

ORIGINAL ARTICLE/ARTYKUŁ ORYGINALNY

Evaluation of the relationship between leptin, resistin, adiponectin and natural regulatory T cells in relapsing-remitting multiple sclerosis

Ocena zależności pomiędzy leptyną, rezystyną i adiponektyną a naturalnymi limfocytami regulatorowymi T w postaci nawracająco-zwalniającej stwardnienia rozsianego

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Abstract

Background and purpose: Data suggest that adipocytokines and natural regulatory T (nTreg) cells play a pivotal role in the immunopathogenesis of multiple sclerosis and the associated inflammation. The purpose of this study was to evaluate selected adipocytokines and nTreg cells and to assess their relationship with relapsing-remitting multiple sclerosis (RRMS).

Material and methods: The study was conducted among 25 patients with RRMS and 25 healthy individuals. Blood samples were collected within two weeks after the beginning of acute relapse of RRMS. The body mass index (BMI) of each patient was calculated. Serum adipocytokine concentrations were determined by ELISA and nTreg cells were evaluated using multicolour flow cytometry.

Results: Patients and controls had similar BMI, regardless of gender. Significantly higher leptin and resistin levels and significantly lower adiponectin levels were found in patients with RRMS in comparison to the control group ($p < 0.0001$). The percentage of nTreg cells ($p < 0.01$) and the mean fluorescence channel (MFC) of FoxP3 were significantly reduced in patients with RRMS ($p < 0.001$). There was an inverse correlation between leptin concentration and MFC of

Streszczenie

Wstęp i cel pracy: Dane z piśmiennictwa sugerują, że w immunopatogenezie i w przebiegu procesu zapalnego w stwardnieniu rozsianym szczególną rolę mogą odgrywać adipocytokiny oraz naturalne limfocyty regulatorowe T. Dlatego celem pracy była ocena wybranych adipocytokin i limfocytów nTreg oraz analiza zależności pomiędzy nimi u chorych na stwardnienie rozsiane w postaci nawracająco-zwalniającej (RRMS).

Materiał i metody: Badania przeprowadzono u 25 chorych na RRMS. Krew pobrano w ciągu 2 tygodni od początku ostrego rzutu choroby. Grupę kontrolną stanowiło 25 zdrowych osób. U każdego pacjenta wyliczono wskaźnik masy ciała (BMI). Stężenie adipocytokin oznaczono w surowicach techniką ELISA. Ocena immunofenotypu limfocytów nTreg wykonano przy użyciu wielokolorowej cytometrii przepływowej.

Wyniki: Nie stwierdzono statystycznie istotnej różnicy we wskaźniku BMI pomiędzy pacjentami z RRMS a grupą kontrolną niezależnie od płci. Wykazano znamienne większe stężenie leptyny i rezystyny oraz znamienne mniejsze stężenie adiponektyny u chorych na RRMS w porównaniu z grupą kontrolną ($p < 0,0001$). Odsetek komórek nTreg ($p < 0,01$) i wskaźnik średniej intensywności fluorescencji (MFC) dla

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the transcription factor Foxp3 nTreg in patients with RRMS ($r = -0.7, p < 0.05$).

Conclusions: Proinflammatory adipocytokine profile and decreased percentage of nTreg cells suggest their implication in the inflammatory response in RRMS regardless of corticosteroid therapy. The correlation between leptin and the MFC of the transcription factor Foxp3 in nTreg cells in patients with RRMS suggests its inhibitory effect on FoxP3 expression.

Key words: multiple sclerosis, leptin, resistin, adiponectin, natural regulatory T cells (nTreg), FoxP3.

Introduction

Multiple sclerosis (MS) is an organ-specific autoimmune disorder with typical multifocal inflammatory lesions (plaques) located mostly in the white matter of the brain and spinal cord, leading to demyelination and neurodegeneration [1,2]. Pathogenesis of the disease is not fully understood but the available data suggest that during the course of MS an autoimmune reaction occurs with autoreactive T cells passing through the blood-brain barrier and entering the central nervous system [3].

It was thought initially that the development of the disease was predominantly related to the CD4⁺ lymphocytes with Th1 cytokine profile, which are responsible for the secretion of proinflammatory cytokines [1,2,4]. However, subsequent studies showed that the immunopathogenesis of MS involves many other lymphocyte subpopulations, e.g. T helper 17 cells (Th17), natural regulatory T cells (nTreg), Treg type 1 (Tr1) cells, as well as mediators secreted by those cells [1,5-7].

nTreg lymphocytes comprise 5-10% of all CD4⁺ T lymphocytes in peripheral blood. They are thought to be the major subpopulation of cells responsible for the maintenance of immunological homeostasis. These lymphocytes can suppress the proliferation and cytokine secretion by effector CD4⁺, CD8⁺, monocytes, NK cells, and dendritic cells. The major discovery related to nTreg lymphocytes was the identification of transcription factor Foxp3, which is believed to be essential for the differentiation and functioning of this subpopulation [8-12]. Many reports suggest that the autoimmune disorders, including MS, are characterized by impaired suppressor function of nTreg CD4⁺ that leads

FoxP3 ($p < 0,001$) były znacząco mniejsze u chorych na RRMS. Stwierdzono ujemną korelację pomiędzy stężeniem leptyny a MFC dla czynnika transkrypcyjnego FoxP3 w limfocytach nTreg u chorych na RRMS ($r = -0,7; p < 0,05$).

Wnioski: Prozapalny profil adipocytokin i zmniejszenie odsetka limfocytów nTreg sugeruje ich udział w przebiegu reakcji zapalnej u chorych na RRMS niezależnie od terapii kortykosteroidami. Korelacja pomiędzy stężeniem leptyny i wskaźnikiem MFC dla czynnika transkrypcyjnego FoxP3 w limfocytach nTreg u chorych na RRMS wskazuje na hamujący wpływ leptyny na jego ekspresję.

Słowa kluczowe: stwardnienie rozsiane, leptyna, rezystyna, adiponektyna, naturalne limfocyty regulatorowe (nTreg), FoxP3.

to the decreased expression of the transcription factor FoxP3 [3,11,13].

Recently, attention has been paid to the close association between inflammation and adipose tissue, where some adipocytokines, namely leptin, resistin and adiponectin, are synthesized [14-17]. The impact of leptin on immunological function is well known. Leptin induces the proliferation of T lymphocytes, production of cytokines, mainly interleukin (IL)-1, IL-12, tumour necrosis factor (TNF)- α , activation of monocytes and macrophages, and phagocytosis [15,17-21]. It was also shown that macrophages and T lymphocytes infiltrating the area of inflammatory process in experimental autoimmune encephalomyelitis (EAE) might be the source of leptin [1,5,16,21].

It is believed that resistin probably comes from monocytes and macrophages, and to a lesser extent, from adipocytes [15,22-24]. Increased concentrations of that protein were found in obese subjects, mostly during acute inflammatory reactions and after stimulation with some cytokines, i.e. TNF- α , IL-1, IL-6 [7,17,22,23].

In contrast to leptin and resistin, adiponectin is thought to be an important anti-inflammatory factor [17,24]. It suppresses the activation and proliferation of T and B lymphocytes, as well as synthesis of proinflammatory cytokines (IL-6, TNF- α , INF- γ), and induces synthesis of the anti-inflammatory cytokine IL-10 [7,15,17].

Recent data suggest that leptin and nTreg may play a pivotal role in the immunopathogenesis of MS and the associated inflammation. Thus, the purpose of this study was to evaluate selected adipocytokines and nTreg cells and to assess their relationship with RRMS.

Table 1. Body mass index (BMI) of patients with relapsing-remitting multiple sclerosis (RRMS) and healthy subjects. Quantitative variables are presented as median, lower and upper quartile

	Patients with RRMS			Controls		
	Total <i>n</i> = 25	Women <i>n</i> = 16	Men <i>n</i> = 9	Total <i>n</i> = 25	Women <i>n</i> = 16	Men <i>n</i> = 9
BMI [kg/m²]	23.8 22.4-24.8	23.8 22.7-25.2	24.2 22.4-24.2	24.3 23.6-24.8	24.3 23.1-24.9	24.3 23.9-24.7

Material and methods

The study included 25 patients (16 women and 9 men, mean age 40.3 ± 13.1 years) and 25 healthy controls (16 women and 9 men, mean age 38.5 ± 6.5 years). Patients were diagnosed with RRMS according to the McDonald criteria, with the use of two-phasic magnetic resonance imaging (MRI) of the head, analysis of the cerebrospinal fluid and assessment of visual evoked potentials [25]. The Expanded Disability Status Scale score in each patient was ≤ 3.5 .

Patients with disorders mimicking MS with their clinical picture or MRI findings, e.g. with vascular collagen disorders, neuroborreliosis and neuroinfections, were excluded. Peripheral blood was drawn for testing within two weeks after the onset of acute MS relapse. MS relapses were treated routinely with corticosteroids (intravenous methylprednisolone, 1 g daily, followed by tapered oral corticosteroids).

The protocol of the study was approved by the Bioethical Committee at the Medical University of Łódź (decision no. RNN/19/09/KE).

Body mass index (BMI) was calculated, based on patients' body weight and height (Table 1).

Concentrations of adipocytokines were measured in sera with ELISA immunoenzymatic tests (leptin – LEPTIN ELISA, DRG Diagnostic, resistin – QUANTIKINE human Resistin, R&D Systems, and adiponectin – QUANTIKINE human Adiponectin, R&D Systems). Colour intensity was measured spectrophotometrically with ELISA reader ELx800 at the wavelength of 450 nm.

Immunophenotyping of nTreg isolated from peripheral blood was performed with eight-colour flow cytometer BD FACS CANTO II. The following monoclonal antibodies were used to stain the nTreg subpopulations: anti-human CD4 conjugated to Pacific Blue (RPA-T4 clone), anti-human CD25 conjugated to FITC (M-A251 clone), anti-human CD127 conjugated to PE (HIL-7R-M21 clone), anti-human FoxP3 conjugated to Alexa 647 (259D/C7 clone). All anti-

bodies were purchased from BD Pharmingen. Staining of the superficial antigens was performed according to the manufacturer's suggested procedure (BD Bioscience). Staining of the intracellular transcription factor FoxP3 was performed after the permeabilization of cells, according to the manufacturer's suggested procedure (BD Bioscience).

Analysis of expression of particular antigens and mean fluorescence intensity measured as mean fluorescence channel (MFC) was performed with the use of FACS DIVA v.6.2 software.

For each tube, lymphocytes were gated according to Forward Scatter (FSC) and Side Scatter (SSC) parameters. The second gate was set for the subpopulation of CD4⁺. Then, the gates for nTreg lymphocytes were set according to the phenotype D4⁺CD25^{high}CD127^{low}FoxP3⁺.

The results were analysed statistically with the STATISTICA v. 8.0 PL software. Variables were assessed regarding the distribution and equality of variances. Quantitative variables were characterized with median, lower and upper quartile. Comparisons of studied features between groups were performed with Mann-Whitney *U*-test. Correlations were assessed with the Spearman correlation rank coefficient. A *p*-value < 0.05 was considered significant.

Results

Anthropometric data showed that the BMI was similar in patients with MS and in controls. No difference regarding BMI was found between patients and controls in relation to sex (Table 1).

The concentration of leptin was significantly, three times greater in patients with RRMS than in controls ($p < 0.0001$) (Table 2). The concentration of resistin in patients with RRMS was two times greater than in controls ($p < 0.0001$) (Table 2). On the other hand, adiponectin concentration was three times smaller in patients with RRMS than in controls ($p < 0.0001$) (Table 2).

The percentage of nTreg was assessed according to the expression of the most specific markers for this subpopulation, namely CD4⁺CD25^{high}CD127^{low}FoxP3⁺. It was significantly lower in patients with RRMS when compared with controls ($p < 0.01$) (Table 3). MFC ratio for FoxP3 transcription factor was also significantly lower in patients with RRMS than in controls ($p < 0.001$) (Table 3).

An inverse correlation between leptin concentration and MFC ratio for FoxP3 transcription factor was found in patients with RRMS (Spearman $r = -0.7$; $p < 0.05$). Significant correlations were found neither between resistin or adiponectin concentration and MFC ratio nor between adipocytokine concentration and the percentage of nTreg in studied groups.

Discussion

Multiple sclerosis with its underlying inflammatory process and autoimmunological disturbances has been a focus of extensive research for many years. The course of MS is most often relapsing-remitting or secondary progressive; rarely, primary progressive or benign form occurs [26]. The disease affects young people, predominantly in developed countries (United States, Canada, Scandinavian countries). MS poses significant social, economic, and most of all clinical problems [2]. Current management includes mainly corticosteroids for relapses and immunomodulation (interferon β , copolymer-1, immunosuppressive agents) for chronic forms. Despite improved methods of treatment, the disease is incurable [2]. Therefore, new possibilities for the modulation of the inflammatory process and the involved cells are actively sought which may lead to new options of targeted intervention.

The influence of adipocytokines on Treg and effector T cells (Teff) has been highlighted [27]. Some of these proteins (leptin, resistin) induce proinflammatory processes, while others (adiponectin) have protective, anti-inflammatory properties [24]. The concentration of adipocytokines in blood is obviously related to the adipose tissue amount, indirectly assessed by BMI. It was shown previously that obesity, especially of the visceral type, accompanied the presence of a chronic subclinical inflammatory process, which is responsible for activation of proinflammatory pathways [7]. In our study, BMI in patients with MS was comparable to that in controls. These findings suggest that the difference in adipocytokine concentrations in patients with MS in comparison to controls cannot be attributed to the adi-

Table 2. Concentrations of adipocytokines in patients with relapsing-remitting multiple sclerosis (RRMS) and in healthy subjects. Quantitative variables are presented as median, lower and upper quartile

Variable	Patients with RRMS n = 25	Controls n = 25
Leptin [ng/mL]	19.9 12.0-21.5	4.5 1.6-8.6
Resistin [ng/mL]	7.1 4.7-9.3	3.5 2.7-4.4
Adiponectin [ng/mL]	41.6 17.8-53.5	119.6 74.3-193.4

Table 3. Percentage of natural regulatory T lymphocytes (nTreg) CD4⁺CD25^{high}CD127^{low}FoxP3⁺ and mean fluorescence channel (MFC) of the transcription factor Foxp3 in patients with relapsing-remitting multiple sclerosis (RRMS) and controls. Variables are presented as median, lower and upper quartile

	Patients with RRMS	Controls
nTreg %	4.4 2.2-4.9	7.5 3.9-9.3
MFI FoxP3	335 260.0-345.0	580 563.0-800.0

pose tissue amount, but instead it may result from the ongoing illness with an intense and active inflammatory background.

It was shown that treatment with corticosteroids can affect the concentration of adipocytokines. However, the available data are equivocal. Cimmino *et al.* found that the observed changes in adiponectin level in MS patients treated with prednisone were related more to the inflammation than to the corticosteroid itself, while the increase of leptin level depended on corticosteroids with the synchronous decrease of inflammatory markers (ESR, CRP, IL-6). Those authors also observed a correlation between the level of endogenous or exogenous steroid and the concentration of leptin [28]. It should be stressed, however, that other authors quite commonly have reported contrary findings; thus, it is thought that the most probable cause of increased adipocytokine levels is the inflammatory process, not corticosteroids, although the influence of corticosteroids cannot be clearly excluded.

Increased leptin concentration in patients with RRMS is in accordance with the findings published previously [5,18,21,27,29]. Frisullo *et al.* [14] noted an increased leptin level in the active phase of RRMS in untreated patients when compared to controls. Matarese *et al.* reported increased levels of that adipocytokine both

in serum and in cerebrospinal fluid of untreated patients with RRMS [21,27]. Batocchi *et al.* showed that the serum concentration of leptin markedly decreased after two-month therapy with interferon beta. Those reports confirm the association between leptin and the activity of the process in patients with MS [18]. Frisullo *et al.* [14] additionally observed a modulatory effect of leptin during the relapses, where its use stimulated production of TNF- α and IL-6.

Studies show that resistin also plays an important role in inflammation, and the major sources of it are mononuclear cells: monocytes and macrophages [16,22-24]. Our study showed that the resistin level was two times greater in patients with RRMS than in controls. There are no reports on the role and significance of that marker in serum or in cerebrospinal fluid in patients with MS. Recently reported studies on the role of resistin in many other acute and chronic conditions related to inflammation (kidney diseases, inflammatory bowel disease, liver diseases) have shown a correlation between the level of this protein and exacerbation of the disorder [16,22,23]. The proinflammatory cytokines IL-1, IL-6 and TNF- α increase expression of mRNA of the resistin gene in mononuclear blood cells. On the other hand, resistin, through activation of the NF- κ B pathway, stimulates the production of those proinflammatory cytokines [15,22].

It is known that adiponectin has potential anti-inflammatory properties. It suppresses the synthesis of proinflammatory cytokines (TNF- α , IL-6, INF- γ) and therefore has some protective function against the development of numerous inflammatory conditions. It was shown that its concentration in obese subjects was small and inversely correlated with markers of inflammation (CRP and IL-6) [7,15,16,24]. Data on the beneficial, anti-inflammatory action of adiponectin come from studies in patients with obesity, coronary heart disease, insulin resistance, and diabetes [15,17,24].

We noted a significantly smaller (three times smaller) concentration of that adipocytokine in patients with RRMS when compared with controls. The direction of those changes probably reflects the imbalance of the immunological status promoting the proinflammatory Th1 cytokine profile during acute relapse of MS.

The observed two times greater resistin concentration and three times smaller adiponectin concentration seem to be the result of the inflammatory process. The impact of therapy with corticosteroids on resistin and adiponectin levels cannot be excluded, but there are no data in the literature related to that problem.

A subpopulation of nTreg CD4⁺CD25^{high}CD127^{low}FoxP3⁺ has become an important target of studies on autoimmune disorders because it is responsible for immunological tolerance. It was confirmed that dysfunction of these cells might have a close relation to the pathogenesis of the autoaggressive disorders. The significance of nTreg in human autoimmunological disorders is not fully understood but several lines of clinical and experimental evidence support its major role in abnormal immunological tolerance. Abnormalities in the nTreg population along with Tr1 and Th3 lymphocyte subpopulations are most commonly reported. The relationship between nTreg dysfunction and the occurrence of the disorder was reported for type 1 diabetes mellitus, gastritis, colitis and thyroiditis [1,4]. The tendency to greater prevalence of autoimmune disorders was also noted in cases with deletion of 22q11 associated with thymus atrophy. Apart from MS, dysfunction of nTreg has been reported in myasthenia, rheumatoid arthritis, psoriasis, and polyglandular autoimmune syndrome type 2 [5]. Our study showed that patients with RRMS had a smaller percentage of Treg CD4⁺CD25^{high}CD127^{low}FoxP3⁺ cells in peripheral blood and a lower MFC value for transcription factor FoxP3. These changes might result in decreased suppressor function.

Data related to the nTreg subpopulation in MS are contradictory. Kumar *et al.* observed an increased size of this lymphocyte subpopulation. Putheti and Feger, on the other hand, did not find important differences in the number of regulatory CD4⁺ T cells in peripheral blood [11,30,31]. Viglietta *et al.* [12] observed both decreased number and dysfunction of Treg in the course of MS. Feger *et al.* [11] noted an increased number of Treg lymphocytes in cerebrospinal fluid of patients with MS. The majority of reports confirm the dysfunction of Treg and decreased expression of FoxP3 transcription factor in nTreg lymphocytes in patients with RRMS; this form of MS involves an important role of adipocytokines as mediators of the inflammatory process. Such changes were not observed in chronic forms of the disease [11,12,32,33].

More and more often, the pathogenesis of autoaggressive disorders, including MS, is being associated with the abnormal regulatory function of induced Tr1 lymphocytes, which is related to the change in expression of the cytoplasmic form Cyt2-CD46 [9,10].

Data on the relationship between adipocytokine concentration and nTreg lymphocytes are scarce. De Rosa *et al.* unequivocally demonstrated an association between leptin and nTreg and increase of the autoaggressive

process. The regulatory lymphocytes themselves can be the source of that adipocytokine, which suggests its autocrine function. Thus, leptin may potentiate the inflammatory process through the induction of synthesis and secretion of IL-2, IL-6, INF- γ , TNF- α [4,7,14,19,29], and, on the other hand, by the induction of anergy of the CD4⁺CD25⁺ lymphocyte subpopulation [5,6,20].

Numerous studies highlight the major role of both leptin and Treg lymphocytes in the autoimmune process with the underlying inflammation. It is true also for RRMS. The obtained results suggest that dysfunction of nTreg and exacerbation of the inflammatory process occur in patients with MS during relapse. Surprisingly, the decreased nTreg subpopulation and decreased expression of FoxP3 transcription factor in nTreg was found despite the therapy with corticosteroids. Both our previous studies and reports of other authors suggest that glucocorticoids cause increased expression of FoxP3 transcription factor [34,35]. Markedly increased concentrations of proinflammatory adipocytokines (leptin and resistin) and decreased concentrations of anti-inflammatory adiponectin were noted simultaneously.

To sum up, this study showed neither a correlation between resistin and adiponectin levels and MFC ratio for FoxP3 transcription factor, nor a correlation between adipocytokine and the percentage of nTreg in the studied groups. The inverse correlation found between leptin concentration and MFC value for FoxP3 transcription factor suggests a possible influence of leptin on FoxP3 expression. The results of our study show an important modulatory potential of adipocytokines and nTreg in RRMS.

Conclusions

1. Proinflammatory adipocytokine profile and decreased percentage of nTreg cells suggest their implication in the inflammatory response in RRMS regardless of corticosteroid therapy.
2. The correlation between leptin and the MFC of the transcription factor Foxp3 in nTreg cells in patients with RRMS suggests its inhibitory effect on FoxP3 expression.

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Disclosure

Authors report no conflict of interest.

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