IL-6 levels are associated with clinical status in patients with Myasthenia Gravis

Os níveis de IL-6 estão associados com o status clínico de pacientes com Miastenia Gravis

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ABSTRACT

Background: Myasthenia gravis (MG) is an autoimmune disease marked by fluctuating course of muscle weakness. **Objectives:** The current study was designed to evaluate plasma levels of cytokines (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A) in patients with MG and controls and to investigate whether cytokines levels are associated with clinical parameters. This study was conducted at the Neuromuscular Diseases Outpatient Clinic, Hospital das Clínicas, Universidade Federal de Minas Gerais (UFMG), Brazil.
Methods: Peripheral blood was drawn, and plasma levels of cytokines were measured by cytometric bead array (CBA) in 80 treated patients with MG and 50 controls. The MG Composite (MGC) was used to evaluate muscle weakness and severity of typical motor symptoms of MG.
Results: Patients with MG undergoing treatment exhibit lower levels of all evaluated cytokines compared to controls. There was a negative correlation between IL-6 levels and the MG Composite score, indicating that higher levels of IL-6 were associated with better control of the disease.
Conclusion: This exploratory study suggests that IL-6 is associated with MG clinical status, as assessed by the MGC.

Keywords: myasthenia gravis; immune system; cytokines; interleukin 6; MG composite.

RESUMO

Introdução: A Miastenia Gravis (MG) é uma doença autoimune caracterizada por fraqueza muscular flutuante.
Objetivos: avaliar os níveis plasmáticos de citocinas (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, e IL-17A) em pacientes com MG e controles e investigar se essas citocinas estão associadas com parâmetros clínicos. Este estudo foi conduzido no ambulatório de doenças neuromusculares do Hospital das Clínicas, Universidade Federal de Minas Gerais (UFMG), Brasil.
Métodos: Foi coletado sangue periféricos e os níveis plasmáticos das citocinas foram medidos por citometria em 80 pacientes com MG tratados e em 50 controles. O MG composite (MGC) foi utilizado para avaliar a fraqueza muscular e a gravidade dos sintomas motores típicos da MG.
Resultados: Os pacientes com MG em tratamento apresentaram menores níveis de todas as citocinas avaliadas comparados ao controle. Houve uma correlação negativa entre os níveis de IL-6 e o MGC, indicando que altos níveis de IL-6 estão associados com melhor controle da doença.
Conclusão: este estudo exploratório sugere que a IL-6 está associada com o status clínico da MG, quando avaliado pelo MGC.

Palavras-chave: miastenia gravis; sistema imune; citocinas; interleucina 6; MG composite.

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INTRODUCTION

Myasthenia gravis (MG) is a chronic autoimmune disease caused by antibodies directed to epitopes located in the postsynaptic membrane at the neuromuscular junction. Three main antigenic targets for disease-inducing antibodies have been described in MG: the acetylcholine receptor (AChR), the muscle specific tyrosine kinase (MuSK) and the lipoprotein receptor-related protein 4 (LRP4). Approximately 80% of MG cases are caused by pathogenic autoantibodies against the AChR [1-3].

The origin of the autoimmune dysfunction in MG patients is unknown but thymic abnormalities and defects in immune regulation and sex hormones seem to play a role in the pathophysiology of MG[4] . The thymus is essential for T-cell differentiation and for the establishment of central immune tolerance. As autoimmune sensitization against AChRs is likely developed in the thymus, pro-inflammatory cytokines may be implicated in MG pathogenesis [5] . For instance, thymic epithelial cells from patients with MG produce more tumor necrosis factor (TNF)-α and interleukin (IL)-6 than controls [6] .

Only a few studies have evaluated the circulating levels of cytokines in MG patients [7-9]. One recent study on inflammatory proteins in MG revealed significantly increased serum levels of cytokines IL-19, IL-20, IL-28A and IL-35 in MG as compared with controls [7]. Molin et al., evaluated 92 proteins associated with inflammation in patients with MG and found that the levels of MMP-10, TGF-, β -NGF, IL-6, IL-8, CCL19, IL-17C, CXCL1, IL-10, IL-17A were significantly elevated compared to healthy controls [9] .

In the current study, we aimed to compare the plasma levels of cytokines in patients with MG and controls. In addition, we investigated whether the levels of circulating cytokines were associated with clinical parameters.

METHODS

Subjects

This study included 80 patients with MG followed at the Neuromuscular Diseases Outpatient Clinic, *Hospital das Clínicas, Universidade Federal de Minas Gerais* (UFMG), Brazil. The diagnosis of MG was based on the Myasthenia Gravis Foundation of America (MGFA) criteria[10], and relied on typical clinical symptoms along with improvement of motor symptoms with acetylcholinesterase inhibitors, decremental muscle response to a set of low frequency repetitive nerve stimuli, or the presence of autoantibodies against AChRs. The patients were clinically stable and had no modification of the treatment for at least two months prior the study. Patients presenting pregnancy or cancer diagnoses were excluded.

A control group comprising age- and sex-matched subjects was also recruited from the local community. Exclusion criteria for controls were pregnancy, presence of any chronic inflammatory diseases, cancer, and current use of systemic glucocorticoids, insulin, statins, growth hormone, anabolic drugs or hormone replacement therapy.

All subjects provided written informed consent before admission to the study. The Research Ethics Committee of UFMG approved this study (Protocol number: CAAE-19045413.0.0000.5149).

Clinical assessment

The MG Composite (MGC) scale was used to evaluate the degree of muscle weakness fluctuation and the severity of typical motor symptoms of MG [11, 12]. This scale comprises 10 items evaluating ocular (3 items), bulbar (3 items), respiratory (1 item), neck (1 item), and limb (2 items) signs and symptoms [11, 12], with higher scores indicating more frequent and/or severe symptoms of MG. Participants were evaluated by a trained researcher.

The cumulative dose of prednisone, i.e., the total prednisone dose used by the patient throughout the course of his disease, was calculated based on medical record data [13, 14] .

Cytokine Assessment

Fasting blood samples were drawn by venipuncture in vacuum tubes containing heparin in the morning of the clinical assessment. Blood was immediately centrifuged at 3,000 rpm for 10 min, 4°C. Plasma was collected and stored at -70°C until assayed.

Multiple cytokines [IL-2, IL-4, IL-6, IL-10, TNF, interferon (IFN)-γ, and IL-17A] were simultaneously measured by flow cytometry using the Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA). Acquisition was performed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The instrument was checked for sensitivity and overall performance with Cytometer Setup and Tracking beads (BD Biosciences) prior to data acquisition. Quantitative results were generated using FCAP Array v1.0.1 software (Soft low Inc., Pecs, Hungary).

Statistical Analysis

All continuous variables were tested for Gaussian distribution by the Kolmogorov-Smirnov normality test. Two groups (patients vs. controls) were compared by Mann–Whitney test since data was determined to not follow a normal distribution. Spearman's correlation analyses were performed to examine the relationship between clinical variables and plasma level of cytokines. All statistical tests were two-tailed and were performed using a significance level of α=0.05. Data were analyzed using the Statistical Package for the Social Sciences® version 20.0 (SPSS; Chicago, IL, USA) and GraphPad Prism® version 5.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

Demographic and clinical features of patients with MG and controls are shown in table 1. Patients had a mean age of 29.10 years at disease onset. Sixty-four (80%) patients were classified as early onset MG subtype (< 40 years of age). Sixty percent of the sample was positive for anti-AChR antibody detection. Thirty-one patients underwent thymectomy, and four showed thymic hyperplasia and two had thymoma. The co-occurrence

of other immune-related diseases (Grave's disease, chronic autoimmune hepatitis) was reported in four patients. Regarding current treatment, 87.5% of the patients were taking symptomatic medication (pyridostigmine), 72.5% were in use of prednisone, 41.3% were taking azathioprine and 8.8% were taking other immunosuppressants. The remaining MG patients (27.5%) have already used glucocorticoids (GC), during the course of their disease. There was no significant difference in cumulative dose of prednisone between the groups currently taking and not taking prednisone.

As shown in table 2, MG patients presented lower levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A in comparison with controls. In subgroup analyses, patients under current GC therapy (N = 58; 1.96 pg/ml ± 1.82) had higher levels of IL-10 than patients not taking GC (N = 22; 1.40 pg/ml ± 0.23) (p = 0.04), without significant differences in the other cytokines. Thymectomy did not influence the levels of cytokines.

We also investigated the potential association between clinical parameters (MGC, disease duration, cumulative GC dose, and current GC dose) and cytokine levels. There was a negative correlation between IL-6 and the MGC (rho = -0.233, p = 0.04).

Table 1. Clinical and demographic features of patients with myasthenia gravis (MG) and controls.

Characteristics	MG (N = 80)	Controls (N = 50)
Sex, N (%)		
Female	60 (75%)	30 (60%)
Male	20 (25%)	20 (40%)
Age in years (mean ± SD)	41.89 ± 14.17	42.94 ± 13.76
Education in years (mean ± SD)	9.29 ± 3.73	-
Age at disease onset in years (mean ± SD)	29.10 ±13.48	-
Length of disease in years (mean ± SD)	13.53 ± 10.05	-
Cumulative glucocorticoid dose in mg (mean ± SD)	38123.48 ± 41895.66	-
Age at thymectomy in years (mean ± SD)	32.50 ± 11.83	-
MG composite score (mean ± SD)	4.96 ± 5.02	-

Abbreviations: MGFA = Myasthenia Gravis Foundation of America Clinical Classification; SD: standard deviation

Table 2. Plasma levels of cytokines in patients with myasthenia gravis (MG) and controls.

Biomarkers (pg/ mL)	**MG (N = 80)** Mean ± SD	**Controls (N = 50)** Mean ± SD	***P* value**
IL-2	2.44 ± 0.38	24.41 ± 23.32	<0.001[a]
IL-4	2.12 ± 0.25	29.95 ± 110.05	<0.001[a]
IL-6	3.70 ± 7.97	9.78 ± 10.03	<0.001[a]
IL-10	1.81 ± 1.59	5.07 ± 3.28	<0.001[a]
IL-17A	12.25 ± 9.43	558.79 ± 262.27	<0.001[a]
IFN-γ	1.20 ± 0.26	19.52 ± 24.65	<0.001[a]
TNF-α	1.39 ± 0.33	16.02 ± 52.05	<0.001[a]

Abbreviations: IL = interleukin; IFN = interferon; SD= standard deviation; TNF = tumor necrosis factor.
[a]Mann-Whitney Test. Significant p values written in bold.

DISCUSSION

In the current study, the plasma levels of all measured cytokines were reduced in treated patients with MG in comparison with controls. This result can be at least in part explained by the immunosuppressant treatment to which patients with MG are submitted. In line with this finding, Yilmaz et al. (2014) evaluated lymphocytes from patients with MG under immunosuppressant treatment and found that IL-6, IL-10 and TNF-α production were down-regulated. Interestingly, patients under GC therapy presented higher levels of IL-10 than patients who were not taking GCs [15]. Previous studies have also shown that IL-10 can be up-regulated by GCs [16,17], and that this cytokine can work synergistically with GC on T lymphocytes[18].

Some recent studies evaluated cytokines in medication-free patients with MG. One study investigated serum levels of 27 cytokines/chemokines in 47 seropositive and untreated MG patients, reporting lower levels of IL-4 and higher levels of IL-15 and vascular endothelial growth factor (VEGF) in MG patients in comparison with controls[7]. There were no significant differences in the plasma concentration of the remaining molecules, including the ones investigated in the current study. Another study evaluated plasma levels of IL-17A in untreated MG patients and healthy controls and observed that women with early disease onset, without thymoma and who had not received immunotherapy presented higher levels of IL-17A than controls[8] . Altogether, these findings suggest that MG is associated with mild changes in the circulating/systemic levels of cytokines.

More recently, Uzawa et al. reported higher serum levels of IL-6 in treatment-naïve MG patients compared to controls, and a positive correlation between IL-6 levels and MG Foundation of America Clinical Classification. After immunosuppressive treatment, IL-6 levels significantly decreased[19]. While the latter finding goes in line with our results showing decrease IL-6 levels in immunosuppressed patients, we found a negative correlation between IL-6 levels and MGC scores, indicating that higher levels of IL-6 were associated with less severe MG symptoms. These discrepant results reflect, at least in part, important methodological differences. While our study involved patients under chronic immunosuppression, Uzawa et al. investigated treatment-naïve patients with a more recent MG diagnosis[19]. The severity of MG was assessed by MGC (that provides a granular assessment of the function of different muscle groups) in our study, while the other used a categorical classification that defines an overall muscle functioning not much sensitive the fluctuating nature of the disease. Moreover, IL-6 is a pleotropic cytokine, exerting both pro- and anti-inflammatory effects[20 21].

IL-6 can also be regarded as a myokine associated with hypertrophic growth of striated muscles and myogenesis, in this latter case through the regulation of the proliferative potential of muscle stem cells[22, 23]. Future studies must investigate the biological meaning of this finding, *i.e.*, reactive increase of IL-6 to minimize or improve muscle fatigue and weakness and confirm the role of IL-6 as a staging biomarker for MG.

We are aware of the limitations of our study. First, the cross-sectional design did not allow us to make any cause-effect inference on the relationship between characteristics of the illness and the plasma levels of cytokines. Second, we did not evaluate GC-naïve patients. By contrast, the sample size and the strict diagnosis/inclusion criteria can be regarded as strengths of the study.

CONCLUSIONS

In conclusion, patients with MG undergoing treatment present decreased levels of cytokines in comparison with controls. As changes in circulating levels of cytokines associated with MG are mild, this finding is mostly due to immunomodulatory treatment. IL-6 levels are associated with MG status, as assessed by MGC.

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