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Thymic TFH cells involved in the pathogenesis of myasthenia gravis with thymoma

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ABSTRACT

Follicular helper CD4+ T (TFH) cells are the specialized providers of B cell help in germinal centers (GCs). Formation of GCs in thymi is the primary thymi characteristic in MG patients. TFH cells are involved in the pathogenic process of many autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis and autoimmune thyroid disease. The role thymic TFH cells played in MG with thymoma has not been elucidated. Here, we analyzed surface markers CXCR5, Bcl-6, ICOS and IL-21 on TFH cells in thymus derived from thymoma patients with ocular MG (OMG), generalized MG (GMG) or without MG using immunohistochemical staining, immunofluorescence, western blotting, and real-time PCR analysis. We show that clinical severity of MG is correlated with higher mRNA expression of the four markers. Indeed, results show higher expression of all four markers in thymoma with GMG patients compared with both OMG and non-MG patients. We found no significant difference in the expression of CXCR5, Bcl-6 and ICOS in OMG compared with non-MG patients. Regression analysis shows a positive correlation between thymic CXCR5, BCL-6, ICOS and IL-21 levels and quantitative MG score (QMGS) in GMG patients. In addition, we found no significant correlation between TFH cell expression and QMGS in OMG patients. Our findings show that higher expression of TFH cells in the thymoma is related to the clinical severity of MG and suggests a role in the pathogenesis of MG. However, the real source of these TFH cells is still uncertain and needs further study.

Introduction

Myasthenia gravis (MG) is a prototypical autoimmune disease that is mediated by antibodies against the acetylcholine receptor (AChR) of skeletal muscle at the neuromuscular junction (NMJ). Initial symptoms of MG usually manifest as ocular MG (OMG) with characteristic extraocular muscle weakness and ocular misalignment. Nearly 90% of OMG patients progress to generalized MG (GMG) within three years (Grob et al., 2008). However, in Asian countries such as China, nearly 50% of MG patients maintain purely ocular manifestations throughout their lifetime (Zhang et al., 2007). GMG causes impairment of skeletal and bulbar muscle, resulting in a more severe phenotype compared with MG. Furthermore, 80–90% of MG patients manifest thymic pathology, showing strong association between thymic alteration and clinical symptoms of MG (Marx et al., 1997). Standard treatment shows that thymectomy leads to a satisfactory improvement of clinical symptoms for MG patients and a suppression of both cellular and humoral immunity, and reduction in AChR antibodies (Remes-Troche et al., 2002). Although the generally accepted hypothesis in MG establishes the thymus as the disease initiating site, the differential immunological responses in thymoma from distinct clinical manifestations of OMG and GMG are still unknown.

An important aspect of a healthy immune response is $CD4^+$ T helper cells. They are grouped into subsets according to their various transcription factor expression, cytokine production, and subsequent immune functions (Huang et al., 2009). Recent studies have shown that a subset of $CD4^+$ T cells, named follicular helper T (TFH) cells, is specialized for helping B cells in germinal centers (GCs) (Fazilleau et al., 2009). Formation of GCs in thymi is the primary thymi characteristic in MG patients (Roxanis et al., 2002; Watanabe, 1971), which indicates an increased number of activated B cells (Ströbel et al., 2004). TFH cells express C-X-C chemokine receptor type 5 (CXCR5), inducible T-cell costimulator (ICOS) and interleukin-21 (IL-21), which are important for optimal TFH cell function (Laurent et al., 2010). The transcriptional repressor B cell lymphoma 6 protein (Bcl-6) is a major regulator of B cell differentiation and intimately associated with TFH cells. Bcl-6 regulates naive T cell development into mature TFH cells (Chtanova et al., 2004).

Evidence has demonstrated that TFH cells are related to the pathogenic process of many autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and autoimmune thyroid disease

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(Vinuesa et al., 2009; Yanaba et al., 2008; Zhu et al., 2012). As a cytokine, IL-21 plays an important role in the development of TFH cells. IL-21 deficiency would lead to a negative regulation of humoral immunity (Linterman et al., 2010; Spolski and Leonard, 2010).

Several studies have shown expansion of the circulating TFH cell population in MG patients (Luo et al., 2013; Matsumoto et al., 2006; Saito et al., 2005). Given that thymi play important roles in the pathogenesis of MG (Cavalcante et al., 2011), reports on TFH cells in thymus in MG are still controversial. The aim of the present study was to explore the immunological protein expression profile of TFH cells in thymoma from different clinical manifestations of OMG and GMG. We found higher expression of CXCR5, Bcl-6, ICOS and IL-21 in thymoma of GMG patients compared with OMG and non-MG patients. In addition, we show a positive correlation between thymic CXCR5, BCL-6, ICOS and IL-21 expressions, and quantitative MG score (QMGS) in GMG patients, but not in OMG patients. These data offer insights into higher TFH cellular expression in autoimmune diseases such as MG. However, we did not ascertain where these TFH cells originate, which may be from the thymoma, the surrounding normal thymus tissue, or the circulation, and that needs further study.

Methods

Samples

A total of 118 patients underwent thymectomy at the Department of Thoracic Surgery of Tangdu Hospital between 2009 and 2011. The experimental group was from 118 patient thymi with either OMG or GMG. The control group was from thymoma without MG (non-MG). All patients gave written informed consent and thymus tissues were used only for research purposes. The study conforms to the Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18 July 1964). The study protocol was approved by the Ethics Committee of Tangdu Hospital. The clinical data of all the subjects are shown in Table 1.

Data and sample collection

The diagnostic criteria of MG were previously described (Zhang et al., 2012). The exclusion criteria included: (1) no previous treatment with immunosuppressive drugs, such as corticosteroids or azathioprine; (2) no previous treatment with plasma exchange or intravenous immunoglobulin; (3) pregnancy; (4) diagnosis with other autoimmune diseases or inflammatory diseases; (5) diagnosis with type C thymoma or thymic hyperplasia; (6) positive to Musk antibody. OMG or GMG was identified according to the Myasthenia Gravis Foundation of America (MGFA) clinical classification (Jaretzki et al., 2000). The clinical data of all the subjects are shown in Table 1. The clinical severity of MG patients are evaluated by the quantitative MG score (QMGS) (Jaretzki et al., 2000).

Immunohistochemical (IHC) staining for Bcl-6 and ICOS

Tissues obtained from thymectomy were treated as previously described (Zhang et al., 2012). Before the immunostaining, all the studied tissue sections had been examined by two back to back pathologists on the H&E staining. The two experts were blind to both the experimental purpose and the experimental grouping. So, we are sure that all tissues studied were from thymoma. In this section, we make lymph node as positive control and lung tissue as negative control.

Antigen retrieval was performed by the ethylene diamine tetraacetic acid (EDTA) microwave vacuum histoprocess. Endogenous peroxide activity was quenched by 3% (*v*/*v*) hydrogen peroxide in methanol. Tissue was blocked 30 min in a drop of 10% (*v*/*v*) donkey non-immune serum.

Sections were incubated at 4 °C overnight with rabbit anti-human Bcl-6 monoclonal antibody (mAb; sc-858, Santa Cruz biotechnology, USA) diluted 1:100 (*v*/*v*) in phosphate buffered saline (PBS); rabbit anti-human ICOS (mAb; sc25585, Santa Cruz biotechnology, USA) diluted 1:100 (*v*/*v*) in PBS. Then, the following antibodies were added: biotinylated second antibody for 20 min; streptavidin/horseradish peroxidase (HRP; Invitrogen, UK, catalog number 85–9043) for 20 min. Color development was performed with a DAB Substrate Kit (Sigma–Aldrich, USA) for 10 min. Positive staining defined as dependent on the proportion of positive staining cells (S1): <5% scored 0, 6–25% scored 1, 26–50% scored 2, 51–75% scored 3, and >75% scored 4; staining intensity (S2): colorless scored 0, flavescent scored 1, yellow scored 2, and brown scored 3. S1 was multiplied by S2 to obtain an estimate of the total IHC score for each molecule.

Double-labeled immunofluorescence (IF) for TFH cell expression

Tissues were obtained, de-waxed, hydrated through a graded ethanol series, and washed. Antigen retrieval and endogenous peroxide activity were assayed similar to IHC staining. Tissue was incubated at 37 °C 4 h with rabbit anti-human CD4 antibody (A0846, assay biotech), washed three times. The tissue was incubated at 37 °C 4 h with mouse anti-human CXCR5 monoclonal antibody (1:100 in PBS, Ab89259, Abcam). Sections were incubated at 37 °C for 2 h with dylight™ 488-conjugated affinipure donkey anti-rabbit IgG (1:1000 in PBS; 711-485-152, 94861, Jackson Immunoresearch, USA). Sections were incubated at 37 °C for 2 h with dylight™ 549-conjugated affinipure donkey anti-mouse IgG (1:1000 in PBS; 715-505-150, 95149; Jackson Immunoresearch, USA). Finally, the sections were washed 3 times in PBS, mounted and coverslipped using SlowFade® Gold antifade reagent (Molecular Probes, USA).

Western blot (WB) analysis for Bcl-6 and ICOS

There were 5 GMG thymoma tissues, 6 OMG thymoma tissues and 6 non-MG thymoma tissues enrolled with western blot analysis. Samples were de-waxed, hydrated through a graded ethanol series. Total protein of lysates was determined by the BCA method. Protein (10 μg) was loaded on 12% Tris–glycine gel and transferred to nitrocellulose membranes. Blots were blocked in 5% milk in PBS with 0.1% Tween-20 PBST for 2 h, then incubated with diluted antibodies (Bcl-6, ICOS) at 4 °C overnight. Secondary antibody was added at 37 °C for 1 h. After washing, blots were developed with the ECL chemiluminescence system (GE Healthcare) and signals were captured on X-ray films.

Table 1
Clinical characteristics of all subjects included in study.

Groups	n	QMGS	Thymoma histology					MGFA				Anti-AChR	
			A	AB	B1	B2	B3	I	II	III	IV	Positive	Negative
GMG	14	18.64	1	5	4	2	2	0	8	5	1	11	3
OMG	18	7.42	1	6	3	5	3	18	0	0	0	10	8
Non-MG	19	N/A	1	6	5	5	2	N/A	N/A	N/A	N/A	N/A	N/A

GMG: generalized myasthenia gravis with thymoma; OMG: ocular myasthenia gravis with thymoma; Non-MG: without myasthenia gravis but with thymoma; QMGS: quantitative MG score; MGFA: Myasthenia Gravis Foundation of America Clinical Classification; Anti-AChR Ab, anti-acetylcholine receptor antibody; N/A, not available.

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