Decreased hydrocortisone sensitivity of T cell function in multiple sclerosis-associated major depression

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Received 27 October 2011; received in revised form 1 March 2012; accepted 1 March 2012

Summary Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the CNS with a high prevalence of depression. Both MS and depression have been linked to elevated cortisol levels and inflammation, indicating disturbed endocrine-immune regulation. An imbalance in mineralocorticoid versus glucocorticoid signaling in the CNS has been proposed as a pathogenetic mechanism of depression. Intriguingly, both receptors are also expressed in lymphocytes, but their role for 'escape' of the immune system from endocrine control is unknown. Using steroid sensitivity of T cell function as a read-out system, we here investigate a potential role of mineralocorticoid receptor (MR) versus glucocorticoid receptor (GR) regulation in the immune system as a biological mechanism underlying MS-associated major depression. Twelve female MS patients meeting diagnostic criteria for current major depressive disorder (MDD) were compared to twelve carefully matched MS patients without depression. We performed lymphocyte phenotyping by flow cytometry. In addition, steroid sensitivity of T cell proliferation was tested using hydrocortisone as well as MR (aldosterone) and GR (dexamethasone) agonists. Sensitivity to hydrocortisone was decreased in T cells from depressed MS patients. Experiments with agonists suggested disturbed MR regulation, but intact GR function. Importantly, there were no differences in lymphocyte composition and frequency of T cell subsets, indicating that the differences in steroid sensitivity are unlikely to be secondary to shifts in the immune compartment. To our knowledge, this study provides first evidence for altered steroid sensitivity of T cells from MS patients with comorbid MDD possibly due to MR dysregulation.

Abbreviations: Aldo, aldosterone; BDI, Beck depression inventory; Cort, hydrocortisone; Dex, dexamethasone; EDSS, expanded disability status scale; GR, glucocorticoid receptor; HSD, hydroxysteroid

dehydrogenase; MDD, major depressive disorder; MR, mineralocorticoid receptor; PHA, phytohemagglutinin; RRMS, relapsing-remitting multiple sclerosis.

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KEYWORDS
Multiple sclerosis; Depression; Cortisol; Steroid resistance; Mineralocorticoid receptor; Hypothalamic— pituitary—adrenal axis

1. Introduction

Multiple sclerosis (MS) is a demyelinating, inflammatory dis- ease of the central nervous system (CNS) with presumed autoimmune origin. In MS, major depressive disorder (MDD) lifetime prevalence rates of up to 50% are commonly found (Siegert and Abernethy, 2005), with 12-month preva- lence as high as 25% (Patten et al., 2003). The biological mechanisms underlying the high prevalence of depression in MS, however, are poorly understood.

One possibility is that depression in MS is directly linked to pathogenetic processes of the disease. Hypothalamic—pitui- tary—adrenal (HPA) axis hyperactivity (Pariante and Light- man, 2008), hippocampal atrophy (Macqueen and Frodl, 2011), and disturbed glucocorticoid (GC) signaling in the immune system (Zunszain et al., 2011) are among the most consistently reproduced biological findings in idiopathic MDD. Intriguingly, HPA axis activation (Heesen et al., 2007), hip- pocampal damage (Aktas and Hartung, 2011), and GC resis- tance in the immune system (L.M. van Winsen et al., 2005; Ysrraelit et al., 2008) are also observed in MS. Furthermore, there is evidence that during the early stages of the disease, neuroendocrine-limbic abnormalities may be specific for MS patients who exhibit depressive symptoms (Fassbender et al., 1998; Gold et al., 2010, 2011; Kern et al., 2011).

The biological effects of cortisol are mainly mediated by two receptors, the high affinity mineralocorticoid receptor (MR) and the low affinity glucocorticoid receptor (GR). An imbalance in these two receptors within the CNS has been proposed as a possible pathogenetic mechanism in mood disorders (de Kloet et al., 2007), although their relative involvement remains a matter of intense debate in the field (de Kloet et al., 2007; Anacker et al., 2011). Intriguingly, both MR and GR are also expressed in human lymphocytes (Armanini et al., 1988). A growing number of preclinical and clinical studies provide evidence for the involvement of immune activation in the pathogenesis of mood disorders (Dantzer et al., 2008). Much of the focus in this area has been on the role of innate immune responses but more recent studies have

provided first evidence that adaptive responses, most prominently T cell dysregulation, may play a key role in depression (Miller, 2010). T cell driven inflammation is a hallmark of MS (Sospedra and Martin, 2005), so that this disease with its high prevalence of depression could serve as a highly informative paradigm to study the role of T cell responses in mood disorders. To date, it is unknown if differ- ential changes in MR/GR sensitivity are linked to steroid resistance in the immune system and how this is related to inflammatory aspects of depression. Here, we investigate T cell regulation by MR and GR agonists as a novel mechanism for immune activation in MS-associated MDD.

2. Materials and methods

2.1. Subjects

Female relapsing-remitting multiple sclerosis (RRMS) patients without depression (n = 12) and female depressive RRMS patients (n = 12) were recruited from the MS outpatient clinic in the University Hospital Hamburg Eppendorf, as described (Gold et al., 2011). They were matched according to Expanded Disability Status Scale Score (EDSS) (Kurtzke, 1983) and age. All patients were diagnosed with RRMS according to Poser et al. (1983). Exclusion criteria were a history of endocrine abnormalities, neoplasms, a relapse during the previous 3 months or treatment with steroids within the last 3 months. All procedures were approved by the Ethics committee of the Medical Board Hamburg and all subjects provided written informed consent prior to enroll- ment in the study.

2.2. Clinical assessments

Patients underwent neurological examination, and disability was assessed using the expanded disability status scale EDSS (Kurtzke, 1983). All patients underwent the Structured Clin- ical Interviews for DSM-IV Axis I disorders administered by a Ph.D. level clinical psychologist (K.J.Z.). Additionally, they completed the self-report questionnaire Beck depression inventory (BDI) (Beck et al., 1961) for quantitative assess- ment of depression severity.

2.3. Flow cytometry

500ml of fresh blood were stained with the following fluorochrome cocktails: (a) Anti-CD3 FITC, anti-CD8 PE, anti-CD45PerCP, anti-CD4 APC; (b) Anti-CD3 FITC, anti- CD16 + CD56 PE, anti-CD45 PerCP, anti-CD19 APC; (c) Anti-CD45RA FITC, anti-HLA-DR PE, anti-CD3 PerCP, anti-CD4 APC; (d) Anti-CD4 APC, anti-CD25 FITC and anti-CD127 PE (BD Pharmigen, San Diego). Stained samples were suspended in FACS buffer and analyzed on a BD FACS- Calibur (Becton Dickinson) flow cytometer. Approximately 15,000 events within the lymphocyte gate were acquired per sample. Samples were analyzed using CellQuest software.

2.4. Proliferation assays

For proliferation assays, peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Hypaque method, aliquoted at a final concentration of 10^7 cells/ml, and cryo- preserved in liquid nitrogen until assayed. All proliferation assays were prepared simultaneously for one subject from each group on the same day. After thawing, PBMCs were seeded on a 96-well plate at a concentration of $2\ 10^6\ ml^{-1}$ [200,000 cells/well] in filtered RPMI-1640 (PAA Laboratories) supplemented with penicillin/streptomycin and 2 mM L-glu- tamine.

To examine steroid receptor function, we used hydrocor- tisone (which binds both MR and GR but has a higher affinity to MR), aldosterone (which selectively binds MR), and dex- amethasone (which predominantly binds GR) (Rupprecht et al., 1993). Cells stimulated with the mitogen phytohe- magglutinin (PHA) (1 mg/ml) served as the individual refer- ence for T cell proliferation. Normalized proliferation of cells stimulated with 1 mg/ml PHA and increasing doses of agonists were then used to estimate steroid sensitivity (see below). The following dose ranges were employed: dexamethasone (10^{-12} M to 10^{-6} M), aldosterone (10^{-11} M to 10^{-5} M), or hydrocortisone (10^{-11} M to 10^{-5} M), based on dexamethasone doses used in previous studies (Ysrraelit et al., 2008). This includes physiological as well as pharmacological doses, and showed sufficient suppression for highly reliable curve fitting for all steroids in preliminary experiments. Cells were incu- bated at 37 8C in 5% CO_2 for 48 h and then pulsed with 1 mCi/ well [methyl-^{3}H]thymidine. After another 24 h of incubation, cells were harvested using a Tomtec Harvester 96 Mach III M. Thymidine incorporation was determined by liquid scintilla- tion counting (Wallac 1450 microbeta, Trilux) and results expressed as counts per minute (cpm). All readings were averaged from triplicate wells.

2.5. Statistical analysis

A sigmoidal curve was used to model the dose response relationship (Prism[1]Software). The model used for the curve fits was $Y = Bottom + (TOP\ Bottom)/(1 + 10^{(X \log IC\text{-}50)})$. The IC-50 represents the data point half way between max- imal (TOP) proliferation in the presence of the agonist (dex- amethasone, aldosterone or hydrocortisone) and maximally inhibited proliferation (Bottom), which should asymptoti- cally equal zero, indicating no further proliferative activity. The two markers used as a measure of steroid sensitivity were IC-50 and TOP values. While the IC-50 values represent a classical measure of steroid receptor sensitivity with higher values indicating greater resistance, TOP values indicate the predicted highest proliferation rates with the respective

agonist present. TOP thus represents a measure of lowest observed agonist-induced anti- proliferative effect and in this way a measure of potential differences in low concentration levels. Curve fits show the decreasing proliferation response of the in vitro PHA-stimulated cells on the ordinate as a function of the logarithmic, increasing molar concentration of the respective agonist on the abscissa. To control for baseline differences, the data were normalized with respect to the positive control condition (PHA stimulated cell pro- liferation without inhibitory steroid present) prior to curve fitting procedure and calculation of IC-50 and TOP values.

All statistical analyses of group differences were con- ducted using PASW Statistics 18.0

software. All variables were tested for distributional Normality. Clinical and demo- graphic descriptors and immune markers were compared between depressed and non-depressed MS patients using independent samples t-test. Welch's t-test was used when- ever Levene's test suggested potential inequality of var- iances ($p < .10$). For all comparisons p-values <.05 were considered significant; p-values <.10 were interpreted as a trend. Due to the relatively small sample size, we also provide estimates of effect size using Cohen's d ($d = (M1\ M2)/H((s1^2 + s2^2)/2)$).

3. Results

The non-depressed MS patients and MS patients with comor- bid depression were well matched for age and EDSS (Table 1). As expected, patients with comorbid MDD displayed much higher BDI scores ($p < .001$). There were no significant group differences in day of menstrual cycle, smoking, body mass index or disease duration (Table 1).

In vitro steroid sensitivity assays revealed group differ- ences in hydrocortisone inhibition of PHA-stimulated T cell proliferation (Fig. 1). RRMS patients with comorbid MDD showed significantly higher proliferation in the presence of hydrocortisone in the low concentration range as repre- sented by TOP values (TOP $p = .022$, $d = 1$, see Table 2), while in higher dose range, reflected by IC-50 values, there was no significant difference. Similar results were observed for the MR-agonist aldosterone, showing a trend towards higher proliferation of T cells from depressed patients in the low concentration range (TOP $p = .084$, $d = .8$, see Table 2). In contrast, no significant differences were seen with the GR agonist dexamethasone. After statistically controlling for

Table 1 Clinical characteristics of non-depressed relapsing-remitting multiple sclerosis (RRMS) patients and RRMS patients with comorbid major depressive disorder (MDD).

Figure 1 Differential steroid regulation of T cell proliferation in multiple sclerosis-associated major depression. Steroid sensitivity of phytohemagglutinin (PHA)-induced Tcell proliferation in relapsing-remitting multiple sclerosis (RRMS) with comorbid major depressive disorder (RRMS + MDD) and matched RRMS patients without depression (RRMS) was measured using [methyl-^{3}H]thymidine incorpo- ration. Graphs show relative proliferation of PHA-stimulated T cells in the presence of increasing doses (given as log molar concentrations) of hydrocortisone (A), dexamethasone (B) and aldosterone (C). Results indicate a lack of hydrocortisone inhibition in the RRMS + MDD group compared to non-depressed MS patients in the low concentration range (A), see also Table 2. While no differences were seen for dexamethasone inhibition (B), regulation by aldosterone (C) showed a trend towards higher normalized proliferation in the low concentration range in depressed MS patients, see also Table 2.

disease-modifying therapy, group comparisons revealed a similar pattern for TOP hydrocortisone ($p = .012$) and TOP aldosterone ($p = .054$). PHA proliferation in the absence of steroid was not significantly different between the groups ($p = .42$, $d = .35$).

Importantly, no group differences could be detected in lymphocyte subset percentages (NK cells,

B cells, T cells, see Table 3). Furthermore, no significant group differences were seen in CD4+ T cell subpopulations, such as percentage of na¨ıve CD4+ T cells (CD45RA+) or regulatory CD4+ T cells (CD127 CD25+).

4. Discussion

To our knowledge, this is the first study to directly assess mineralocorticoid and glucocorticoid signaling within the immune system in the context of depression. We found preliminary evidence for decreased sensitivity to hydrocor- tisone (which has a stronger affinity to MR than GR) in depressed MS patients, as well as a trend pointing into the same direction for the MR agonist aldosterone. However, no significant differences were seen in experiments using the GR agonist dexamethasone. In addition, all patients were assessed during the remission phase of MS and no group differences could be detected in lymphocyte composition or CD4+ T cell subsets. Thus, the differences in steroid sensitivity are unlikely to be secondary to T cell shifts. We believe that therefore our findings reflect a true functional difference in T cells from depressed and non-depressed MS patients and not simply an epiphenomenon of shifts in cir- culating cells with different steroid sensitivity.

Our results suggest that MS-associated depression (com- pared to non-depressed MS patients) is linked to disturbances in MR signaling but intact GR signaling in the immune system. Indirect evidence from our previous studies in MS-depression also points towards MR-dysfunction: We have recently shown that circadian cortisol profiles in depressed MS patients are characterized by elevated nadir levels but normal morning levels compared to non-depressed MS patients as well as healthy controls (Gold et al., 2010, 2011). In humans, the circadian trough of cortisol secretion is largely mediated via the high-affinity mineralocorticoid receptor (MR), while cir- cadian peak levels in the morning are more strongly depen- dent on low-affinity glucocorticoid receptors (GR) (Kellner and Wiedemann, 2008). Intriguingly, these flattened cortisol slopes in MS-depression were significantly associated with smaller volumes in the cornu ammonis (CA) 2—3 and dentate gyrus subfields of the hippocampus (Gold et al., 2010), where MR are highly expressed (Seckl et al., 1991). The indirect evidence for evening cortisol elevations and subregional hippocampal involvement together with the direct evidence

Table 2 Markers of steroid sensitivity of T cell proliferation in non-depressed relapsing-remitting multiple sclerosis (RRMS) patients and RRMS patients with comorbid major depressive disorder (MDD). The inhibitory concentration (IC) 50 values represent levels of the respective agonist necessary to suppress 50% of the phytohemagglutinin (PHA)-induced proliferation. TOP values indicate the pre- dicted highest proliferation rates with low concentrations of the respective agonist present (normalized to the individ- ual's PHA-stimulated proliferation without agonist).

Table 3 Lymphocyte subsets in non-depressed relapsing-remitting multiple sclerosis (RRMS) patients and RRMS patients with comorbid major depressive disorder (MDD).

for MR dysfunction in MS depression presented in this paper suggests that MR dysfunction but intact GR regulation may be linked to MS-associated depression. Whether or not this is a unique feature of MS depression remains to be elucidated in direct comparisons with patients suffering from MDD but not MS as well as with healthy controls.

A non-mutually exclusive explanation to the postulated shift in MR/GR function may be that our results in part reflect changes in pre-receptor steroid regulation. We found group differences for hydrocortisone, which can be inactivated by the enzyme 11b-hydroxysteroid dehydrogenase 2 (11b- HSD2), but not dexamethasone, a weak 11b-HSD substrate (Diederich et al., 2002). The enzymes 11b-HSD1 and 2 play a pivotal role for local, tissue-specific regulation of steroid effects (Tomlinson et al., 2004) and are expressed in the immune system (Chapman et al., 2009). Importantly, changes in 11b-HSD2 activity may play a role in autoimmunity: 11b- HSD2 is expressed in active MS lesions (Heidbrink et al., 2010) and increased 11b-HSD2 activity has been found in other inflammatory conditions such as rheumatoid arthritis (RA) (Schmidt et al., 2005), potentially contributing to limited anti-inflammatory effects of endogenous glucocorticoids in these diseases. In addition, we have previously reported a higher frequency of TNFa-producing CD8+ Tcells upon ex vivo stimulation in depressed MS patients compared to matched patients without comorbid depression (Gold et al., 2011). While it is unclear if this results in higher levels of this cytokine in the circulation or the CNS, TNFa can induce NFkB and thereby cause inactivation of GR (Pace and Miller, 2009). However, effect sizes for dexamethasone (which selectively binds GR) were small in our study, suggesting that strictly GR dependent mechanisms may play a relatively minor role in MS-depression. Further studies that directly assess molecular mechanisms such as receptor expression, transrepression, coactivation or intracellular bioconversion in MS-depression as well as patients with idiopathic MDD will help to dissect the contribution of receptor-dependent versus pre-receptor reg- ulation to steroid resistance in these disorders.

Some limitations have to be considered. Although clini- cally well-characterized and carefully matched for important potential confounds, the sample in our study was compara- tively small. Thus, the results require replication in larger samples. The study was adequately powered to detect sig- nificant differences for hydrocortisone. The lack of statistical differences in aldosterone, however, may be due to the small sample size given the reported effect size. On the other

hand, the small sample size carries the risk of false positive findings and despite the large effect size, the biological difference observable in the inhibition curve appears to be modest. Effect sizes for dexamethasone, on the other hand, were small, indicating that the lack of statistical differences in dexamethasone responses is unlikely to be due to insuffi- cient statistical power.

It should be noted that the doses where the strongest group differences in steroid sensitivity were seen are only in the low range for hydrocortisone (10^{11} M to 10^{9} M) and aldosterone (10^{9} M to 10^{8} M) as indicated by TOP values and not for increased doses of the agonists as reflected by IC-50 values. For aldosterone, this approximately reflects con- centrations previously used in immune cell culture models to approximate physiological levels (Wehling et al., 1987), although circulating levels in humans are usually below 10^{9} M. For cortisol, circulating levels are

typically higher than the concentrations where the biggest differences were found in our study. Whether or not the functional steroid resistance in vitro reflects steroid resistance in vivo however is difficult to judge from our assays.

Steroid sensitivity has been shown to be linked to gluco- corticoid receptor variants (DeRijk et al., 2002) and our findings in PBMCs may therefore potentially reflect a genetic disposition. However, in MS, steroid sensitivity of immune cells may be more strongly influenced by non-genetic factors (L.L. van Winsen et al., 2005). Whether or not our findings reflect a global genetic trait or a more tissue-specific state remains to be determined.

Lastly, our study did not include a healthy control group, as our main interest was to investigate biological substrates of depression within the MS population. Not all MS patients develop depression and MS itself is associated with altera- tions in stress response systems (Gold et al., 2005). Future studies should also enroll healthy controls as well as patients with idiopathic MDD who do not suffer from inflammatory or autoimmune disorders to determine the specificity of the observed effects for MS depression.

Taken together, this study provides empirical evidence that steroid resistance of T cells could play a role for multiple sclerosis-associated depression. In addition, in line with indirect evidence from neuroendocrine and neuroimaging studies, our results indicate that steroid resistance in this population more strongly affects MR signaling with relatively intact GR function, at least in the immune system. If this indeed represents a differential biological substrate in comparison with idiopathic MDD remains to be determined in future studies.

Role of the funding source

The funding agencies supporting this research project had no role in the acquisition and interpretation of data and the preparation of the manuscript.

Conflict of interest statement

None of the authors reports any biomedical financial interests or potential conflict of interest.

Acknowledgements

The research leading to these results has received funding from the European Union Seventh Framework Program (FP7/ 2007-2013) under grant agreement n8 [268381]. The inims is supported by the Gemeinn¨utzige Hertie-Stiftung. The results from this study have been presented as a poster at the 41st annual meeting of the International Society for Psychoneur-oendocrinology (ISPNE) in Berlin, Germany, August 4—6, 2011.

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