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Original research article

The impact of multiple sclerosis relapse treatment on migration of effector T cells – Preliminary study

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ARTICLE INFO

Article history:
Received 3 December 2015
Accepted 10 February 2016
Available online 20 February 2016

Keywords:
Methylprednisolone
Multiple sclerosis
Migration
Chemokines
Chemokine receptors

ABSTRACT

Migration of inflammatory cells from the blood to the central nervous system (CNS) is crucial for development of multiple sclerosis (MS). Inhibition of this process would allow to control disease activity. The first step confirming this approach would be the analysis of the impact of effective MS relapse therapy on migration of effector T cells. The aim of the study was to analyze the influence of methylprednisolone (MP) on the migratory activity of effector CD4+ T cells from MS patients. Moreover, to study the potential mechanism of this process we studied expression of chemokine receptors on migrating cells.

Material and methods: Peripheral blood samples were obtained from relapsing-remitting MS (RR-MS) patients during relapse ($n = 23$) and from control group ($n = 23$). After isolation CD4+ T cells were incubated with various concentrations of MP. Then they were stimulated in chemotaxis assay with chemokines CCL3 or CXCL10 or were used to CCR1 and CXCR3 expression analysis.

Results: CXCL10- and CCL3-stimulated migration of CD4+ T cells was significantly increased in MS. MP was able to reduce *in vitro* migration of effector T cells induced by CXCL10, but not by CCL3. Inhibition by MP was dose-dependent. Expression of analyzed chemokine receptors was unaltered after MP incubation.

Conclusions: MP reduced CD4+ T cells migration induced by CXCL10 without affecting CXCR3 expression. These observations demonstrate one of the potential mechanisms of MP action in MS, distinct from inducing cell apoptosis, and suggests the new targets for development of more effective MS treatments.

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http://dx.doi.org/10.1016/j.pjnns.2016.02.003

1. Introduction

Multiple sclerosis (MS) is considered as an autoimmune disease of the central nervous system (CNS) [1]. Although the initial process leading to its development is still not known, it is highly suggested that the transmigration of effector cells, especially T cells and monocytes, through the blood–brain barrier (BBB) to the CNS, is one of the most important events. The extravasation of inflammatory cells from the blood to the CNS is regulated by adhesion molecules, cytokines and chemokines [2]. The attack of the inflammatory effector cells on the CNS leads to demyelination, gliosis, axonal degeneration and development of MS clinical signs [3].

Chemokines constitute a large family of small chemoattractant cytokines that share important structural features and ability to attract leukocytes [4]. Chemokines function through specific interaction with chemokine receptors, which belong to the seven-transmembrane G protein-coupled receptor family mediating a variety of leukocyte responses including chemotaxis and immune activation [5]. It is confirmed that differential spatial and temporal chemokine production by specific cell types serves as an important effector and regulatory mechanism in the pathogenesis of experimental allergic encephalomyelitis (EAE), an animal model of MS, by directing mononuclear cell infiltration and trafficking within the CNS [6]. CCL3 is one of the chemokines produced in the CNS perivascular space that direct inflammatory cell migration toward inflammatory foci. Migrating cells produce other cytokines that stimulate astrocytes localized at the glial limitans to produce other chemokines, like CXCL10, guiding leukocytes further toward the brain parenchyma [6]. CXCL10 production by activated astrocytes is postulated to be an important factor involved also in the induction of antigen specific T cells responsible for epitope spreading and monocytes/macrophages that are the end stage effector cells responsible for axon demyelination and direct axonal transection [6].

The main treatment goal for MS is to reduce number of disease relapses, prevent new attacks as well as to inhibit disability progression [1]. In the last several years there has been a major progress in development of new MS treatments but their effectiveness is still unsatisfactory. The standard and effective drug used in MS relapse is still methylprednisolone (MP) [7]. The mechanism of MP action in MS is complex and requires further studies. MP is a steroid immunosuppressant that reduces inflammatory tissue edema and myelin breakdown [8]. One of its main mechanism of action is induction of T cell apoptosis [9,10]. Moreover, MP may switch the immunological response from Th1 to Th2 cytokine profile [11]. However, it was shown recently that MP may exert its therapeutic effect independently from apoptosis, through the alteration of migratory capacity of T cells toward chemokines, like CXCL12 [12].

The aim of this study was to analyze the ability of MP to modulate the migratory potential of inflammatory effector cells during active MS. Particularly, we analyzed the impact of MP on CXCL10- and CCL3-induced migration of CD4+ T cells isolated from relapsing-remitting MS patients (RR-MS). Moreover, to study the mechanism of this influence, expression of chemokine receptors for these ligands (respectively CXCR3 and CCR1) was also studied.

2. Material and methods

2.1. *Patients*

All procedures conducted in this study involving human participants were approved by the Local Bioethics Committee. Informed consent was obtained from all individual participants at the beginning of the study.

Peripheral blood samples were obtained from untreated patients with RR-MS during relapse (total of 23) and patients with other non-inflammatory neurological disorders (OND) (total of 23, control group). The control OND group consisted of patients with stroke, subarachnoid hemorrhage, epilepsy, brain trauma, migraine, Parkinson's disease, vertigo and vertebral column degeneration. All patients were hospitalized at the Department of Propedeutics of Neurology and the Department of Neurology and Stroke, Poland. Blood samples were collected into vacuum tubes containing heparin as an anticoagulant.

2.2. *PBMCs and CD4+ T cell isolation*

Collected blood was diluted (1:1) with phosphate buffered saline (PBS, Biomed, Lublin, Poland). Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation using Histopaque-1077 (Sigma–Aldrich, Poznan, Poland) (450 × *g*, 25 min, 20 °C. The upper phase was removed, PBMCs from the middle phase were collected into clean tube and the red blood cells (RBCs) buffer was added for red blood cell lysis by hypotonic shock (NH_4Cl, $KHCO_3$ and $Na_2EDTA{\cdot}2H_2O$ buffer, pH 7.2, Sigma, Poznan, Poland) for 5 min at 4 °C. Then PBMCs were washed with PBS by centrifugation (350 × *g*, 10 min, 4 °C). PBMCs were resuspended in PBS, counted using Bürker chamber (Merck, Warsaw, Poland) under a light microscope (Carl Zeiss, Axio Observer.A1, Poznan, Poland) and used for isolation of CD4+ T cells with CD4+ T Cell Isolation Kit II human from Miltenyi Biotec (Bergish Gladbach, Germany).

2.3. In vitro *incubation of cells with MP*

CD4+ T cells isolated from patients with RR-MS and OND were cultured in RPMI 1640 medium (PAA, Pasching, Austria) containing L-glutamine supplemented with 5% fetal calf serum (FCS) (Sigma, Poznan, Poland), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, Poznan, Poland) at 37 °C in 5% of CO_2. Various concentrations of MP (0.002 mg/ml, 0.02 mg/ml or 0.2 mg/ml) were added to 0.8 mln of CD4+ T cells resuspended in 1 ml of medium and cultured for 18 h. Control cells (without addition of MP to the medium) were also cultured for 18 h. After incubation cells were collected into the clean tube. 5 μl of CD4+ T cells were resuspended in 40 μl of PBS and stained with Trypan Blue (5 μl) (Sigma, Poznan, Poland). Cells were counted under light microscope using Bürker chamber. Subsequently, appropriate number of cells was used in chemotaxis assay or flow cytometry analysis.

2.4. Chemotaxis assay

Chemotaxis assay was conducted in Corning Transwell Polycarbonate Membrane Insert (Kaysville, UT, USA) consisting of 2 compartments separated by a polyethylene membrane filter (10.5 mm in diameter with 5-μm pores). 4×10^5 viable CD4+ T cells from MS or OND patients were resuspended in 100 μl of medium (RPMI 1640 with L-glutamine, 5% FCS – fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin) and were added to each well of the upper chamber. The lower chamber was filled with 600 μl of the same medium per well. Recombinant human CCL3 (100 ng/ml) (MIP-1alpha, macrophage inflammatory protein 1 alpha) or CXCL10 (50 ng/ml) (IP-10, gamma Interferon inducible Protein 10) were also added to lower chamber (R&D Systems, Inc., Minneapolis, MN, USA) after optimization of their concentrations. For each concentration of MP CD4+ T cells from MS or OND patients have migrated to CCL3 (n for MS and OND = 7) or CXCL10 (n for MS and OND = 6) and medium (negative control, n for MS and OND = 13). The migration was conducted in 37 °C at 5% CO_2 for 3 h. After this time the inserts were removed and CD4+ T cells were collected into small tubes (1.5 ml, Falcon, BD Bioscience, San Jose, CA, USA). After centrifugation, the supernatant was discarded and cell pellet was resuspended in 40 μl of PBS and stained with Trypan Blue (5 μl) (Sigma, Poznan, Poland). All cells were counted under light microscope using Bürker chamber. The results of *in vitro* chemotaxis assay were presented as the chemotactic index (CI) calculated as chemokine-induced migration normalized to spontaneous migration of CD4+ T cells. Medium alone served as a negative control. All measurements were done in duplicate.

2.5. Chemokine receptors analysis using flow cytometry

CD4+ T cells (0.5×10^6) isolated from patients with MS (n = 10) and OND (n = 10) and incubated as described in Section 2.3 with 0.2 mg/ml MP were analyzed. CD4+ T cells without MP incubation served as a control. Obtained T cells were resuspended in 100 μl of PBS and incubated with "FcBlock" (BD Bioscience, San Jose, CA, USA) for 15 min at 4 °C. Then specific mouse antibodies to human CD45-PE-Cy7, CD4-Pacific Blue, CCR1-APC and CXCR3-FITC (BD Bioscience, San Jose, CA, USA) were added at a predetermined optimal concentration and incubated for 15 min at 4 °C. Cells were washed with 1 ml of PBS, fixed in 200 μl of 0.4% formaldehyde/PBS and stored at 4 °C until further analysis. The analysis was carried out on FACS Canto II Instrument (BD Bioscience, Warsaw, Poland) and the data were analyzed using FACSDiva 6.0 software (BD Bioscience, Warsaw, Poland).

2.6. Statistical analysis

For statistical analysis, non-parametric Mann–Whitney test with Bonferroni correction (when needed) was applied. Correlation coefficient was used to determine correlation of chemotactic index with concentration of analyzed drug. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using Statistica v.8 Software.

3. Results

3.1. Chemokine-induced migration of CD4+ T cells is increased in MS patients

CXCL10-induced migration of CD4+ T cells from MS patients was significantly higher (chemotactic index (CI) = 2.75) than in control group (OND patients; CI = 1.9) (Mann–Whitney test, p = 0.045). Similarly, CCL3-stimulated migration of CD4+ T cells from MS patients was higher (CI = 1.5) than in OND patients (CI = 0.8) (Mann–Whitney test, p = 0.017) (Fig. 1).

3.2. MP reduces CXCL10-induced migration of CD4+ T cells

In vitro preincubation of CD4+ T cells with the highest dose of MP (0.2 mg/ml) significantly reduced their migration in response to CXCL10 in MS group (p = 0.004, Mann–Whitney test) (Fig. 2a). Similarly, only the highest dose of MP was able to reduce migration of this T cell subpopulation from OND group (p = 0.004, Mann–Whitney test) (Fig. 2b).

3.3. Reduction of CXCL10-induced migration by MP is dose-dependent

CXCL10-stimulated migration of CD4+ T cells from MS patients negatively correlated with concentration of MP in the culture medium (correlation coefficient = −0.44; p = 0.034) (Fig. 3a). Similar results were seen for OND group (correlation coefficient = −0.46; p = 0.02) (Fig. 3b).

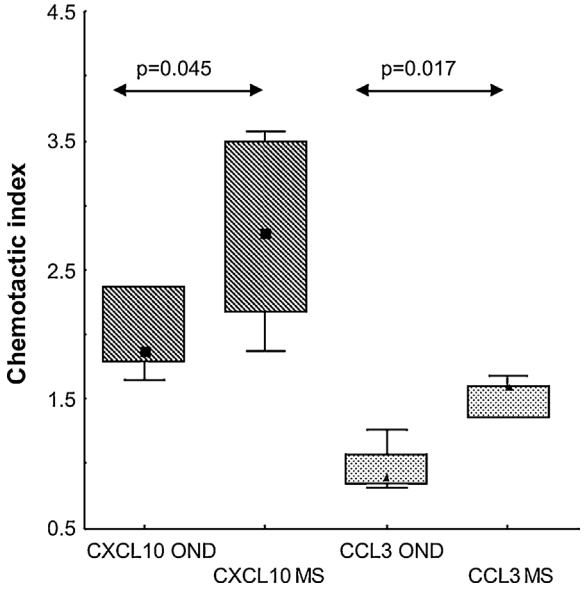

Fig. 1 – CXCL10- and CCL3-induced chemotaxis of CD4+ T cells from the blood of MS and control OND patients (n for MS and OND = 13). Cells were cultured for 18 h in medium and then *in vitro* migration stimulated by CXCL10 or CCL3 was analyzed in the transwell system for 3 h as described in Section 2.

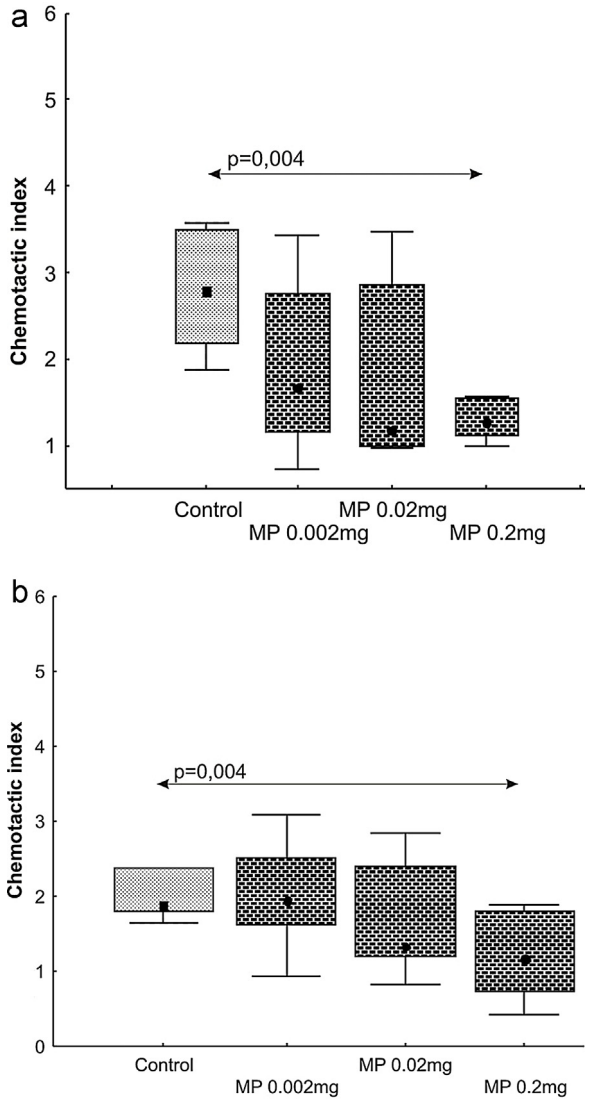

Fig. 2 – Reduction of CXCL10-induced chemotaxis of CD4+ T cells from MS (a) and OND (b) patients (*n* for MS and OND = 6) by MP. Before chemotaxis assay cells were preincubated for 18 h with methylprednisolone (MP). "Control" – migration of CD4+ T cells without preincubation with MP. *In vitro* migration was analyzed in transwell system for 3 h under stimulation with CXCL10. CD4+ T cells were isolated as described in Section 2.

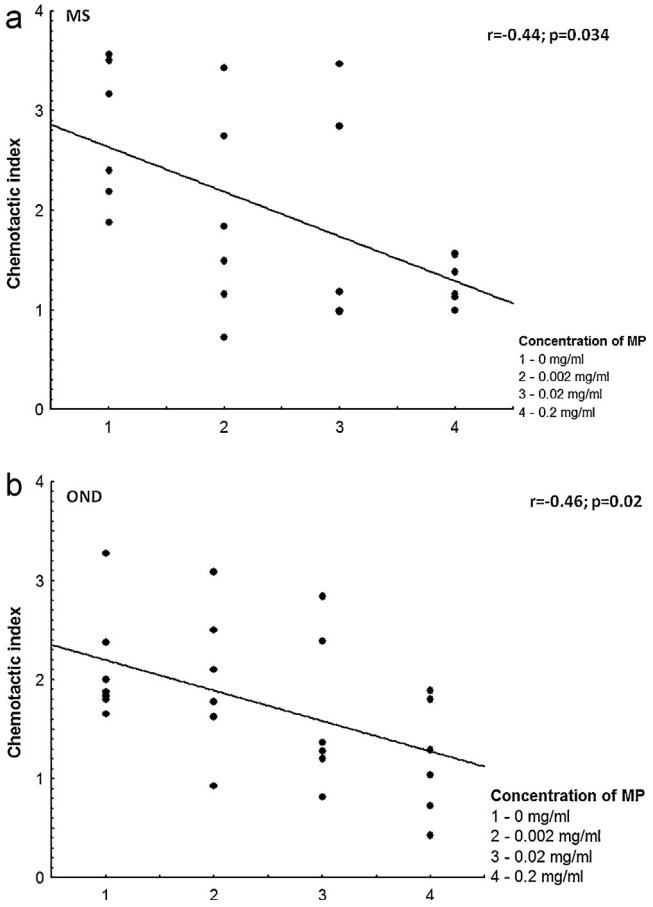

Fig. 3 – Correlation between the concentration of MP in culture medium and inhibition of CXCL10-induced migration of CD4+ T cells from MS (a) and OND (b) patients (*n* for MS and OND = 6). Cells were obtained from the peripheral blood and preincubated with MP for 18 h. Then *in vitro* migration was analyzed in transwell system for 3 h under stimulation with CXCL10 as described in Section 2 (correlation coefficient: MS (a) = −0.44; $p = 0.034$) and control (b) = −0.46; $p = 0.02$).

3.4. *MP do not influence CCL3-induced migration of CD4+ T cells*

Chemotactic indexes of CCL3-stimulated CD4+ T cells from MS (Fig. 4a) and OND patients (Fig. 4b) preincubated with MP did not differ significantly.

3.5. *MP do not influence CCR1 and CXCR3 expression on CD4+ T cells*

Chemokine receptors (CCR1 and CXCR3) analyzed in this study did not show any significant alterations in their expression on CD4+ T cells from MS and OND patients after cell incubation with MP (Fig. 5a, b).

4. Discussion

The blood–brain barrier (BBB) damage and migration of effector CD4+ T cells from the blood to the central nervous system (CNS) are the fundamental events in the immunopathogenesis of MS [1]. The process of inflammatory cells recruitment to the CNS comprises several steps from weak adhesion and rolling on the BBB endothelium, through the firm arrest on the luminal side of the endothelium to cell diapedesis across the BBB to brain parenchyma [13]. Chemokines are involved in several of these steps, including induction and activation of leukocyte adhesion molecules that mediate firm adhesion of migrating cells to the endothelium, formation of a chemotactic concentration gradient resulting in migration of those cells across the endothelial monolayer, and the

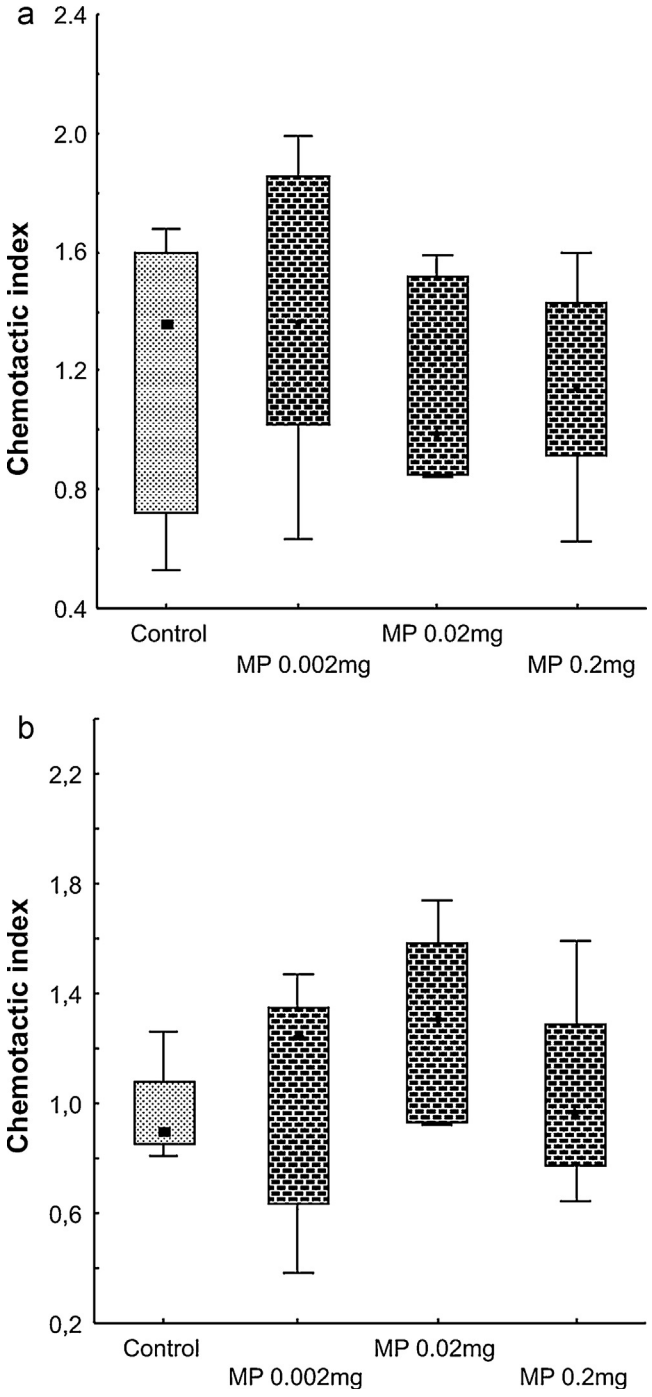

Fig. 4 – CCL3-induced chemotaxis of CD4+ T cells from MS (a) and OND (b) patients (*n* for MS and OND = 7). Cells were preincubated with methylprednisolone (MP) for 18 h before the chemotaxis assay. "Control" – migration of CD4+ T cells without preincubation with MP. *In vitro* migration was analyzed in the transwell system for 3 h under stimulation with CCL3. CD4+ T cells were isolated as described in Section 2.

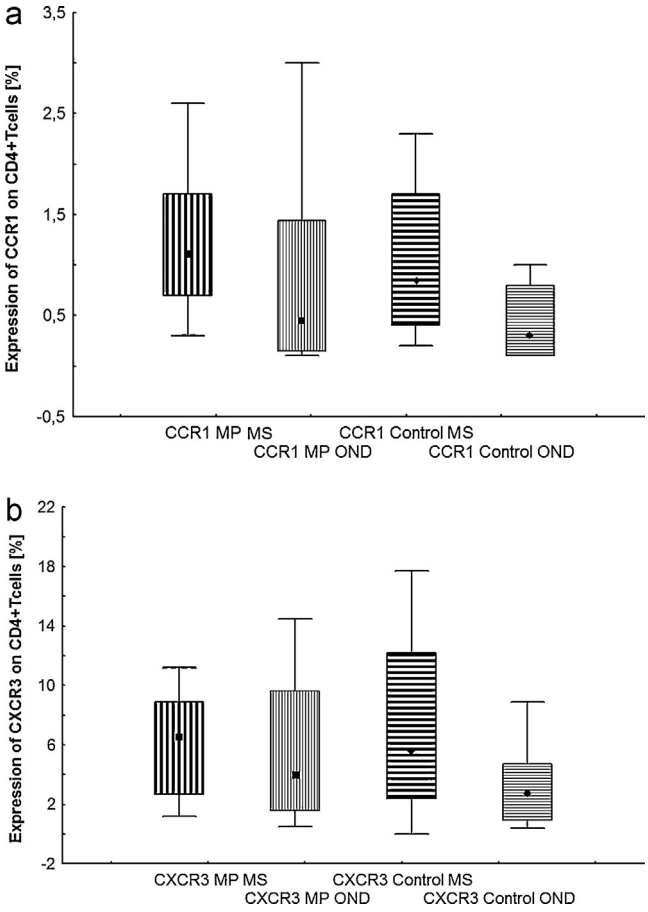

Fig. 5 – Effect of methylprednisolone (0.2 mg) on the protein expression of CCR1 (a) and CXCR3 (b) analyzed on CD4+ T cells isolated from multiple sclerosis (MS) patients and patients with other neurological disorders (OND) (*n* for MS and OND = 10). The cells were cultured as described in Section 2. "Control" means that CD4+ T cells were cultured without this drug.

induction of proteolytic enzymes involved in the breakdown of extracellular matrix proteins in the CNS parenchyma [14].

One of the crucial chemokine involved in this process is CXCL10. Several years ago we and others showed that CXCL10 plays an important role in the pathogenesis of animal model of MS – experimental allergic encephalomyelitis (EAE), mainly in the recruitment and accumulation of inflammatory mononuclear cells in the CNS [15–18]. Fife et al. [18] observed that increasing production of CXCL10 in the CNS precedes the development of clinical EAE symptoms and maintains high levels during peak clinical disease. Administration of anti-CXCL10 antibodies decreased clinical and histological disease incidence, severity, as well as infiltration of mononuclear cells into the CNS. Anti-CXCL10 treatment specifically decreased the accumulation of encephalitogenic $PLP_{139–151}$ antigen-specific CD4+ T cells in the CNS compared to control-treated animals, but did not affect the activation of encephalitogenic T cells [18]. Also in the viral model of MS Liu et al. [19] indicated that CXCL10 promotes CNS demyelination by attracting CD4+ T lymphocytes into the CNS. Further studies conducted on MS patients also suggested the important role of this chemokine in the pathogenesis of this disease. The high concentration of CXCL10 has been detected in the cerebrospinal fluid (CSF) of MS patients during relapse [20]. In addition, postmortem

analyses of MS brains revealed the presence of T lymphocytes bearing CXCL10 receptor – CXCR3 within inflammatory CNS lesions undergoing demyelination [20].

CCL3 is another crucial player involved in the migration of inflammatory cells to the CNS during development of MS. CCL3 expression in the CNS has been associated with acute disease symptoms in both rat [21] and mouse [16] EAE models. Administration of anti-CCL3 antibodies prevented the development of acute and relapsing EAE as well as infiltration of lymphocytes into the CNS which was re-initiated by the adoptive transfer of antigen-specific effector T cells [22]. CCL3 have been shown to be present not only within inflammatory CNS MS lesions, but also in cerebrospinal fluid of patients with relapsing-remitting MS [20,23,24]. It is postulated that CCL3 together with CCL5 provide a signal for T cell activation, thus facilitating antigen presentation [25].

Several studies have suggested that prevention of inflammatory CD4+ T cells migration from the blood to the CNS would allow the control of MS activity and should be a target for future more effective therapies of this disease. An important step to confirm this approach would be analysis of the impact of MS relapse treatment on migration of effector T cells. At present, the standard and relatively effective drug for MS relapse treatment is methylprednisolone (MP). In this study we analyzed the potential impact of this drug on *in vitro* migratory activity of CD4+ T cells from MS patients induced by chemokines CXCL10 and CCL3. Moreover, to analyze the mechanism of this activity we studied the impact of MP on the expression of CCR1 and CXCR3 (receptors for those chemokines) on migrating cells.

CXCL10-induced migration of CD4+ T cells from MS patients was significantly higher than in control patients. Moreover, we observed that this migration was significantly inhibited after cell pre-incubation with MP. However, this effect was not specific only for MS patients, as it was observed also in OND group. Only the highest analyzed concentrations of MP were effective. We observed that the decrease of migratory activity of effector T cells after incubation with MP has not been followed by changes of analyzed chemokine receptors expression. The decrease of migratory activity of CXCL10-stimulated CD4+ T cells detected after MP incubation did not induce any change in CXCR3 expression, the only receptor for CXCL10. This observation suggests that the mechanism of MP action in MS may be distinct from the induction of T cells' apoptosis [9,10]. These results may be a consequence of blocking of some specific domains of receptor for CXCL10 on CD4+ T cells by the drug or changing the cell response machinery to receptor activation by the ligand, what may result in altered migratory activity of T cells. This explanation is in line with the study showing that administration of anti-CXCL10 antibodies decreased clinical and histological disease activity in MS model, as well as specifically decreased the accumulation of effector encephalitogenic CD4+ T cells in the CNS [18]. As increase in the concentration of CXCL10 was observed in MS serum [26], CD4+ T cells could be constantly stimulated in the course of active disease. This stimulation may increase their ability to cross BBB as we know that stimulated T cell can easily transmigrate to the CNS. We confirmed this possibility showing increased migration of CXCL10-induced CD4+ T cells from MS patients over controls. Another confirmation of our results comes from the study of Schweingruber et al. [12]. They have demonstrated increased CXCL12-induced T cell migration after glucocorticoids (GCs) treatment both in EAE and MS patients. Enhanced T cells migration to the CXCL12 may results in their accumulation in the perivascular space, secondary lymphoid and non-lymphoid organs instead of brain parenchyma [27–29]. As in our study, Schweingruber and co-workers did not observe any concurrent changes in CXCR4 surface levels. The authors' explanation for such phenomenon is that glucocorticoids (GCs) are able to enhance CXCR4 signaling, rather than its expression. They have found that GCs have altered cytoskeleton rearrangement through the focal adhesion kinase (FAK) phosphorylation leading to enhanced T cell migration [12]. Importantly, FAK is a part of the CXCR4 signaling pathway [30,31].

In our study the anti-migratory effect of MP was specific for CXCL10-attracted CD4+ T cells. We did not observe any significant influence of MP on *in vitro* migration of T cells after stimulation by CCL3. Furthermore, in our previous study we have observed significant reduction in lymphocytes' migration induced by CCL5 between cells obtained from MS patients treated with MP, untreated MS patients or healthy controls [32]. These data could be the result of some mentioned above differences in functions of CXCL10, CCL3 and CCL5 in development of perivascular inflammatory foci in the CNS in active phase of MS. They may be also the effect of various impact of MP on different chemokines.

We have shown that MP has significant impact on migration of activated CD4+ T cells. The mechanism of MP action is complex so it can target different pathological processes in MS development. Studies on the effect of MP on molecules mediating transmigration of inflammatory cells through the BBB showed down-regulation of adhesion molecules L-selectin, VLA-4 and ICAM. In contrast, expression of LFA-1 (leukocyte function associated antigen), CD29, CD25, CD69 and HLA-DR, has not been changed significantly [33]. Our results are partially in line with the observation that MP treatment can block the BBB crossing by inflammatory T cells through inhibition of their adhesion molecules expression [33]. However, as for CCL3-induced migration of CD4+ T cells we did not observe any significant changes after MP incubation, it is rather not the main mechanism of MP action.

Polarization of activated T cells is an important process in efficient cell migration and adhesion. In polarized cells lamellipodiae are formed on the leading edge and an uropod at the opposing pole. Part of the surface molecules, such as specific surface receptors, integrins and chemokine receptors are distributed within these two poles [34]. Such structural rearrangements are essential for proper T cell movement [35]. Polarized morphology is necessary also for cell–cell interaction and directional secretion of mediators. By the application of 2-photon microscopy it was discovered that encephalitogenic T cells change their phenotype to ''migratory'' one, allowing cell migration to the target site, the phenomenon important for EAE development [36]. Müller et al. [37] have shown that inhibition of T cell migration caused by GCs like MP is related to morphological alterations. They indicated that GCs induce T cell depolarization through modulation of cytoskeletal architecture. In that study T cells were incubated only for

3 h, so they suggested that depolarization is an early result of GCs action on T cells [37]. In our study T cell incubation with MP lasted for 18 h so another intracellular mechanisms could be activated leading to inhibition of CXCL10-specific effector cell migration. Additionally, studies by Schweingruber et al. [12] also have indicated that GCs-induced depolarization of T cells may not be a general process, as they observed various migratory activity of effector cells depending on the chemoattractant used in the experiment. They have shown that GCs inhibited T cell migration toward CCL19, but enhanced CXCL12-induced chemotaxis [12].

The overall conclusion from the findings presented here is that MP used as the standard treatment for MS relapse is able to reduce migratory activity of CD4+ MS T cells induced by CXCL10. This suggests that effectiveness of MP may be partially a consequence of its impact on migration of effector T cells to the CNS. These results give additional insight into the mechanisms of therapeutic action of MP in MS, however this phenomenon should be further confirmed in the larger MS group as the sample size is the main limitation of our study. It also suggests that targeting migratory activity of CD4+ T cells is a promising way for development of more effective future MS therapies.

Conflict of interest

None declared.

Acknowledgement and financial support

This work was sponsored by grant from Polpharma Foundation no. 15/IV/2005.

The funding institution had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; Uniform Requirements for manuscripts submitted to Biomedical journals.

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