Soluble CD163 as a Marker of Macrophage Activity in Newly Diagnosed Patients with Multiple Sclerosis

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Abstract

Background: Soluble CD163 (sCD163) is a macrophage specific protein known to be up-regulated in serum from patients with multiple sclerosis (MS).

Objective: To investigate sCD163 in serum and CSF (cerebrospinal fluid) from patients undergoing MS diagnostic work-up and analyse its potential as a diagnostic biomarker.

Methods: After a full MS diagnostic work-up, including collection of paired samples of CSF and serum, 183 patients were evaluated for inclusion in this study. Patients were divided into groups based on their diagnosis. Patients with normal clinical and paraclinical findings were grouped as symptomatic controls. Serum and CSF levels of sCD163 were determined by enzyme-linked immunosorbent assay (ELISA).

Results: sCD163 could be measured in all serum and CSF samples. A high sCD163 CSF/serum ratio in relation to molecular weight was found, strongly indicating local production in the CNS. Median levels of sCD163 were significantly decreased in serum and significantly elevated in CSF in patients with relapsing-remitting, and primary- progressive MS. There were, however, some overlaps of the measures between groups. In a receiver operating characteristic (ROC) analysis sCD163 CSF/serum ratio had an area under the curve of 0.72.

Conclusion: The sCD163 CSF/serum ratio was significantly increased in patients with MS and may reflect macrophage activation in MS lesions. These results suggest that primary progressive MS also is driven by inflammation in which the innate immune system plays a pivotal role.

Introduction

Multiple sclerosis (MS) is the most common, debilitating immune-mediated disease of the central nervous system in young adults. The cause of the disease is unknown, yet it is generally assumed to be a complex interaction of genetic susceptibility and environmental factors [1–3]. MS pathology is characterised by lesions in the central nervous system. These MS lesions, plaques, are infiltrated by immune cells and constitute foci of distinct patterns of demyelination, inflammation, axonal damage, and gliosis [4,3]. The underlying mechanism is currently thought to be an autoimmune dysfunction involving both the adaptive and the innate immune system [6,7].

Macrophages are abundantly present in inflammatory MS lesions [4]. Macrophages are tissue specific monocytes with versatile roles including phagocytosis, antigen presentation and lymphocyte stimulation. They orchestrate the inflammatory response in a balanced manner with flexible phenotypic responses to local environmental signals. It has also been demonstrated that macrophages in both stages of activation (pro-inflammatory (M1), and anti-inflammatory and growth promoting (M2)) are present in active MS lesions [8,9].

CD163 is a receptor for haptoglobin - hemoglobin complexes and a monocyte/macrophage specific membrane protein. It is found on macrophages in the CNS, including perivascular macrophages (PVMs) and microglia [10]. Membrane bound CD163 (mCD163) is considered a marker for the M2 pathway, although mCD163 is also up-regulated in chronically inflamed tissues [11–13].

CD163 can be found in a soluble form (sCD163) in plasma and other body fluids such as cerebrospinal fluid (CSF) [14]. In the CNS, sCD163 is probably shed by macrophages and microglia, triggered by complex immune modulating mechanisms in the microenvironment. In a study of 129 random blood samples, sCD163 has been shown to be inversely related to the plasma levels of sCD163 [15] and in another study proteolytic shedding of
CD163 has been shown to be increased in serum of MS patients [16].
sCD163 can be measured in serum by enzyme linked immuno-
sorbent assay (ELISA) [17], and changes in sCD163 has been associated with a number of inflammatory disorders, as recently reviewed [18].

In this study we aimed to investigate whether sCD163 is measurable in CSF and serum of newly diagnosed MS patients. We wanted to estimate the intrathecal production of sCD163 and investigate putative diagnostic qualities of sCD163 in a large cohort of patients with newly diagnosed remitting relapsing MS (RRMS), primary progressive MS (PPMS), secondary progressive MS (SPMS), clinically isolated syndrome (CIS), and compare with a symptomatic control (SC) group [19].

Materials and Methods

Ethics statement

The study was conducted in accordance with the Ethical Declaration of Helsinki and all patients gave written, informed consent as approved by The Central Denmark Region Committee on Biomedical Research Ethics (journal number: 20090210)

Patients

From September 2009 to December 2011 patients admitted for further diagnostic investigations to the MS clinic, at the Department of Neurology, Aarhus University Hospital, were consecutively included in the study. Diagnostic procedures consisted of anamnesis, clinical examination, MRI of the entire neural axis, CSF analyses (cells, protein, immunoglobulin G (IgG) index), and evoked potentials as recommended [20]. EDSS (extended disability status scale) was assessed according to Kurtzke [21]. MRI and cerebrospinal fluid examination were evaluated according to the latest classification of MS [20].

Patient demographics and clinical data are presented in Table 1. A total of 183 patients agreed to participate and serum and CSF samples were collected and frozen at -70°C for later examination in accordance with consensus guidelines [22].

The inclusion criteria were met by 130 patients and the inclusion and exclusion of patients are illustrated in Figure 1. Patients were excluded if they had other neurologic disease, a medical disease, had been undergoing immune modulating treatment within the month preceding sampling, had paraclinical isolated syndromes, or if data were missing. Patients included in the study cohort were divided into five subgroups: RRMS (n = 45), PPMS (n = 15), SPMS (n = 4), CIS (n = 27) and, SC (n = 39) with either normal or unspecific MRI. For further details on exclusion please see Data S1.

The diagnoses were established before the results of sCD163 analysis were received.

ELISA for sCD163 measurement in serum and CSF

sCD163 was measured by ELISA essentially as described [17]. Briefly, rabbit anti-CD163 (2 mg/L, SK Moestrup Aarhus University) was coated onto microtitre wells. Serum samples (diluted 1:101) or CSF samples (diluted 1:4) was added in duplicates and incubated for 1 h at RT. Monoclonal anti-CD163 (clone number: GHI/61,3 μg/mL) was added followed by incubation for 1 h at RT with horseradish peroxidase-labelled goat anti-mouse antibodies (0.125 μg/mL, Dako, Glostrup, Denmark). The assay was calibrated using serum traceable to purified human CD163, with the lowest calibrator being 6.25 μg/L.

Validation of ELISA for CSF measurements

In a pilot experiment, results were compared with a commercial sCD163 assay (Macro163TM, IQ-products Groningen NL), showing good correlation, however with a bias due to lack of assay standardisation as described earlier for serum samples [18]. The intra-assay precision was determined in two pools of cerebrospinal fluid with low and high sCD163 concentrations. The intra-assay precision (coefficient of variation) was 3.0% at a level of 0.123 mg/L (n = 24) and 7.9% at a level of 0.073 mg/L (n = 24). The total imprecision was determined from diluted serum-control samples included in each plate and ranged from 5.3–12.9% (n = 15). Linearity was confirmed by measuring a diluted (2, 4, 8, and 16 times) pool of CSF in quadruplicate. Aliquots of pools of samples were kept at -80°C and showed stable concentrations for >1 year.

Results

Measurement of sCD163 by ELISA

Soluble sCD163 was detectable in CSF and serum in all individuals (Figure 2a and 2b, Table 2). For the SC group the median level of sCD163 in serum was 1.70 mg/L (range 0.97–3.73 mg/L). This corresponds well with the sCD163 serum reference interval for healthy subjects (0.7–3.9 mg/L) [18]. The median level of sCD163 in CSF for the SC group was 0.076 mg/L, as compared with 1.70 mg/L in serum, yielding a median CSF/serum ratio of 0.041. We calculated that ~87% of sCD163 CSF, in the SC group, was produced intratheically. The sCD163 CSF/serum ratio and sCD163 Index values are shown in Figure 2c and 2d. Figures 2a-2d show some overlaps in levels between patient groups, and symptomatic controls.
Correlation analysis of sCD163 measurements and demographic data

Patients in the PPMS and SPMS groups were significantly older than patients in the RRMS, CIS and SC groups; and levels of sCD163 in both serum and CSF were found to correlate with age. Consequently, all regression analyses were performed on data adjusted for age as described in the statistical analysis section.

The levels of sCD163 in serum and CSF were not correlated to gender or any of the clinical measures: time since last attack, EDSS, mean no. of relapses, mean disease duration, enhancing lesions or the total number of MRI white matter lesions (TNL). TNL and EDSS were correlated to group as patients with CIS had fewer lesions and lower EDSS than patients with RRMS and PPMS.

Age adjusted linear regression

Results from the age adjusted linear regression analysis are presented in Table 3 and in the Data S1. Interestingly, the age adjusted geometric means (GM) of sCD163 in CSF were significantly elevated for patients in the RRMS (p = 0.026), PPMS (p = 0.011), and CIS (p = 0.014) groups compared with the SC group. In serum, the GM levels of sCD163 were significantly lower in the RRMS (p = 0.006) and PPMS (p = 0.008) groups compared with the SC group. The GM values of the sCD163 CSF/serum ratio was significantly increased in RRMS (p < 0.001), CIS (p = 0.011), and especially the PPMS group in which the GM value was elevated by 56% (p < 0.001). The GM value of sCD163 index was significantly elevated by 41% (p = 0.022) in the PPMS group.
group compared with the SC group. Due to the small number of patients with SPMS in the study cohort, this group was omitted from the figures 2a–2d and also from the linear regression analysis.

Receiver Operating Curve analysis of sCD163 diagnostic properties

Results of ROC analyses of sCD163 CSF, sCD163 serum, sCD163 CSF/serum ratio, and sCD163 index are shown in
Figure 3a–3e. Also, this figure displays a small table with area under curve (AUC) and corresponding 95% Confident Interval (CI). As a diagnostic marker the sCD163-ratio performed better than measurement in CSF alone (AUC 0.72 (95% CI: 0.63–0.81) vs. AUC 0.69 (95% CI: 0.60–0.78)). However, the IgG index was superior as a diagnostic marker to sCD163 CSF, sCD163 serum, sCD163 CSF/serum ratio, and sCD163 Index measurements with an AUC of 0.91 (95% CI: 0.86–0.95).

Discussion

Soluble CD163 could be measured in serum and CSF in all persons of the study cohort.

The intrathecal synthesis of sCD163 in the SC group of this study was more than 80% and this is in agreement with results from investigations in healthy controls [14] and places sCD163 with other molecules produced in CNS, that show a higher concentration than would be expected from the molecular weight [24]. The intrathecal production may originate from activated perivascular macrophages and microglia known to express CD163 in MS. The function of sCD163 in the CNS is unknown. A number of inflammatory stimuli, such as oxidative stress and microbial toxins are known to mediate sCD163 shedding, which in turn may modulate macrophage activation. sCD163 has also been shown directly to inhibit T-cell activation [25,26].

In patients with a demyelinating disease in the CNS we found significantly elevated levels of sCD163 in the CSF. This is in agreement with neuropathological investigations of active lesions in patients with RRMS. In these active lesions macrophages were densely present and could also be seen in chronically, active lesions [27]. In chronic inflammatory lesions, macrophages are probably activated by complex mechanisms involving both pro- and anti-inflammatory stimuli. Increased mCD163 is considered to be an anti-inflammatory response to prolonged inflammation in diseases such as macrophage activation syndrome, sepsis, and liver disease [16,28–31].

A small group of patients in the present patient cohort were diagnosed with SPMS. They were diagnosed a long time after their first relapse (15 to 38 years, see Table 1) and had slow progression of symptoms. Essentially, they represent the benign course of MS. These patients are not representative for patients with SPMS in general and were omitted from figure 2 and the linear regression analysis.

Table 2. Median levels of sCD163 in serum and CSF samples as determined by ELISA.

| Characteristics | RRMS | PPMS | SPMS | CIS | SC |
|-----------------|------|------|------|-----|----|
| No. of Subjects (total = 130) | n = 45 | n = 15 | n = 4 | n = 27 | n = 39 |
| Serum sCD163 mg/L (range) | 1.49 | 1.58 | 2.0 | 1.62 | 1.70 |
| CSF sCD163 mg/L (range) | 0.084 | 0.089 | 0.097 | 0.081 | 0.076 |
| sCD163 CSF/serum ratio (range) | 0.054 | 0.062 | 0.048 | 0.057 | 0.041 |
| sCD163 index | 11.2 | 13.6 | 7.7 | 11.9 | 9.4 |

Table 2 shows the median levels of sCD163 in serum, CSF and, sCD163 CSF/serum ratio, and sCD163 index. Abbreviations: RRMS (relapsing-remitting MS), PPMS (primary-progressive MS), CIS (clinically isolated syndrome), SC (symptomatic controls), n (number of persons), CSF (cerebrospinal fluid).

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Table 3. Results from linear regression analyses of differences between patients with MS/CIS and SC.

| Characteristics | RRMS | PPMS | CIS | SC |
|-----------------|------|------|-----|----|
| No. of Subjects (total = 130) | n = 45 | n = 15 | n = 27 | n = 39 |
| Serum sCD163, difference in percentages from SC group (p-values) | −19% | −22% | −11% | 1.49 |
| CSF sCD163, difference in percentages from SC group (p-values) | 12% | 22% | 19% | 0.059 |
| sCD163 ratio, difference in percentages from SC group (p-values) | 38% | 56% | 21% | 0.040 |
| sCD163 Index, difference in percentages from SC group (p-values) | 20% | 41% | 21% | 11.18 |

Table 3 shows the results of linear regression on four different parameters: serum sCD163, CSF sCD163, sCD163 CSF/serum ratio, and sCD163 index. The regression analyses are done on log transformed, age adjusted data. Differences are shown in percentages for RRMS, PPMS, and CIS compared with the SC group. The level of significance is shown by the p-value and * marks a significant difference between groups. The SC column shows the age corrected geometric means for the SC group used in the regression analysis. Abbreviations: RRMS (relapsing-remitting MS), PPMS (primary-progressive MS), CIS (clinically isolated syndrome), SC (symptomatic controls), n (number of persons), CSF (cerebrospinal fluid).

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In serum we found that sCD163 levels were significantly decreased when comparing patients with RRMS and PPMS with patients in the SC group. In a study by Fabriek et al sCD163 was shown to be up-regulated in MS patients when compared to healthy controls [16]. One explanation for the discrepancy is likely to be differences in the patient cohorts. The patients in the present study were newly diagnosed with MS, they had lower EDSS, and they were younger. Additionally, patients who had been treated with methylprednisolone, or other immunomodulating agents were excluded from the present study. Finally, our control group consisted of symptomatic controls, not healthy controls, and an explanation for the unspecific symptoms in these individuals could be a general increased inflammatory activity.

In progressive courses of MS, absence of infiltration of inflammatory cells has been reported in several studies [8,9,32]. However, recently studies have shown that inflammation and neurodegeneration can be found in all lesions and stages of MS [33] and additionally, inflammatory biomarkers can be measured in the CSF from patients with progressive MS [34]. This is in agreement with our present findings of macrophage activity in PPMS similar to that of patients with a relapsing course of MS. Actually, both sCD163 CSF/serum ratio and index were high in PPMS in agreement with pathological investigations demonstrating continuous inflammation-mediated tissue injury [6]. It has also recently been shown that activated macrophages and microglia in active lesions express CD40 (M1 marker) while mannose receptor (MR) and CD163 (M2 markers) are expressed by myelin-laden macrophages in active lesions and perivascular macrophages. Double staining showed that approximately 70% of the CD40-positive macrophages in MS lesions also expressed MR, indicating that the majority of infiltrating macrophages and activated microglial cells display an intermediate activation status [9,12]. It could therefore be argued, that high levels of sCD163 in the CSF should be associated with high disability score as is indeed substantiated in the present study where there is a trend for a declivity in sCD163 levels with higher levels in PPMS, intermediate levels in RRMS and lower levels in CIS.

sCD163 is especially elevated in the patients with PPMS. This is particularly interesting since sCD163 may originate from M2 macrophages, notably involved in chronic inflammation. However, several factors could potentially contribute to the increased sCD163 levels in MS. It could be a consequence of an anti-inflammatory response, and mCD163 is strongly upregulated by IL-10 and glucocorticoid. It has been shown that CD163 macrophages are most abundant in acute active lesions and markedly decrease in number in chronic active lesions and further to chronic inactive lesions [8]. Furthermore, sCD163 has been shown to actively inhibit T-cell mediated immune reactions in vitro, thus exhibiting anti-inflammatory functions [26]. The close interactions between T cells and MHC class II+ macrophages have also been reported in a study of parenchymal lesions in the spinal cord and meningeal lesions pointing towards a role in diffuse axonal loss [35]. This apposition of T cells and macrophages was seen in patients with progressive disease and could suggest antigen presentation and thus a mechanism for chronic inflammation. Even though the patients in the present study cohort have explicitly not been treated with glucocorticoids, it is possible that the local microenvironment could trigger a
similar reactivity [12,36]. One such trigger could be iron deposition which several studies have shown is increased in the plaques of patients with MS [37,38]. A recent study by Hametner et al [39] has shown an age-related increase of iron in the white matter of MS patients and this study suggests that the degeneration of cells in MS lesions leads to iron liberation from oligodendrocytes. To our knowledge there is no study of iron deposition related to CD163 expression in MS lesions but we suggest that a relationship is likely and that CSF sCD163 could be marker for this relationship. The PPMS patients have the highest mean age in our cohort and this corresponds well with the age-dependent increase of iron release in the white matter seen in the above mentioned study [39]. The elevated levels of sCD163 could be a mechanism of tissue homeostasis and repair and thus CD163 could be a marker of immune modulatory functions regarding not only degeneration and anti-inflammation but also tissue repair [40,41].

The potential role of sCD163 in MS is presently unclear. sCD163 in CSF and serum was not correlated significantly to EDSS, duration of disease, time since last attack or number of attacks, but the sCD163 ratio was significantly increased in patients with MS/CIS. The membrane bound form of CD163 is up-regulated in MS lesions, and, as sCD163 is seen to be shed in other inflammatory conditions, we find that our results are highly suggestive of increased macrophage activation in patients with MS disease, particularly in patients with PPMS. These findings suggest that PPMS pathology is at least partly driven by inflammation. Previous studies [42,43] have shown that MS immune-modulating treatment, to some extent, have an effect on monocytes. Whether the level of sCD163 is affected by immune-modulating treatment and thus could function as a longitudinal disease activity marker for treatment efficacy has yet to be addressed.

The ROC analysis indicated the sCD163 CSF/serum ratio as only a fair diagnostic marker [44], and adding the results from sCD163 to that of IgG index (not shown) did not add further to the diagnostic properties of the IgG index.

In conclusion, soluble CD163 was measurable in CSF and serum from patients both with and without neurological deficits, and the sCD163 CSF/serum ratio was significantly increased in patients with inflammatory demyelinating disease. However, overlap of the levels of sCD163 between the groups and a ROC analysis that indicated sCD163 as only a fair marker makes the diagnostic utility of sCD163 limited. The sCD163 CSF/serum ratio was significantly increased in all groups of patients with MS and may reflect macrophage activation in MS lesions. These results suggest that primary progressive MS also is driven by inflammation in which the innate immune system plays a pivotal role.

Supporting Information

Data S1  File contains: Table S1 Table S1 includes Excel file and (double click to activate) contains all basic data for this paper. Abbreviations: RRMS (relapsing-remitting MS), PPMS (primary-progressive MS), SPMS (secondary-progressive MS), CIS (clini-
cally isolated syndrome), SC (symptomatic controls with normal or abnormal MRI), n (number of persons), CSF (cerebrospinal fluid), y (years), d (days), Gender (1 = male; 2 = female), OND (other neurological disease), OMD (other medical disease).

Table S2 Table S2 lists proteins, their respective molecular weight and ratio CSF/serum. The table is adapted from Nockher et al [22]. Abbreviations: kDA (kilo Dalton). Table S3 Table S3 shows the output of our regression analysis on log transformed sCD163 serum values ([Serum] Table S4 Table S4 shows the output of our regression analysis on log transformed sCD163 CSF values (lcsv). Table S5 Table S5 shows the output of our regression analysis on log transformed sCD163 CSF/serum values (lRatio). Table S6 Table S6 shows the output of our regression analysis on log transformed sCD163 index values (lIndex) Figure S1 Figure S1 shows the distribution of proteins and their CSF/serum ratios plotted against their molecular weight (in kD). This figure is adapted from Nockher et al [22]. Proteins marked with (α) originate solely from the blood compartment (see appendix table 2 below) and proteins marked by (α) are suspected to be produced intracellularly.

(OCX)

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Author Contributions

Conceived and designed the experiments: MS AKR TC HJM PVR TP. Performed the experiments: AKR MS HJM. Analyzed the data: MS HJM TP. Contributed reagents/materials/analysis tools: MS AKR HJM TP. Wrote the paper: MS AKR TC HJM PVR TP.

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