行政院國家科學委員會專題研究計畫　成果報告

苗勒氏肌在甲狀腺上眼瞼攣縮致病機轉的角色

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Purpose: Among the clinical manifestations, upper lid retraction is the most common ocular sign of Graves' ophthalmology (GO). Although several mechanisms have been postulated, the exact pathology of Müller's muscle in pathogenesis of upper lid retraction is not fully elucidated. To study the relation between tissue pathology and the severity of upper lid retraction, we have created an innovative methodology to quantify the pathological changes.

Methods & Materials: Twenty GO Müller's muscles were consecutively collected, and the severity of upper lid retraction measured by MRD_1 was also recorded. The excised Müller's muscle was fixed in formalin and sectioned in 3-μm thickness, which was stained serially with H&E, Toluidine blue, Masson's trichrome stain, and several immunohistochemistries including LCA, CD3, CD20, CD68, HLA-DR, caspase 3, and TSHr.

Results & Conclusion: We showed that the degree of fibrosis in the diseased Müller's muscle correlated with the severity of upper lid retraction especially in patients with severe GO. In addition, normal muscle volume was decreased and replaced by fat and fibrosis infiltration. However, there was no evidence of apoptosis in Müller's muscle. On the other hand, the number of macrophages increased in most of diseased Müller's muscles with predominant fibrotic changes, providing a role for macrophage during fibrogenesis.

二、緣由與目的

Manifestations of Graves' disease include thyrotoxicosis and several extra-thyroidal signs including ophthalmopathy, dermopathy, and acropachy.[1] Approximately 25-50% of patients may have ocular complications, termed Graves' ophthalmopathy (GO) or thyroid eye disease (TED). Although it is widely accepted that Graves' disease is an autoimmune disease and that the primary antigen is the thyrotropin receptor (TSHr),[2,3,4] the pathogenesis of GO remains poorly understood. Most believe that it is also autoimmune and involves shared autoantigen(s) in thyroid and orbital tissues.[5,6,7,8,9]

Nearly 90% of patients with Graves' disease manifest upper lid retraction, which is the most common ocular sign[10]. Nevertheless, much debate exists concerning whether pathological changes of Müller's muscle are a consistent finding in GO patients. Although fatty and fibrotic changes have been found in GO Müller's muscle, the correlation between the pathological changes and clinical severity of upper lid retraction is lacking.[11] Therefore, in this study, we aimed to elucidate the molecular pathology occuring in Müller's muscle in GO and to address the relation between histopathological changes and the severity of upper lid retraction.

三、Methods & Materials

Subjects and Collection of Müller's Muscle

Twenty subjects (2 males and 18 females) with GO who received Müller ectomy were consecutively collected. Müller's muscle was collected from a scheduled operation for thyroid lid retraction of GO patients who are stable in thyroid and ophthalmic state for at least 3 to 6 months before operation. Another group of normal Müller's muscle was collected from 5 patients (4 female, 1 male), ages from 45 to 73 years, who underwent a correction of ptosis due to non-thyroidal causes.

Special Stain and Immunohistochemistry

The excised Müller's muscle was dipped immediately into 10% buffered formalin for overnight at 4℃. After a gradient dehydration, the tissue was then embedded in paraffin. Sections of 3-μm thickness were stained with hematoxyline and eosin (H&E), Toluidine blue, Masson's trichrome stain, and several immunohistochemical analyses. These include LCA, CD3, CD20, CD68, HLA-DR, caspase 3, and TSHr. Incubation period for each primary antibody is dependent on manufactures' recommendation. To quantify the inflammatory cells, the number of cells with positive stain for Toluidine blue, LCA, CD3, CD20, CD68, TSHr, HLA-DR, and caspase 3 was counted manually using a light microscope at ×200 high power fields by two independent pathologists.

Measurement of the severity of upper lid retraction in GO patients

The degree of upper lid retraction was measured by documenting the distance from the corneal light reflex to the upper lid margin, which exhibited as the upper marginal reflex distance ($MRD_{1,}$ mm).

Quantification of the Pathological Compositions in Müller's Muscle

Using Masson's trichrome stain, mainly pathological components can be distinguished, i.e. red to purple for muscle, blue for fibrosis. Using Adobe Photoshop, version 7.0.1 [3900×3090 pixels] (Adobe Systems, San Jose, CA), different pathological composition could be quantified based on a discrepant character of marked color for each component.

Statistical analyses

Data were presented in means and SD. The correlation between different pathological composition and the severity of upper lid retraction was evaluated using Pearson's correlation.. The differences of MRD_1 and the differences in pathological composition were analyzed by Wilcoxon Rank-Sum test. All statistical analyses were performed with SAS software (SAS Institute, Cary, North Carolina). A $p< 0.05$ was considered significant statistically.

四、Results

Quantitatively Pathological Analyses of Müller's Muscle

Using Masson's trichrome, there was no existence of fibrotic composition in normal Müller's muscle (Fig. 1A). While a variable degree of fat and fibrosis, as well as, a decrease in normal muscle volume, were noted in the thyroid Müller's muscle (Fig. 1B). There were significant differences of an increase fibrosis ($p=0.006$) and a decrease in muscle ($p<0.001$) composition between the two groups, compared with those in normal Müller's muscle.

As we analyzed the data of patients with more severe upper lid retraction, the value of MRD_1 was $\geq$ 8 mm, and the pathological composition contains plenty of fibrosis tissues. It disclosed that the marked fibrosis in Müller's muscle might correlate with more severe upper lid retraction. The analytical results are summarized in Table 1.

Semi-quantification of Inflammatory Cells and Immunohistochemistry

The results of special stains and immunohistochemistry are illustrated in Table 2. The numbers of T cells, in normal Müller's muscle, were also higher than those in the thyroid group; however, this did not achieve a significant level, statistically ($p=0.161$). Neither cells stained positive of CD20 or TSHr or HLA-DR in both groups. To determine the possibility of decreased muscle composition correlating to an apoptotic process, we tried to stain the specimens with caspase 3, which is a negative result.

The Relationship Between Infiltrated Inflammatory Cells and the Pathological Compositions and Severity of Upper Lid Retraction

In association analyses, a positive correlation between mast cells counts and the fat composition was noted (r=0.56, p=0.019), in contrast, a negative correlation exited between macrophage counts and fat composition (r=-0.389, p=0.123). (Fig.2A and 2B) Conversely, a positive correlation between macrophage counts and the fibrotic composition (r=0207, p=0.405), and a negative correlation between the fibrotic composition and mast cell counts were obtained (r=-0.727, p=0.001). (Fig.3A and 3B) There was no significantly positive correlation between macrophages and fibrosis, which was almost observed in specimens with predominant fibrosis. Under further analyses by collecting simultaneously two inflammatory cells, the relationship between increased macrophage counts and more severe fibrosis became obvious and significant as fibrotic percentage increased up to 30% in specimens (p=0.006), and this association remained in the predominant fibrotic group (p=0.004).

五、結果與討論

To cope with pathological heterogeneity, which pervaded within Müller's muscle, we have developed an innovative design using an image processing system of Adobe Photoshop software to analyze the pathological composition more precisely and objectively. Mosedale et al[12,13] attempted to postulate the concept of quantitative

immunohistochemistry (Q-IHC), using single-channel grayscale that was transformed from the 256 separate shades of red, green, and blue as working in 24-bit RGB color, to avoid inter- or intra-observer variation of the traditional IHC. Using this method, the pathological changes in the diseased Müller's muscle have been confirmed and the severity of upper lid retraction could be correlated with the degree of fibrosis composition especially in a more severe subgroup.

On the cellular level, the macrophage counts increased in most of diseased Müller's muscle specimens and were higher than that of mast cells in predominantly fibrotic specimens, suggesting an association between macrophage propagation and fibrogenesis. Using IHC, there was no evidence of cells stained with TSHr, a potential autoantigen shared between the thyroid gland and orbital tissues. Our data suggest that there were two possibilities for this finding: (1) a low abundance of TSHr contained in thyroid Müller's muscle, which can not be detected with IHC, (2) TSHr is not the major autoantigen in thyroid Müller's muscle. The existence of TSHr involving thyroid Müller's muscle, using PCR or quantitative PCR amplification, remains to be studied in the near future.

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Table 1. Correlation between the severity of upper lid retraction and pathological composition of fibrosis

	Differences of composition in fat and fibrosis proportion * (%)		P value †
	N	Mean ± SD	
$MRD_1 < 8$ mm	15	-11.17 ± 26.96	0.208
$MRD_1 \geqq 8$ mm	16	-12.24 ± 19.24	**0.044**
P value [c]		0.767	

[*] The value was obtained from, which the fibrosis proportion was subtracted from fat proportion individually

[†] Within the group, based on Wilcoxon Signed-Rank test

[c] Between the group, based on Wilcoxon Rank-Sum test

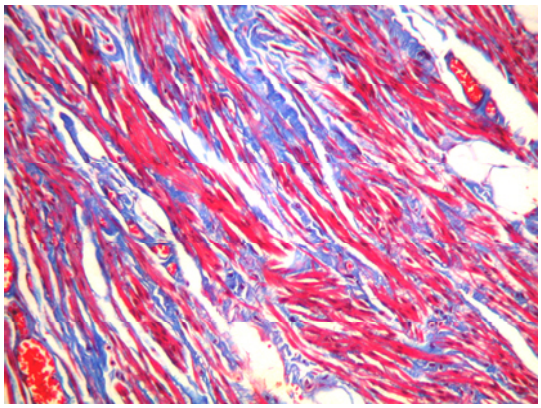
Table 2. Summary of inflammatory cells and immunohistochemistry in the two groups

	Mast cells	CD 68	CD3	CD20	LCA	HLA-DR	TSHr	Caspase 3
Disease group	29.58±14.03 (5.00~60.00)	30.71±14.95 (12.00~65.00)	8.24±6.41 (0.00~25.00)	(-)	36.29±14.03 (15.00~68.00)	(-)	(-)	(-)
Control group	60.00±18.76 (40.00~97.00)	14.57±3.64 (10.00~20.00)	10.14±1.95 (8.00~13.00)	(-)	54.14±17.24 (20.00~70.00)	(-)	(-)	(-)
P value*	**<0.001**	**0.004**	0.161		**0.023**			

* Based on Wilcoxin Rank-Sum test

Fig. 1

(A)



(B)

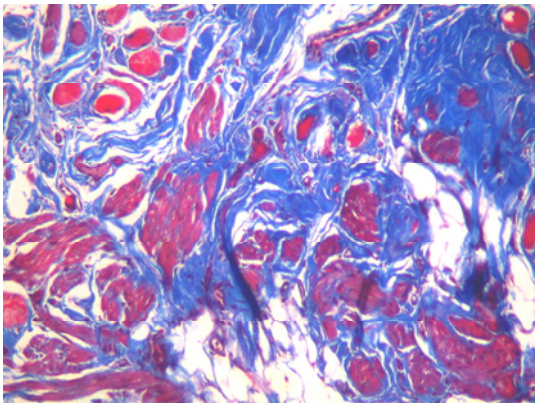
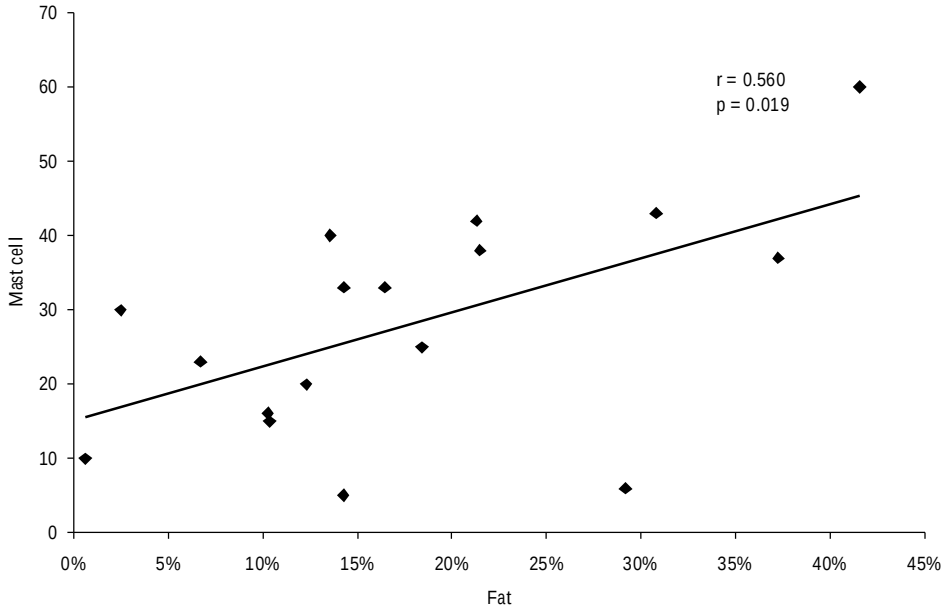


Fig. 2

(A)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p = 0.019
Mast cell
Fat
0
10
20
30
40
50
60
70
0%
5%
10%
15%
20%
25%
30%
35%
40%
45%

(B)

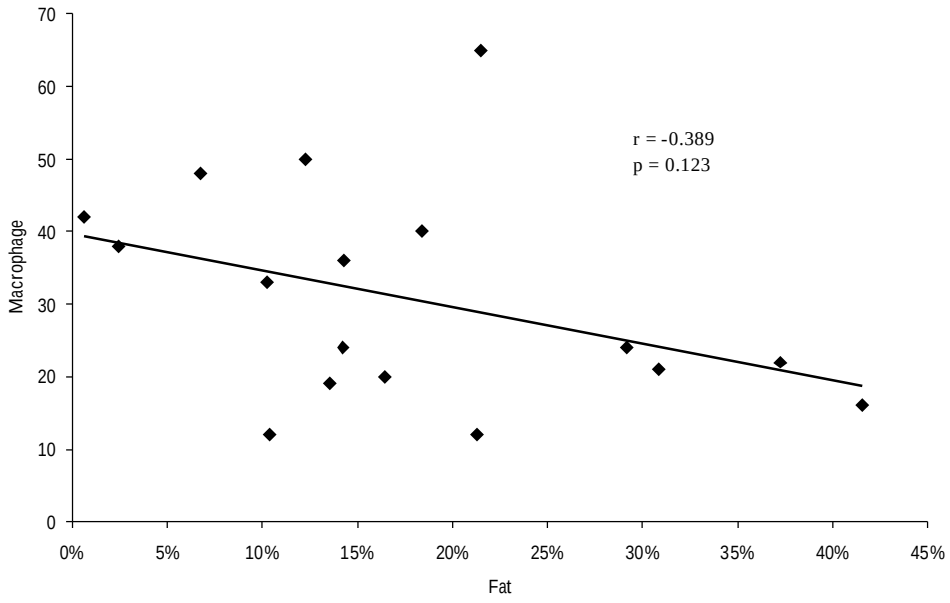
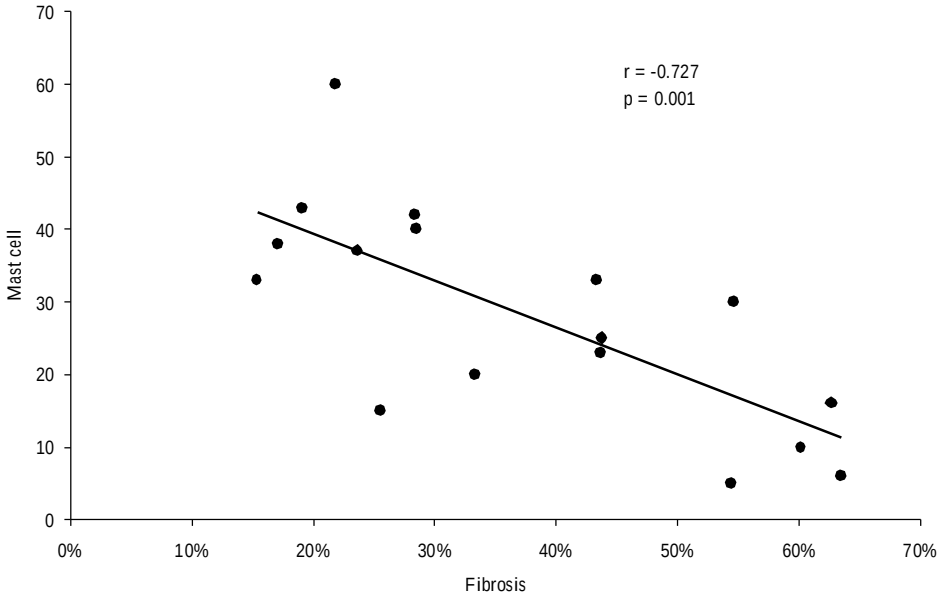

r = -0.389
p = 0.123
Macrophage
Fat
0
10
20
30
40
50
60
70
0%
5%
10%
15%
20%
25%
30%
35%
40%
45%

Fig. 3.

(A)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p = 0.001
Mast cell
Fibrosis
0
10
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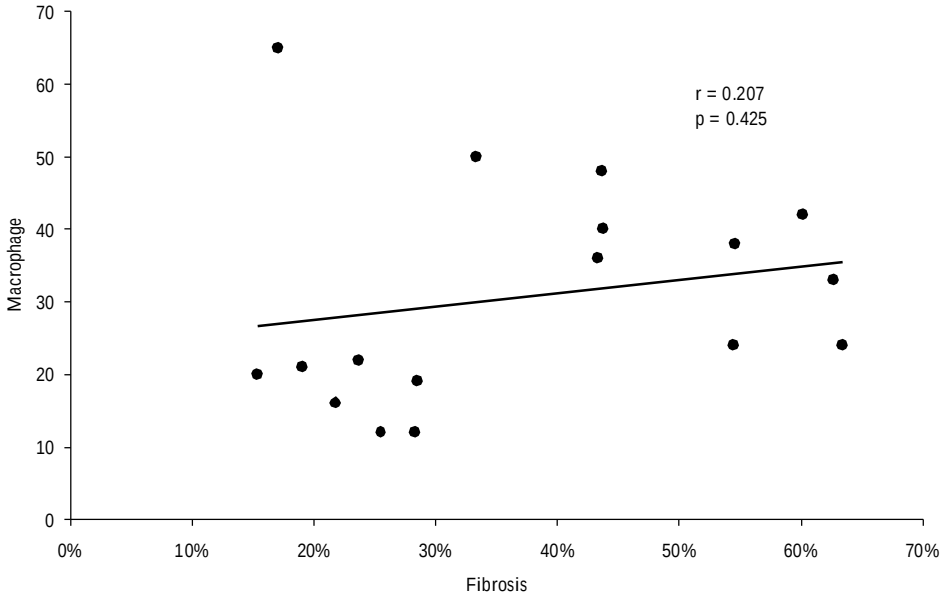
30
40
50
60
70
0%
10%
20%
30%
40%
50%
60%
70%

(B)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20%
30%
40%
50%
60%
70%