* concentrations. Why did it still work? The answer may involve the positive feedback between C5a and inflammation *in vivo*. On one hand, uncontrolled complement activation can lead to excessive inflammation, tissue damage and elevation of many cytokines [31]. On the other hand, particularly after stimulation with cytokines, glial cells and neurones can produce almost all the complement proteins (including C5) [32], and activation and over-expression of C5a can be detected during inflammatory processes [33]. Exogenous administration of C5a will promote this feedback and generate more endogenic C5a. Therefore, the C5a that really played a role *in vivo* might come from two sources: exogenous administration and endogenic generation.

Conclusion

C5a has been thought to be a chemotactic factor that participates in inflammation and results in the secondary injury after SCI. However, our study indicated that a certain concentration of C5a administrated 24 h after injury could reduce neurone apoptosis, promote neurite outgrowth and improve functional recovery after SCI. These data suggest that C5a may have opposite functions in a time- and concentration-dependent manner after SCI. The dual roles of C5a have to be taken into account when measures to inhibit complement activation are taken in order to promote regeneration after SCI.

Acknowledgements

This study was based on the original idea of Qiang Guo and Cheng Zhong. Qiang Guo and Junlin Cheng contributed equally to this work. Qiang Guo and Junlin Cheng carried out the spinal cord injury, C5a injection, locomotor function analysis and neurone culture studies. Jiqiang Zhang carried out the histology analysis. Bingyin Su drafted the manuscript and Jiqiang Zhang proofread the manuscript. Chen Bian and Sen Lin carried out the data analysis. Qiang

Guo and Cheng Zhong were responsible for supervising the experiments, data analyses and writing of the manuscript. All authors read and approved the final manuscript. This work was supported by the National Natural Science Foundation of China (No. 30900572).

Disclosure

The authors have no conflicts of interest to declare.

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