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# Comparison of histology and immunohistochemistry of thymus between young- and elderly-onset myasthenia gravis without thymoma

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**Running title**: Elderly-onset MG without thymoma

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## Abstract

We performed histological and immunohistochemical analyses of the removed thymus in 20 elderly (onset age >60 years) and 23 young (onset age <40 years) patients with myasthenia gravis (MG) who showed positive anti-acetylcholine receptor (AChR) antibodies in serum without associated thymoma. In the elderly group nine (45%) demonstrated accumulations of lymphocytes indicating atrophied thymus without the basic structure. The elderly MG patients with atrophied thymic tissues showed higher titers of the anti-AChR antibody (59.6±81.0 nmol/l) than those with adipose tissue alone (20.1±20.9 nmol/l). In immunohistochemistry both the young group and elderly patients with atrophied thymic tissues showed significantly higher levels of CD20 on image analysis than the age-matched controls ($p<0.005$). Atrophied thymic tissues as often seen immunohistochemically in young MG patients might be found also in the elderly ones, particularly in those with high titers of the anti-AChR antibody, even though fat infiltration is remarkable.

**Keywords**: Elderly patient, Immunohistochemistry, Lymphocytes, Lymphofollicular hyperplasia, Myasthenia gravis, Thymus

## Introduction

Myasthenia gravis (MG) is an autoimmune disorder in neuromuscular junctions mainly resulting from pathognomonic activity of anti-acetylcholine receptor (AChR) antibodies in serum (1, 2). In this disease the thymus plays an important role in the induction of the anti-AChR antibodies, and at the present time total thymectomy is accepted as a standard therapy irrespective of associated thymoma, particularly for the generalized type (1, 2). Although typical MG is likely to develop in the second to fourth decades (1), the number of elderly patients with this disease has recently increased in Japan as well as in Europe and the United States (3-6). Because elderly-onset MG patients without thymoma are considered to commonly show extensive fat infiltration in the thymus, it remains unclear whether total thymectomy is the best therapeutic option for these patients or not. To retrospectively address this clinical problem, we carried out histological and immunohistochemical analyses of the thymus removed from both young- and elderly-onset MG patients without thymoma. Approximately a half of the elderly-onset MG patients showed accumulations of lymphocytes indicating atrophied thymic tissues with abundant fat infiltration on histopathology, and immunohistochemistry demonstrated a significant increase in CD3, CD4, CD8, CD20 and CD79a compared with the age-matched controls. A significantly elevated level of CD20 was seen also in the young-onset MG patients irrespective of the presence of hyperplasia in comparison with the age-matched controls. We postulate that thymic abnormality may be present both in the young-onset MG group and also in the elderly-onset group, although the frequency in the latter is much lower than in the former.

## Materials and methods

Patients

Twenty-three young MG patients (onset age <40 years; 7 men and 16 women; age

range, 16 to 39 years; mean, 30.1±7.3 years) and twenty elderly (onset age >60 years; 6 men and 14 women; age range, 61 to 79 years; mean, 68.3±6.2 years) were enrolled in this study. All patients were positive for the anti-AChR antibody in serum on admission to our hospital, and treated with transsternal extended thymectomy within one year after onset between 1992 and 2002. Clinical information, including grade of disease according to the established clinical classification (7) and anti-AChR antibody titers, was obtained from their medical records. As age-matched controls we used the thymuses obtained at autopsy from 6 young (2 men and 4 women, mean age: 29.7±9.6 years) and 6 elderly patients (3 men and 3 women, mean age: 71.5±3.6 years) who had died of non-immunological disorders.

Pathology of the thymus

Serial three-μm-thick sections prepared from the removed thymus were stained with hematoxylin-eosin, and a skilled pathologist (JN) made pathological diagnoses. There are three histopathological diagnoses of the thymus in MG without thymoma: lymphofollicular hyperplasia, thymic atrophy and adipose tissue alone. Hyperplasia is characterized by the appearance of lymphoid follicles, which are usually round-shaped accumulations of lymphocytes with or without a germinal center on microscopic examination, while thymic atrophy shows residual thymus with a varying degree of fat infiltration. When lymphocyte accumulations could not be found even by intensive examination of multiple sections, the thymus was histologically diagnosed as having adipose tissue alone.

The rest of the serial sections were simultaneously immunostained with a monoclonal antibody using a Super Sensitive detection kit (BioGenex Laboratories, San Romon, CA) based on the biotin-streptavidin-peroxidase method (8). The following monoclonal antibodies were employed in our study: anti-CD3 (PC3/188A, DAKO, Glostrup, Denmark), CD4 (1F6, Novocastra Laboratories, Newcastle, UK) and CD8 (C8/144B, DAKO) as T cell markers, anti-CD20 (L26, DAKO) and CD79a (HM57,

DAKO) as B cell markers, and anti-APO-1/Fas (APO-1, DAKO) as an apoptosis-related marker. Briefly, deparaffinized tissue sections were treated with 0.3% $H_2O_2$ solution in methanol and then blocked with 1% normal goat serum in Tris-buffered saline (TBS). Sections were incubated with each antibody for 1.5 hrs. After washing with TBS they were incubated with biotinylated anti-mouse IgG (DAKO) and then with horse radish peroxidase-labeled streptavidin (DAKO). The peroxidase reaction was developed with a diaminobenzidine/ $H_2O_2$ solution and counterstaining was performed with hematoxylin. After washing with running tap water, sections were mounted with Glycergel (DAKO). In control experiments performed by replacing the primary antibody with normal mouse serum, no specific staining was found.

Quantitative evaluation of immunohistochemical stainings

Areas positive for immunohistochemical stainings were evaluated using an image analyzing system (BX50, Olympus, Tokyo) and commercially available software (Adobe Photoshop for Macintosh; Adobe Systems, San Jose, CA, USA, and MacSCOPE; Mitani, Fukui, Japan). The image of each immunostained section divided into squares of 3.5 $mm^2$ was transferred to a TV screen through the microscope, and the positive and negative reactions were separately identified according to the color tone. The ratio of positive reaction area to the total area (positive and negative reaction area) was automatically determined in 10 visual fields in each section.

Statistics

To determine statistically significant differences between the patients and age-matched controls, Mann-Whitney's U test was employed for anti-AChR antibody titers before thymectomy and positive area ratios on immunohistochemical stainings. The results represent the mean$\pm$standard deviation where applicable, and a p-level less than 0.05 was considered to be statistically significant. Commercially available statistics software was used for data analysis (StatView for Macintosh, Abacus Concepts, Berkeley, CA, USA).

## Results

Clinical profiles and histopathology of the thymus

Clinical profiles and histology of the thymus in MG patients and controls are summarized in Table 1. The most common grade of disease was IIa in both the young and elderly MG patients. As an initial symptom blepharoptosis was most commonly seen in both the young and elderly MG patients, and more than 90% of patients showed ocular manifestations in each group. There was no significant difference in anti-AChR antibody titers before thymectomy between the young and elderly MG patients. All patients were treated with anti-cholinesterase drugs alone before thymectomy. Soon after staring oral prednisolone at an initial dose of 30 mg/day following the thymectomy, clinical symptoms of MG clearly improved in all the patients.

On histopathology lymphofollicular hyperplasia was seen in 15 young MG patients (65.3%) but not in any elderly ones. Eight young (34.7%) and 9 elderly (45.0%) MG patients showed accumulations of lymphocytes indicating thymic atrophy with a varying degree of fat infiltration. The thymus was completely replaced by adipose tissue without detectable accumulation of lymphocytes in the rest of the elderly MG patients. All of the young and elderly controls showed thymic atrophy and adipose tissue alone, respectively. In the young groups, comprising MG and age-matched controls, the basic structure of the thymus was well preserved, and the distinction between the cortex and the medulla was relatively clear. In all thymic tissues from the elderly MG group the basic structure of the thymus could not be recognized in any specimen. The anti-AChR antibody showed higher titers in lymphofollicular hyperplasia (182.1±371.4 nmol/l) than in thymic atrophy (138.2±269.5 nmol/l) in the young MG patients, and in thymic atrophy (59.6±81.0 nmol/l) than in adipose tissue alone (20.1±20.9 nmol/l) in the elderly ones, although there were no significant differences.

Immunohistochemistry of the thymus

In the young MG patients and age-matched controls CD3-, CD4- and CD8-positive cells were diffusely distributed in the thymus, but predominantly so in the cortex. CD20- and CD79a-positive cells were mainly observed in the medulla but also in lymphoid follicles in the MG patients with lymphofollicular hyperplasia (Fig. 1). A few APO-1/Fas-positive cells were present in the thymus. In the elderly MG patients with thymic atrophy no positive cells showed a specific distribution (Fig. 1).

Results of image analysis of immunohistochemical stainings are demonstrated in Table 2. CD20 was significantly higher in the young MG patients with lymphofollicular hyperplasia ($p<0.001$) or thymic atrophy ($p<0.005$) than in the age-matched controls. The young MG patients with lymphofollicular hyperplasia showed a significant increase in CD20 also in comparison with those with thymic atrophy ($p<0.001$). There was no significant difference in any other positive cells between the young MG patients and age-matched controls. In the elderly MG patients with thymic atrophy, a significant increase was seen in CD3, CD4, CD8, CD20 and CD79a compared with those with adipose tissue alone ($p<0.0005$) or the age-matched controls ($p<0.005$).

## Discussion

In general the thymus is gradually replaced by fat tissues after birth, and results in atrophy in parallel with aging in normal individuals. Also in MG patients without thymoma the thymus frequently seems atrophic in both microscopic and gross appearance, particularly in elderly patients, despite the presence of architectural changes such as distended perivascular space and disrupted basement membrane (9-11). In the present study severely atrophic thymus with abundant fat infiltration was seen in nine of the elderly MG patients (45%), and this positive rate was similar to that previously described in postmortem examination (12), while all of the age-matched controls showed adipose tissue alone. Young MG patients showed thymic tissues at a varying degree with or without hyperplasia. The anti-AChR antibody titers before treatment

were higher in the elderly MG patients with atrophic thymus than in those with adipose tissue alone, although there was no significant difference. These results suggest that lymphocyte accumulations indicating residual thymus are seen in young-onset MG patients but also sometimes in elderly ones, and may contribute to the immunopathogenesis and progression of MG as a possible site for the induction of anti-AChR antibodies (1, 2). The therapeutic efficacy of thymectomy in the elderly-onset MG could not be evaluated in the present study because administration of oral prednisolone was started soon after surgical treatment in all the patients.

The most important finding in the present study is the results of immunohistochemical analysis of the thymus in the elderly MG patients. The precise pathogenetic mechanism of MG without thymoma remains unclear, but it is thought that anti-AChR antibodies are induced mainly in thymic tissues by the interaction of several cellular components, including lymphocytes, myoid cells, interdigitating cells and epithelial cells [2]. Among these, lymphocytes are considered to be central to the production of anti-AChR antibodies based on the findings that the thymus contains many T and B cells specifically reactive to AChR (13-15). Several recent reports have demonstrated that numerous B cells are present in the medullary area and lymphoid follicles in MG thymus, and often show surface markers suggestive of activation, including CD23 and L29 (16-18). In the present study also, both the young group and elderly MG patients with atrophied thymic tissues showed a significant increase in B cells as indicated by CD20 and/or CD79a compared with the age-matched controls. Considering the coexistence of a significant increase in T cell-markers such as CD3, CD4 and CD8, interaction of T and B cells in the thymus might have contributed to the development and disease progression of MG also in the elderly patients with atrophied thymic tissues. These results suggest that the thymus in MG without thymoma is largely different from that in controls irrespective of the onset age in its immunohistochemical aspects, even though fat infiltration progresses in parallel with aging. With respect to the

sustained production of anti-AChR antibodies, several apoptosis-related molecules have recently been investigated in MG thymus, and the expression of APO-1/Fas remains controversial (19-22). In the present study no significant difference was seen in this surface molecule between age-matched controls and either the young or the elderly MG patients.

In conclusion, the thymus in elderly MG patients rarely shows lymphofollicular hyperplasia, but sometimes demonstrates atrophied thymic tissues with a varying degree of fat infiltration, particularly in patients with high titers of the anti-AChR antibody in serum. This pathology is probably related to the pathogenesis of elderly-onset MG, and thymectomy might be a therapeutic option when clinical symptoms cannot be sufficiently controlled only by immunomodulatory treatments such as immunosuppressants and plasmapheresis.

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**Figure legends**

Figure 1: Immunohistochemical stainings of the thymus in representative cases of young- (left column) and elderly-onset (right column) myasthenia gravis (MG). In the young MG patient with lymphofollicular hyperplasia CD3-, CD4- and CD8-positive cells were diffusely distributed in the thymus but predominantly in the cortex (arrows), whereas CD20- and CD79a-positive cells were mainly observed in the medulla but also in lymphoid follicles (arrowheads). A few APO-1/Fas-positive cells were present in the thymus. In the elderly MG patient with thymic atrophy no positive cells (arrows) showed a specific distribution. Bar =100 μm.