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Up-regulated Expression of Fas Antigen in Peripheral T cell Subsets in Patients with Myasthenia Gravis

Abstract

Purpose: Recent reports have linked various autoimmune diseases to defective Fas-mediated apoptosis or Fas expression. Here we aimed to determine whether Fas-mediated apoptosis is involved in the pathogenesis of myasthenia gravis (MG).

Methods: The expression of Fas antigen in peripheral T cell subsets from 17 Chinese patients with MG and 13 healthy individuals was determined by flow cytometry, and its associations with clinical classification, thymus pathology, the concomitance with hyperthyroidism (HT) and corticosteroid treatment were investigated.

Results: Compared with normal controls, a significantly up-regulated expression of Fas antigen was observed in the peripheral CD4⁺, CD4⁺CD8⁻ and CD4⁻CD8⁻ T cell subsets from patients with MG. Fas expression in CD4⁺CD8⁺ T cells of MG patients with normal thymus was significantly higher than that of patients with thymoma. Fas expressions in CD4⁺CD8⁺ T cells in MG patients with HT was significantly higher than controls and the ones without HT. Enhanced Fas expressions was found in CD4⁺CD8⁺ and CD4⁻CD8⁻ T cells of MG patients with corticosteroid treatment, but no significant difference of Fas expression in peripheral T cells between patients with ocular MG (OMG) and general MG (GMG) was observed.

Conclusion: Fas antigen may play a role in the pathogenesis of MG. It may be involved in the mechanisms of corticosteroid treatment, and with the occurrence of HT. OMG may represent a systemic disease, similar to that of GMG.

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Myasthenia gravis (MG) is a T cell-dependent autoimmune disease mediated by antibodies against acetylcholine receptor (AChR) at the neuromuscular junction. These autoantibodies existing in more than 85% of MG patients are produced with the assistance of major histocompatibility class (MHC) II-restricted CD4⁺ helper T lymphocytes. [1]

Fas/CD95 is a cell surface molecule belonging to the tumor necrosis factor family. By binding to Fas ligand (FasL), Fas can induce apoptosis of the Fas-bearing cells. There are two potential mechanisms involved in the Fas/FasL system: T-cell cytotoxicity [2] and activation-induced cell death [3]. It was reported that malfunction of the Fas system could cause or accelerate some autoimmune diseases such as systemic lupus erythematosus (SLE) and lymphoproliferative syndrome [4,5,6].

A few studies have suggested that Fas-mediated apoptosis may be also involved in the pathogenesis of MG. Fas and AChR antigens were demonstrated to share homologous sequences of nucleotide and amino acids, and to co-localize in the thymic epithelial cells of patients with MG, indicating that these two antigens may play a role in triggering the autoimmunity of MG [7,8]. Another study reported that Fas/FasL-mediated apoptosis was one of the mechanisms in the high-dose AChR alpha 146–162 peptide-induced tolerance in the experimental autoimmune myasthenia gravis (EAMG), a classical MG animal model [9]. The Fas expression on thymocyte or peripheral blood lymphocyte (PBL) in MG patients is still controversial. It was reported that the expressions of Fas antigen and mRNA were decreased in the thymuses of patients with MG [10], while another study showed that there was no mutation of mRNA for the cytoplasmic domain of Fas antigen, and its expression was not reduced in the thymuses from patients with MG [11]. It was also reported that the proportion of Fas^{hi} thymocytes was markedly increased in MG patients with anti-AChR antibodies, but that the proportion of Fas^{hi} lymphocytes in the peripheral blood was not much modified, regardless of the anti-AChR antibody titer [12].

MG patients often have associated abnormalities of the thymus: 10-15% MG patients have thymoma, and 50-60% have hyperplasia of thymus [13]. It was reported that Fas expression in MG patients with thymoma was significantly higher than in patients without thymoma [14]. Fas expression on PBL from MG patients with different thymus pathologies has not been described.

It is known that the prevalence of hyperthyroidism (HT) in MG varies from 2 to 17.5%, which, on average, is higher than that in the general population [15]. Previous studies have suggested that HLA-DQ3 and elevated thyroid-related Ab levels may play a potential pathogenic role in the concomitant

development of the two diseases [16,17], but the exact mechanism is still undefined. Whether Fas-mediated apoptosis is involved in it is not known.

MG can be classified into ocular MG (OMG) and general MG (GMG), according to different affected muscles. Whether OMG and GMG are separate diseases with different pathogenesis, or whether OMG is a systemic disease, similar to GMG, is still undefined. Therefore, we conducted this study to investigate Fas expression in peripheral T cell subsets of patients with MG, and its association with clinical classification, thymus pathology, the presence of HT and corticosteroid treatment. We aimed to provide evidence that Fas-mediated

Table 1: Demographic and clinical characteristics of patients with MG and controls

		MG (n=17)	Controls (n=13)
Gender, F/M		11/6	9/4
Age (year) ^a		46.47±14.35	32.15±6.03
Disease duration (year) ^a		6.43±5.15	
MGFA classification			
I		4	
II	IIa	3	
	IIb	1	
III	IIIa	2	
	IIIb	4	
IV	IVa	0	
	IVb	2	
V		1	
Thymus pathology			
normal thymus		7	
thymic hyperplasia		7	
thymoma		3	
Corticosteroid treatment			
Yes		6	
No		11	
Accompanied by Hyperthyreosis			
Yes		5	
No		12	

^aMean ±S.D

MG: myasthenia gravis; MGFA: Myasthenia Gravis Foundation of America.

apoptosis is involved in the pathogenesis of MG, as well as the mechanism of corticosteroid treatment, and the occurrence of HT.

Patients and Methods

Subjects

Studies were carried out on 17 Chinese patients with MG in the neurology department of the Fifth Affiliated Hospitals of Sun Yat-sen University between October 2007 and December 2009. All patients were diagnosed based on typical clinical manifestation, neostigmine test and repetitive nerve stimulation (RNS). Disease severity was evaluated by MGFA (Myasthenia Gravis Foundation of America) classification. The demographic and clinical characteristics of all MG patients were shown in Table I. None of MG patients had concomitant infectious diseases or other autoimmune diseases, and none had received immunosuppressant treatment within the previous three months. Thirteen healthy individuals (4 males and 9 females, mean age 32.15 ± 6.03 years) were enrolled as controls. Heparinized venous blood samples were obtained from patients with MG and healthy controls, with informed consent. The study was approved by the Local Ethical Committee in the Fifth Affiliated Hospital of Sun Yat-sen University.

Procedures

The expression of Fas antigen in peripheral T cell subsets was analyzed using flow cytometry analysis. The fresh heparinized blood was stained with the following monoclonal antibodies: PE-labelled anti-Fas (CD95), PerCP-labelled anti-CD4 and FITC-labelled anti-CD8 (Becton Dickinson, USA), incubated for 20 min at room temperature in the dark. PE, PerCP and FITC-labelled mouse IgG1 monoclonal antibodies were used as isotype controls. After incubation, 100 μ l erythrolysin (BD Bioscience, USA) were added, and samples were incubated for 10 min in the dark and then centrifuged for 10 min (1000 rpm/min). Supernatant was removed and the precipitate was washed twice with PBS. Paraformaldehyde (1%) was added and samples incubated for 10 min in the dark. Finally, cell labeling was analyzed on a FACScan flow cytometer (Becton-Dickinson, USA) using Cell Quest (Simultest™) software. A gate for lymphocyte population was defined by forward and side light scatter characteristics. PerCP- or FITC-strongly positive areas were further gated as CD4⁺ or CD8⁺ T cell populations for subsequent analysis for Fas expression.

Statistical analysis

Differences among groups were compared by non-parametric Kruskal-Wallis test and the comparison was represented by the Boxplot. All statistical procedures were performed using the statistical package of the social sciences 16.0 (SPSS16.0) and $P < 0.05$ was considered to be significant.

Results

T cell subsets and Fas/CD95 expression between patients with MG and controls

There was no significant difference of peripheral T cell subsets between patients with MG and controls ($P > 0.05$). As shown in Fig. 1 and 2, Fas expressions in peripheral CD4⁺ (Mean Rank: 18.65 vs. 11.38, Chi-square: 5.013, $P = 0.025$), CD4⁺CD8⁻ (Mean Rank: 18.29 vs. 11.85, Chi-square: 3.952, $P = 0.047$) and CD4⁻CD8⁻ (Mean Rank: 18.47 vs. 11.62, Chi-square: 4.467, $P = 0.035$) T cell subsets were significant higher in patients with MG than in controls.

Association with thymus pathology

As shown in Fig. 3, Fas expression in the peripheral CD4⁺CD8⁺ T cells was significantly higher in MG patients with normal thymus than in patients with thymoma (Mean Rank: 7.00 vs. 2.00, Chi-square: 5.727, $P = 0.017$). Fas expressions in CD4⁺, CD8⁺ and CD4⁻CD8⁻ T cell subsets were much higher in MG patients with normal thymus than that in controls (CD4⁺: Mean Rank: 14.29 vs. 8.46, Chi-square: 4.410, $P = 0.036$; CD8⁺: Mean Rank: 14.86 vs. 8.15, Chi-square: 5.841, $P = 0.016$; CD4⁻CD8⁻: Mean Rank: 14.57 vs. 8.31, Chi-square: 5.100, $P = 0.024$). Fas expressions in CD4⁺ and CD4⁺CD8⁺ T cell subsets were significantly higher in MG patients with thymoma than in controls (CD4⁺: Mean Rank: 13.67 vs. 7.31, Chi-square: 4.348, $P = 0.037$; CD4⁺CD8⁺: Mean Rank: 14.00 vs. 7.23, Chi-square: 4.928, $P = 0.026$).

Association of MG with the occurrence of hyperthyroidism (HT)

As shown in Fig. 4, Fas expressions in CD4⁺CD8⁺ T cells in MG patients with HT was significantly higher than the ones without (Mean Rank: 13.20 vs. 7.25, Chi-square: 4.906, $P = 0.027$). Fas expressions in CD4⁺ and CD4⁺CD8⁺ T cells in MG patients with HT were significantly higher than that in controls (CD4⁺: Mean Rank: 14.20 vs. 7.69, Chi-square: 5.366, $P = 0.021$; CD4⁺CD8⁺: Mean Rank: 14.60 vs. 7.54, Chi-square: 6.318, $P = 0.012$).

FIGURE 1. Fas/CD95 expression in peripheral T cell subsets from patients with MG and control. The fresh heparinized blood samples from patients with MG and controls were analyzed for the expression of Fas by flow cytometry using monoclonal antibody PE-labelled anti-Fas (CD95). Fas expression in peripheral CD4+, CD4+CD8- and CD4-CD8- T cell subsets from patients with MG was significant higher than in controls ($P=0.025$, 0.047 and 0.035).

FIGURE 2. Representative profiles of T cell subsets and Fas antigen in peripheral T cell subsets from a patient with MG (A1, A2, A3) and a healthy control (B1, B2, B3). The fresh heparinized blood was stained with PE-labeled anti-Fas (CD95), PerCP-labeled anti-CD4 and FITC-labeled anti-CD8. Fas expression in peripheral CD4+, CD8+ T cell subsets from patients with MG was significant higher than in controls (A2 vs. B2, A3 vs. B3).

FIGURE 3. Fas/CD95 expression in peripheral T cell subsets from MG patients with different thymus pathology and controls. The fresh heparinized blood samples from patients with MG and controls were analyzed for the expression of Fas by flow cytometry using monoclonal antibody PE-labelled anti-Fas (CD95). Fas expression in peripheral CD4-CD8+ T cells from MG patients with normal thymus was significant higher than in patients with thymoma ($P=0.017$). Fas expression in CD4+, CD8+ and CD4-CD8- T cell subsets was much higher in MG patients with normal thymus than in controls ($P=0.036$, 0.016 and 0.024). Fas expression in CD4+ and CD4+CD8+ T cell subsets was significantly higher in MG patients with thymoma than in controls ($P=0.037$ and 0.026).

FIGURE 4. Fas/CD95 expression in peripheral T cell subsets from MG patients with or without hyperthyroidism and controls. The fresh heparinized blood samples from patients with MG and controls were analyzed for the expression of Fas by flow cytometry using monoclonal antibody PE-labelled anti-Fas (CD95). Fas expressions in CD4+CD8+ T cells in MG patients with hyperthyroidism was significantly higher than in the ones without ($P=0.027$). Fas expressions in CD4+ and CD4+CD8+ T cells in MG patients with hyperthyroidism were significantly higher than in controls ($P=0.021$ and 0.012).

FIGURE 5. Fas/CD95 expression in peripheral T cell subsets from MG patients with or without corticosteroid treatment and controls. The fresh heparinized blood samples from patients with MG and controls were analyzed for the expression of Fas by flow cytometry using monoclonal antibody PE-labelled anti-Fas (CD95). Fas expressions in CD4-CD8⁺ and CD4-CD8⁻ T cells from MG patients with corticosteroid treatment were significant higher than in patients without treatment ($P=0.016$ and 0.035).

Association with clinical classification

There was no significant difference of Fas expression in peripheral T cells between patients with OMG and GMG ($P>0.05$).

Association with corticosteroid treatment

As shown in Fig. 5, Fas expressions in CD4⁺CD8⁺ and CD4⁺CD8⁻ T cells were significantly higher in MG patients with corticosteroid treatment than the ones without (CD4⁺CD8⁺: Mean Rank: 13.00 vs. 6.82, Chi-square: 5.818, $P=0.016$; CD4⁺CD8⁻: Mean Rank: 12.50 vs. 7.09 Chi-square: 4.455, $P=0.035$). Fas expressions in CD8⁺, CD4⁺CD8⁺ and CD4⁺CD8⁻ T cell subsets were significantly higher in MG patients with corticosteroid treatment than controls (CD8⁺: Mean Rank: 14.17 vs. 8.08, Chi-square: 4.808, $P=0.028$; CD4⁺CD8⁺: Mean Rank: 14.17 vs. 8.08, Chi-square: 4.808, $P=0.028$; CD4⁺CD8⁻: Mean Rank: 15.33 vs. 7.54 Chi-square: 7.877, $P=0.005$).

Discussion

In this study, Fas expression in the peripheral CD4⁺, CD4⁺CD8⁻ and CD4⁺CD8⁺ T cell subsets were observed to be up-regulated in patients with MG. Our data were not consistent with those from Moulian et al., which showed an in-

creased proportion of Fas^{hi} cell in thymocytes of MG patients with anti-AChR antibodies but not in the PBL [12]. Our results were in agreement with the observations in other systemic autoimmune disorders such as SLE [18,19,20] and RA [19] where Fas expression was up-regulated in the peripheral CD4⁺ and CD8⁺ T cell subsets.

The possible mechanism for the up-regulated Fas expression in the peripheral T cells in MG patients can be explained as follows. Firstly, the increased Fas expression in the peripheral T cells reflects lymphocyte activation in vivo. It has been demonstrated that activation of peripheral lymphocytes in vitro by mitogens or antigen receptor ligation can induce an increased Fas expression [21,22], and some T cell activation markers (e.g. MHC II, CD25, CD71, HLA-DR) were increased in the peripheral T cells of SLE patients [18,23,24]. The sensitivity or resistance of activated cells to Fas-mediated apoptosis depends on the activation state [25]. In addition, similar to what is observed in normal individuals [22], Fas expression was detected exclusively in the previously activated T cell subsets in SLE patients, which supports the concept that an increased Fas expression reflects an activation of lymphocyte. Secondly, the increased Fas expression in the peripheral CD8⁺ T cells may be functionally important in MG pathogenesis. It has been suggested that FasL was involved in the Ca²⁺-independent T cell-mediated cytotoxicity [26]. An increased Fas expression in

CD8⁺ T cells in MG patients may be associated with the expression of FasL, which probably promotes tissue injury by Fas⁺CD8⁺ T cell-mediated cytotoxicity. Furthermore, it was shown in vitro that CD8⁺ T cells are necessary for spontaneous IgG production by B cells of SLE patients, and CD8⁺ T cells in SLE may play an unusual assistant function due to abnormal cytokine production [27]. Thirdly, the increased Fas expression in the peripheral T cells may be due to a failure to eliminate peripheral Fas⁺ cells. It was shown that FasL plays a prominent role in the elimination of autoreactive cells in the periphery [3, 28]. In patients with SLE, the level of soluble FasL was elevated, which could inhibit Fas-mediated apoptosis [29]. Moreover, it was reported that recombinant IL-12 was able to inhibit Fas-mediated apoptosis in human peripheral CD4⁺ lymphocytes [30]. The accumulation of Fas⁺ cells in MG patients could be related to an increased level of some cytokines.

HT levels are higher in patients with MG than in the general population [15]. This association of MG and HT is more than a coincidence. Recent studies have suggested that HLA-DQ3 and elevated Thyroid-related Ab level may play a potential pathogenic role in the concomitant development of the two diseases [16, 17], but the exact mechanism is still undefined. In the current study, Fas expressions in peripheral CD4⁺CD8⁺ T cells in MG patients with HT was found to be significantly higher than in patients without HT, and Fas expression in CD4⁺ and CD4⁺CD8⁺ T cells in MG patients with HT were significantly higher than that in controls. Our results are consistent with the observations in Graves' disease and Hashimoto's thyroiditis [31,32], suggesting that increased Fas expression in the peripheral CD4⁺ cells may be one possible mechanism for the concomitant development of MG and HT.

According to different affected muscles, MG can be classified into OMG and GMG. Epidemiological, clinical, and serological studies suggested that OMG and GMG may be separate diseases with different pathogenesis [33,34,35]. In recent studies, no differences in MMP2, MMP3, MMP9 and anti-Hsp70 antibodies levels were observed between GMG and OMG patients. The similarities between GMG and OMG support OMG as a systemic disease, similar to GMG. In this study, no difference in Fas expression in peripheral T cells were found between OMG and GMG patients; an observation that strengthens the hypothesis that OMG represents a systemic disease, similar to GMG.

It was suggested that the use of corticosteroid could induce apoptosis in thymocyte [36] and PBL [37], and Fas/FasL system may be one of the possible pathways [36,38]. In this study, we observed that Fas expression in CD4⁺CD8⁺ and CD4⁺

CD8⁺ T cells of MG patients with corticosteroid treatment were up-regulated, which supports the concept that Fas-mediated apoptosis may be one of the possible mechanisms of corticosteroid treatment in MG.

In conclusion, our data provided evidence that the Fas antigen may play a role in the pathogenesis of MG and may be involved in the mechanisms of action of corticosteroid treatment and in the development of HT. OMG appears to represent a systemic disease, similar to GMG. Further studies will focus on how Fas controls apoptosis in MG and how autoantibodies, lymphokines and similar molecules contribute to the balance of activation, survival and death of lymphocyte populations.

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