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Thymoma-associated myasthenia gravis: On the search for a pathogen signature

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ABSTRACT

Myasthenia gravis (MG) is an autoimmune disease mainly mediated by anti-acetylcholine receptor (AChR) antibodies. In the late onset, a thymoma, tumor of the thymus, is quite frequent. However, the events leading to thymoma and MG are not understood. As thymoma-associated MG (MG-T) patients also display anti-interferon type I (IFN-I) neutralizing antibodies, we investigated if MG-T could be associated with an anti-viral signature.

RT-PCR analyses demonstrated huge increases of IFN-I subtypes, IFN-α2, -α8, -ω and -β, in thymoma-associated MG but not in thymomas without MG or in control thymuses. Next, we investigated if dsRNA signaling pathway involvement could be observed in MG-T, as recently observed in early-onset MG. We observed an abnormal regulation of dsRNA-sensing molecules with an increase of toll-like receptor 3 (TLR3), and a decrease of protein kinase R (PKR) and dsRNA helicases (RIG-I and MDA5) in thymoma from MG patients. We also detected a decreased expression of p53, the tumor suppressor that is known to be down-regulated by dsRNA. Altogether, these results strongly suggest that MG-T could be linked to a viral infection.

As p16 (CDKN2A), a marker of HPV infections, was up-regulated in MG-T, we thus screened DNA from thymomas for human papillomavirus (HPV) by real-time PCR using HPV consensus SPF10 primers. RT-PCR results were negative for all samples tested. We confirmed the absence of HPV DNA detection by end point PCR using FAP primers to amplify a larger panel of HPV genotypes.

Our data clearly demonstrate INF-I overexpression together with the activation of innate immunity pathways in thymoma-associated MG suggesting that MG might develop after a pathogen infection. We were not able to relate thymoma to HPV infections and the implication of other pathogens is discussed.

1. Introduction

Acquired Myasthenia gravis (MG) is a neurological autoimmune disease mainly caused by autoantibodies against the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction. These autoantibodies cause the loss of functional AChR, disturb neuromuscular transmission and lead to excessive muscle fatigability [1,2]. In MG, if the target organ is the muscle, the effector organ is the thymus which is often abnormal. Thymus can display a thymic hyperplasia characterized by ectopic germinal center development or a thymoma [3,4].

Abbreviations: AChR, acetylcholine receptor; AIRE, autoimmune regulator; dsRNA, double-stranded RNA; EBV, Epstein–Barr virus; IFN, interferon; IFNAR, IFN-I receptor; ISG, interferon-stimulated gene; HPV, human papillomavirus; MDA5, melanoma differentiation-associated gene 5; EOMG, early-onset myasthenia gravis (without thymoma); MG-T, myasthenia gravis with thymoma; PKR, protein kinase R; Poly(I:C), polyinosinic-polycytidylic acid; RIG-I, retinoic acid-inducible gene I; TEC, thymic epithelial cell; TLR, toll-like receptor.

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Thymomas are neoplasms of thymic epithelial cells (TECs). More than 90% of patients with thymoma can develop autoimmune diseases but mainly MG [5]. The high incidence of autoimmune diseases development might be due to the defective expression by TECs of HLA class II molecules known to play an essential role in intrathymic T-cell differentiation [6], and/or of the autoimmune regulator (AIRE) [7]. Indeed, AIRE normally controls the expression of tissue-specific antigens, such as the α-AChR subunit, necessary to avoid the emergence of autoreactive T cells [8,9]. In thymomas, maturation and export of regulatory T cells are also impaired [10].

MG with thymoma are especially associated with antibodies against AChR, and antibodies against the muscle specific kinase (MuSK) are extremely rare. Patients can also display autoantibodies against intracellular muscle protein such as actin, myosin, ryanodine, and titin but their role in the pathogenesis of MG-T is unclear [11]. In MG-T, the absence of thymic myoid cells, that probably play a role in the induction of immunological tolerance towards muscle proteins, could explain the presence of these autoantibodies [4]. Surprisingly, MG-T patients have also very high titers of neutralizing antibodies against interferon type I (IFN-I) or interleukin (IL)-12 [12]. These autoantibodies against IFN-I subtypes can also be observed spontaneously in systemic lupus erythematosus [13] or in multiple sclerosis patients in response to IFN-β treatments [14] and in response to viral infections [15,12]. IFN-I molecules are known to play a central role in innate immunity. They are expressed by various cell types upon pathogen infection and orchestrate the expression of numerous IFN-I-stimulated genes that aim at eliminating the infection. The thymus is a common target organ for infectious diseases [16]. We also recently demonstrated that Poly(I:C), mimicking dsRNA from viral infection, induced specifically thymic overexpression of α-AChR through the release of IFN-β, and induced an anti-AChR autoimmune response including anti-AChR antibodies [17]. We hypothesized that inappropriate thymic release of IFN-I subtypes in response to pathogen infection could elicit MG. We then searched for a viral signature in MG-T thymus and a link with human papillomavirus (HPV) infection.

2. Materials and methods

2.1. Human thymic samples

Control thymic biopsies were obtained from patients undergoing cardiovascular surgery: infants (under 3-year-old) for thymic epithelial cell (TEC) cultures or adults (21–67 years-old) for RNA and DNA extractions. Thymoma biopsies came from thymectomy of patients (28–69 years-old) with a thymoma associated with MG (MG-T, $n = 18$) or without MG (T, $n = 6$) and details are given in Supplementary Table S1. The WHO classification of a thymoma is based on the nature of the cortical or medullary epithelial cells involved in the tumor: Type A (medullary thymoma), type B1 or B2 (mainly or entirely cortical thymoma), type AB (mixed medullary and cortical thymoma) or type B3 (atypical thymoma, squamoid thymoma, and well-differentiated thymic carcinoma). Our study includes thymoma of B1, B2 and AB types only. Type B2 is the most frequently associated with MG-T, followed by AB and B1 types [18]. Thymoma or adjacent thymic biopsies were used in these analyses (Table S1). All the studies on thymuses were approved by the local Ethics Committee (CCP agreement N°C09-36 – France).

2.2. Human thymic epithelial cell (TEC) cultures

All reagents used for cell cultures were from Invitrogen (Cergy-Pontoise, France) unless otherwise specified. TECs were obtained from thymic explant cultures as fully detailed in Cufi et al. [17]. For all experiments, TECs were seeded (1.4×10^5 cells/cm^2) in RPMI-5% horse serum (Eurobio, Les Ulis, France) and, after 24 h, treated in RPMI-0.5% horse serum for 24 h with Polyinosinic-polycytidylic acid (Poly(I:C), Invivogen, Toulouse, France) 100 μg/ml or IFN-I subtypes 1000 UI/ml: recombinant human IFN-I (11200), recombinant human IFN-β (11415) both from R&D Systems (Lille, France), and recombinant human IFN-α2b (Intron A injection kit, Schering-Plough (Belgium)).

2.3. Reverse transcription and real-time PCR

As previously described [19], total RNA extracted from cells or tissues was reverse-transcribed and analyzed by real-time PCR on the LightCycler 480 system using primers listed in Supplementary Table S2.

2.4. HPV genotyping

We used the SPF10 primers which are part of the INNO-LiPA HPV Genotyping *Extra* AMP (Innogenetics, Les Ulis, France) kit and real-time PCR assay protocol to detect HPV DNA in human thymic samples. HPV (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 69, 70, 73, 74, 82) are detectable with this approach [20]. Briefly, SYBR green-based real-time PCR was performed on a final reaction volume of 20 μl containing 10 μl SYBR green PCR Master mix (Applied Biosystems, Life Technologies, Saint Aubin, France), 0.15 μM of each forward and reverse SPF10 set of primers and 2 μl of template. The target DNA was amplified by real-time PCR (Applied Biosystems 7500) with SYBR green dye for detection and consists on 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 56 °C. The PCR products were subjected to melting curve analysis to determine the presence of HPV DNA. In addition, to increase the panel of HPV genotypes detected, end point PCR using FAP59 forward: 5′TAACWGTIGGICAYCCWTATT3′ and FAP64 reverse: 5′CCWATATCWVHCATITCICCATC3′ primers were used.

2.5. Statistical analyses

In the text or in bar graphs, results are expressed as means of different experiments or samples, or means of duplicate values when a representative experiment is displayed. Error bars represent SEM. For 2-by-2 comparisons, non-parametric Mann–Whitney tests were applied.

3. Results

3.1. Overexpression of IFN-I subtypes in the thymus of MG-T patients

Patients with thymoma are characterized by the presence of anti-IFN-I antibodies, mainly IFN-α2 and IFN-ω [12]. Accordingly, we investigated the level of mRNA expression for these IFN-I subtypes in thymomas compared to thymus of non-MG adult donors. We observed a high overexpression of IFN-α2 (around 92 and 47 times higher in type AB and B MG-T, respectively) and IFN-ω (around 33–35 times higher in both type AB and B MG-T) but also of IFN-α8 (around 146 and 156 times higher in type AB and B MG-T, respectively). These increases were observed in all thymomas with MG but not in those without MG (Fig. 1A–C). Comparing the levels of expression of IFN-I subtypes in thymoma and adjacent thymic biopsies for the same MG-T patient, we observed that the increases were specifically observed in thymoma but not in the surrounding thymic tissue (Fig. 2A–C). Along with this observation, Shiono et al. observed that thymic cells extracted from thymoma of MG patients spontaneously produce more anti-IFN-α antibodies than thymic remnants [21].