Complement Receptor 2 is increased in cerebrospinal fluid of multiple sclerosis patients and regulates C3 function

Rickard P.F. Lindblom, Shahin Aeinehband, Mikael Ström, Faiez Al Nimer, Kerstin Sandholm, Mohsen Khademi, Bo Nilsson, Fredrik Piehl, Kristina N. Ekdahl

Article history:
Received 23 March 2016
accepted with revision 8 April 2016
Available online 13 April 2016

Abstract

Besides its vital role in immunity, the complement system also contributes to the shaping of the synaptic circuitry of the brain. We recently described that soluble Complement Receptor 2 (sCR2) is part of the nerve injury response in rodents. We here study CR2 in context of multiple sclerosis (MS) and explore the molecular effects of CR2 on C3 activation.

Significant increases in sCR2 levels were evident in cerebrospinal fluid (CSF) from both patients with relapsing-remitting MS (n = 33; 6.2 ng/mL) and secondary-progressive MS (n = 9; 7.0 ng/mL) as compared to controls (n = 18; 4.1 ng/mL). Furthermore, CSF sCR2 levels correlated significantly both with CSF C3 and C1q as well as to a disease severity measure. In vitro, sCR2 inhibited the cleavage and down regulation of C3b to iC3b, suggesting that it exerts a modulatory role in complement activation downstream of C3.

These results propose a novel function for CR2/sCR2 in human neuroinflammatory conditions.

© 2016 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

The complement system, an important part of the innate immune system, is activated in conditions of neuroinflammation where it conveys a range of effects comprising cell-lysis, chemotaxis, opsonization and immune cell stimulation [1,2], but also contributes to tissue damage [3–6]. All these functions result in clearance of debris and foreign materials. The complement system consists of a large number of components, many of which derive from the liver, but where some are also expressed by immune/inflammatory cells such as macrophages, other cells in various epithelium, endothelium, and intrinsic cells of the central nervous system (CNS) such as neurons and glia [7,8]. Complement activation is the result of a cascade of interacting processes, a structure that enables fine-tuning and adaptation, but also introduces multiple levels where activation can be dysregulated [9].

A factor crucial for the dexterity of the complement system is cellular responses mediated by several complement receptors present on a range of cell types including macrophages [10], T- and B-lymphocytes [11,12], microglia and astrocytes [13,14], which are either constitutively or conditionally present in the neurological system. Many of the complement receptors belong to a superprotein family (regulators of complement activation, RCA) that contains the main regulators of complement e.g. factor H and C4BP [9]. Also, many of the receptors exist in both secreted and membrane bound forms, for instance complement receptor 1 (CR1) and 2 (CR2) exist in soluble forms, e.g. sCR1 and sCR2 (also known as sCD21) [15,16]. This has functional implications, since soluble complement receptors can function as inhibitors instead of activators [9], which are applied in complement directed therapies [17].

The RCA proteins can act as regulators by influencing the convertases by either decay acceleration of the convertases and/or by acting as co-factors to factor I, which leads to downregulation of
convertase activity. As co-factors they provide help to factor I to cleave C3b to iC3b and thereafter iC3b to C3dg. C3b is the only fragment that can trigger activation of C5 (and subsequent generation of the membrane attack complex, C5b-9). iC3b works mainly as a ligand to CR3 and CR4, while C3dg is a ligand to CR2.

In the CNS an increasing body of evidence suggests that certain parts of the complement system play important roles for shaping synaptic networks during normal development and ageing, as well as being implicated in different disease processes. For example, transgenic mice lacking either C1q or C3 display aberrant innervation of visual pathways and levels of C1q are greatly increased in both the ageing mouse and human brain and correlate to cognitive decline [18–20]. However, the intricate interplay between different complement components and their interacting partners both during physiological conditions and in different disease states is far from clarified in detail.

We previously found considerable strain-dependent differences in the local expression of several complement components in the spinal cord of nerve-injured rats, and identified distinct regulatory pathways [21,22]. Recently, using the same standardized rat nerve injury model, we also demonstrated strain-dependent differences in the local expression of several complement receptors in the spinal cord [23]. Interestingly, the most conspicuous finding was that of large differences in CR2 both regarding tissue mRNA expression and presence of soluble protein in cerebrospinal fluid (CSF). A possible functional role for CR2 was suggested by a reduced elimination of synaptic connections as a result of axonal injury in mice lacking functional protein. The aim of the current study was to extend these observations to human neuromembrane disease and further characterize the function of CR2 at the molecular level.

2. Material and methods

2.1. Ethics, consent and permissions

The study was approved by the regional ethical committee in Stockholm (ethical permit 2009/2107-31/2) and written informed consent was obtained from all patients.

2.2. Patients and CSF CR2/C3 determinations

CSF samples were collected during routine visits to the neurology clinic at Karolinska University Hospital. Samples were centrifuged immediately after lumbar puncture at 440g for 10 min at room temperature to separate cells from the CSF supernatant. The supernatants were subsequently batched and stored at −80 °C until use. Patients were subdivided into relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and other neurological disease (OND) controls lacking signs of inflammatory components on magnetic resonance imaging and established markers of immune activation in the CSF (pleocytosis, oligoclonal bands, increased albumin quotient and/or increased IgG index). The OND controls were; psychosis n = 6; functional

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographics and clinical characteristics of the patient cohort.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnose</td>
<td>Sex (% females)</td>
</tr>
<tr>
<td>OND</td>
<td>18</td>
</tr>
<tr>
<td>RRMS</td>
<td>33</td>
</tr>
<tr>
<td>SPMS</td>
<td>9</td>
</tr>
<tr>
<td>All MS</td>
<td>42</td>
</tr>
</tbody>
</table>

RRMS, relapsing-remitting Multiple Sclerosis; SPMS, secondary progressive MS; OND, other non-inflammatory neurological/psychiatric conditions; EDSS, Expanded Disability Status Scale; MSSS, MS Severity Score.
2.4. Statistical analysis

The software program R 2.6.0 was used to carry out statistical analyses and create all graphs. One-way ANOVA calculated with GraphPad Prism 5.0 (San Diego, CA) were carried out on protein data, results are represented as mean ± SEM. Correlations between protein levels in clinical samples, were calculated using Pearson’s algorithm assuming equal distribution, and visualized graphically using linear regression plots, also in GraphPad Prism 5.0. p < 0.05 was considered statistically significant.

3. Results

3.1. CR2 exists in a soluble form in the intrathecal compartment

In order to replicate our previous finding from rats, we determined levels of sCR2 in CSF from 33 RRMS patients, 9 SPMS patients and a control group consisting of patients with non-inflammatory neurological/psychiatric diseases (OND). This demonstrated significantly increased levels of sCR2 in patients compared to controls (All MS: 6.40 ± 0.33, OND: 4.12 ± 0.37) (A). The levels of C1q were also increased in the MS group (329.4 ± 11.4 μg/L) compared to controls (251.6 ± 9.8 μg/L) (B). CSF levels of sCR2 correlated with C3, suggesting that increased it may serve a counter-regulatory role in situations where C3 activation is increased (C). The levels of sCR2 also correlated with C1q (D).

Furthermore, we found that sCR2 levels displayed a strong positive correlation to both C3 (Fig. 1C) and C1q (Fig. 1D). sCR2 also correlated with albumin quotient (Fig. 2A, Table 2). In contrast, there was no significant correlation between sCR2 and NFL (Fig. 2B, Table 2), a commonly used surrogate marker of ongoing neuroaxonal degeneration in MS [30]. When correlating the sCR2 levels with the clinical parameters at time of sampling we saw a significant correlation between Multiple Sclerosis Severity Score (MSSS), a measure of disease severity, and sCR2 (Fig. 3A, Table 1). The pattern was similar, but not significant for sCR2 and Extended Disability Status Scale (EDSS), a measure of neurological disability, (Fig. 3B, Table 1). However, when stratifying into the group with higher EDSS (4 and above) the levels of sCR2 were significantly higher in the group with higher EDSS (Fig. 3C).

3.2. CR2 correlates with levels of C3 as well as to Multiple Sclerosis Severity Score

CR2 inhibits generation of iC3b by interfering with factor H

C3b is cleaved by the plasma protease fl in three positions. The first two cleavages give rise to iC3b and a third subsequent cleavage to C3d,g [31]. The cleavage process occurs only in the presence of a co-factor binding to C3b and thereby causing a conformational change, which makes the cleavage sites accessible for fl. The co-factor activity can be measured by the relative reduction in the band corresponding to the 101 kDa α-chain of C3b and the corresponding increase of the cleaved product, the 67 kDa band of iC3b. The third cleavage will reduce...
Thus, in 2007 Stevens and co-workers reported that mice lacking C1q regulatory functions on the shaping of neuronal networks is of recent date. Elimination of synaptic elements in the injured area of transgenic mice lacking functional CR1/2, which are transcribed from the same gene in the spinal cord of rats subjected to a standardized nerve injury, which resulted in decreased degradation of C3b, as well as smaller break-down products. As a subsequent step we studied the effect of CR2 in the presence of both fl and fH. Interestingly, increasing concentrations of CR2 now resulted in almost total disappearance of the 101 kDa band and appearance of a strong 67 kDa iC3b band, as well as smaller break-down products.

As a subsequent step we studied the effect of CR2 in the presence of both fl and fH. Interestingly, increasing concentrations of CR2 now resulted in almost total disappearance of the 101 kDa band and appearance of a strong 67 kDa iC3b band, as well as smaller break-down products

4. Discussion

We here demonstrate that sCR2 is detectable in human CSF and that levels are upregulated in patients with MS compared to non-inflammatory controls. At the molecular levels we find evidence that CR2 regulates the function of C3. These findings extend our previous observations of a strong genetic influence on the local expression of CR2 in the spinal cord of rats subjected to a standardized nerve injury, which also was reflected in the levels of sCR2 in CSF [23]. Furthermore, a functional role for CR2 was suggested by the observation of an increased elimination of synaptic elements in the injured area of transgenic mice lacking functional CR1/2, which are transcribed from the same gene in mice but not rats or humans, compared to wild type [23].

The notion that the complement system may exert important regulatory functions on the shaping of neuronal networks is of recent date. Thus, in 2007 Stevens and co-workers reported that mice lacking C1q or C3 displayed defective developmental elimination of synaptic connections in the visual pathway [18]. Subsequent studies have revealed that a dynamic interplay between neurons and microglia mediated by immune factors such as C1q and C3 play an important role for the shaping and homeostatic plasticity of the brain synaptic circuitry [19]. Thus, microglia have been implicated in the removal of synapses occurring after CNS injury, where complement is known to increase their phagocytic properties through receptors such as CR3 (CD11b/CD18), the expression of which characterizes microglia with phagocytic potential [19,32]. The relevance of C3-CR3 communication is supported by the finding that mice deficient for CR3 display reduced loss of synapses during development of the visual system, i.e. a phenotype that is similar to that of mice lacking C3 [18,19].

Complement mediated synaptic plasticity is not limited to physiological functions during normal development, but may also constitute an important biological substrate for chronic neurodegenerative diseases, since failure to regulate the system may lead to excessive loss of synaptic connectivity, in turn representing an early disease related phenomenon in models of neuroinflammation and neurodegeneration [33,34]. Signs of complement activation are readily evident in neurodegenerative diseases such as Alzheimer’s (AD) and Parkinson’s diseases, but also in MS [5,35]. An important question is if dysregulation of the complement system merely reflects downstream effects of an inherent neurodegenerative process. However, the fact that genetic variability in both Clusterin and CR1 genes are associated to risk of late-onset AD [36,37] suggests that this is not the case, but rather that excessive activation may indeed trigger or exaggerate neurodegenerative processes.

CR2 has mostly been studied in context of B cell and follicular dendritic cell immunology, where CR2 forms a co-receptor complex together with CD19 and CD81, and CR2 binds opsonized C3d,g and antigen-bound IgM that in turn results in a more efficient humoral immune response [38]. In contrast, any possible function of CR2 in the CNS has received little attention, even if expression of CR2 by activated astrocytes has been reported [14]. More recently CR2 has also been shown to regulate neurogenesis in the mouse [39]. In another recent study CR1/CR2

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>sCR2 average (SEM)</th>
<th>C3 average (SEM)</th>
<th>C1q average (SEM)</th>
<th>NFL average (SEM)</th>
<th>Albumin quotient (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OND</td>
<td>18</td>
<td>4.12 (0.37)</td>
<td>1853 (170.8)</td>
<td>251.6 (9.8)</td>
<td>233.2 (26.85)</td>
<td>4.53 (0.51)</td>
</tr>
<tr>
<td>RRMS</td>
<td>33</td>
<td>6.24 (0.36)</td>
<td>2320 (142.9)</td>
<td>318.7 (13.2)</td>
<td>2019 (334.0)</td>
<td>4.84 (0.36)</td>
</tr>
<tr>
<td>SPMS</td>
<td>9</td>
<td>6.96 (0.81)</td>
<td>3148 (313.1)</td>
<td>368.5 (16.9)</td>
<td>1378 (457.6)</td>
<td>6.50 (1.22)</td>
</tr>
<tr>
<td>All MS</td>
<td>42</td>
<td>6.40 (0.33)</td>
<td>2498 (139.4)</td>
<td>329.4 (11.4)</td>
<td>1882 (280.8)</td>
<td>5.18 (0.39)</td>
</tr>
</tbody>
</table>

RRMS, relapsing-remitting Multiple Sclerosis; SPMS, secondary progressive MS; OND, other non-inflammatory neurological/psychiatric conditions. The normal reference value for the CSF albumin quotient is <7.
The authors also reported that the deposition of C3 in the brain tissue was reduced in animals lacking CR1/CR2. The fact that both receptors are transcribed from the same gene in mice, however, makes extrapolations to human conditions uncertain. Still, the findings provide some support for a functional role for CR2 in the response to nerve injury.

The existence mainly of a soluble form of CR2 in the CNS could explain why immunohistochemical detection has proven difficult [39]. In fact, while tissue stainings were weak in the rat, scR2 was readily detectable in CSF and levels increased following a standardized nerve injury leading to localized inflammatory activation in the spinal cord, but with very little influx of blood derived immune cells [23]. Possibly increased scR2 may not only be regulated at the transcriptional levels, but can also be the result of increased shedding of the CR2 ectodomain due to oxidative stress [41,42]. We here demonstrate the presence of scR2 also in human CSF and that levels increase in conditions of inflammation. Interestingly, decreased serum scR2 levels have been reported in MS [43], alike multiple other autoimmune diseases [44–46]. If this is due to reduced production or increased consumption during autoimmune inflammation is not known.

We also found a significant correlation between CSF C1q/C3 and scR2 levels. The role of complement in MS has received increasing attention, with a suggested role in the neurodegenerative processes [47]. For example, certain types of MS lesions are characterized by prominent complement activation [48], and elevated complement levels have been demonstrated in serum of MS patients [35,49], as well as in the CSF in protein profiling studies [45,50]. In addition, we previously reported that C3 levels in CSF of MS patients correlate with degree of neurological disability as well as CSF levels of NFL [27]. C3 is a large and complex molecule, with multiple active breakdown products. Therefore, elevated scR2 could reflect an intrinsic regulatory mechanism in the CNS, where up regulation and/or increased shedding of CR2 serves to modulate increased C3 activity. In fact, we here found molecular evidence supporting this notion, since CR2 in vitro inhibited cleavage of C3b into iC3b. This is likely of importance, since C3b (in contrast to iC3b) is able to amplify the alternative pathway amplification loop leading to deposition of C3b at sites of inflammation and to trigger C5 activation and C5a/C5b-9 associated damage and inflammation, i.e., anaphylaxis and cell lysis. Reduced generation of iC3b will impair the clearance of debris from the inflammatory site, since this fragment is the primary ligand for CR3 (CD11b/CD18) [51], whereas C3b is not bound by CR3 [52] but instead by CR1 [53]. Thus, it may be speculated that increased levels of scR2 in context of nerve injury limit iC3b generation, in turn reducing iC3b-CR3 mediated microglial synaptic removal [19]. This hypothesis is interesting in the light of previous postulations of the neurodegenerative disease progression, where loss of synaptic connectivity constitutes an early process [33,34].

**Fig. 3.** Correlations between sCR2 and clinical parameters in MS patients. The levels of soluble CR2 (sCR2) at the time of sampling were correlated with the two most frequently used clinical scoring scales in MS; Multiple Sclerosis Severity Score (MSSS) and Extended Disability Status Scale (EDSS). sCR2 levels correlated with MSSS (A) but not with EDSS (B). However, when stratified into high and low EDSS, the high EDSS group had increased sCR2 levels (C). n = 18 OND; 33 RRMS; 9 SPMS patients respectively. The results are represented as mean ± SEM.
5. Conclusions

We here find that M5 patients display elevated CSF levels of sCR2, which correlate both with C3 and C1q, but less well with biomarkers of nerve injury. We also provide novel evidence that sCR2 impairs the cleavage of C3b into active metabolites in vitro suggesting that it has a modulatory function in situations of complement activation. Further work is needed to explore if CR2/sCR2 treatment is feasible and beneficial in conditions characterized by loss of nerve terminals and dysregulated expression of complement, for example chronic neurodegenerative disorders.

Competing interests

None of the authors has any potential financial conflict of interest related to this manuscript.

Acknowledgements

This work was supported by the 6th Framework Program of the European Union, NeuproMoSe. ISHM-CT-2005-018637, by the Swedish Research Council, the King Gustaf V’s 80-years foundation, Dr. Åke Olssons Stiftelse for utbildning, faculty grants from the Linnaeus University and the Swedish Association of Persons with Neurological Disabilities. The funders had no role in study design, data collection and analysis, preparation of the manuscript or decision to publish.

References


