The role of gangliosides in pathogenesis of Graves' ophthalmopathy

Koung Hoon Kook

Department of Medicine

The Graduate School, Yonsei University

The role of gangliosides in pathogenesis of Graves' ophthalmopathy

Directed by Professor Sang Yeul Lee

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Koung Hoon Kook

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This certifies that the Doctoral Dissertation of
Koung Hoon Kook is approved.

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Thesis Supervisor: Sang Yeul Lee

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Thesis Committee Member #1

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Thesis Committee Member #2

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Thesis Committee Member #3

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Thesis Committee Member #4

The Graduate School
Yonsei University

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I dedicate this thesis to my family.

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<ABSTRACT>

The role of gangliosides in pathogenesis of Graves' ophthalmopathy

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Graves' ophthalmopathy is an orbital disease, a component of Graves' disease which is the thyroid disease related with pathology of autoimmunity. Although the detailed pathologic mechanisms are yet to be known, orbital fibroblasts which exist in orbital connective tissue are believed to play a major role in the process. Gangliosides are sialic acid-containing glycosphingolipids, which widely distributed in the plasma membranes of all vertebrate tissue having roles in cell differentiation, proliferation and signal transduction. In Graves' disease, altered pattern of ganglioside profile in thyroid membranes and increased activity of sialyltransferase as well as increased GD1a in thyroid tissue from patients with Graves' disease have been reported. Therefore, it appears to be highly possible that gangliosides play a role in pathogenesis of Graves' ophthalmopathy. However, few studies have determined the role of

ganglioside in Graves' ophthalmopathy. The purpose of this study was to investigate the role of gangliosides in pathogenesis of Graves' ophthalmopathy. Orbital tissues from patients with Graves' ophthalmopathy as well as normal subjects were obtained during surgery, and orbital fibroblasts were primarily cultured from each obtained tissue. The expressions of gangliosides and sialyltransferase were examined, and the effect of ganglioside on orbital fibroblast in resting state from normal subjects was studied. GT1b was expressed in orbital tissue of patients with Graves' ophthalmopathy, which was not detected in orbital tissue of normal subjects. Moreover, the expression of sialyltransferase-4a mRNA was also increased in orbital fibroblast from patients with Graves' ophthalmopathy. The treatment with GD1a or GT1b induced the differentiation of orbital fibroblasts in resting state, those from subjects without Graves' ophthalmopathy or other inflammatory disease, into adipocyte, while GM1 showed no noticeable effect. GT1b showed more potency to induce the differentiation of orbital fibroblast into adipocyte than GD1a, and this differentiation was most obvious when treated with ganglioside mixture, whereas dermal fibroblast and preadipocyte were irresponsive to the same treatment. Ganglioside mixture increased the amount of hyaluronic acid released and enhanced the expression of mRNA levels of inflammatory cytokines and chemokines, including COX-2, in orbital fibroblast. These results together suggest that gangliosides may have a role in pathologic mechanisms of Graves' ophthalmopathy.

Key words : adipocyte differentiation, ganglioside, Graves' ophthalmopathy, hyaluronic acid, inflammation, orbital fibroblast,

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I. INTRODUCTION

Graves' ophthalmopathy is a component of Graves' disease, a common autoimmune disorder of the thyroid in which stimulatory autoantibodies bind to the thyrotropin receptor and activate glandular function resulting in hyperthyroidism.[1] About 25% to 50% of patients with Graves' disease develop clinical manifestation of Graves' ophthalmopathy.[2]

The clinical symptoms and signs of Graves' ophthalmopathy in terms of the pattern of presentation vary depending on the disease process. The majority of Graves'

ophthalmopathy patients experience only mild ocular discomfort followed by spontaneous regression, however, approximately 5% of the patients have severe morbidity, including proptosis, diplopia related to limitation of ocular movement, or even loss of vision.[3] Currently, therapeutic strategy such as corticosteroid is widely used, targeting the inflammation which is considered as the major process in early stage of disease. However, when the disease course turns to later fibrotic stage, very few therapeutic options can be offered.

Although the pathogenesis of Graves' ophthalmopathy is yet to be cleared, it is widely accepted that Graves' ophthalmopathy is also an autoimmune disease like Graves' disease, and that it is not believed to result directly from the metabolic perturbations caused by thyroid hormone overproduction.[4, 5] Clinically, most of signs and symptoms can be explained by the volumetric expansion of both orbital fatty connective tissues and extraocular muscles.[2] Histopathologic study showed the edematous changes of orbital fatty connective tissue as well as the expansion of fat tissue,[6] and the edema of extraocular muscles without pathology of myocytes themselves.[7] These edematous changes are closely related with increased production of hyaluronan, which has been shown to be one of major substances accumulated in orbital tissue from patients with Graves' ophthalmopathy, resulting in edema by attracting water.[8-10]

Orbital fibroblasts, which are abundantly distributed in orbital connective tissue including orbital fatty compartment, are currently believed to play a major role in the pathologic processes of Graves' ophthalmopathy. Orbital fibroblasts synthesize high levels of hyaluronan when activated by interleukin-1 beta (IL-1β) and other proinflammatory cytokines. The subset of orbital fibroblasts which have no

expression of Thy-1 (CD90), has a potential of differentiation into mature adipocyte when treated with cyclic adenosine monophosphate (cAMP) enhancing agents as well as peroxisome proliferator activated receptor-γ (PPARγ) agonists.[11-15] In addition, cytokines such as IL- 1β, leukoregulin and CD154 induce prostaglandin G/H synthase-2 (PGHS-2) in orbital fibroblast,[16-18] consequently resulting in increased production of prostaglandin E_2 ($PGE_{2)}$[16] which has a potential to aggravate immune response in orbit. When compared to other site-specific fibroblasts such as dermal or truncal fibroblast, these findings are unique characteristics of orbital fibroblast.

Gangliosides are sialic acid (NeuAc)-containing glycosphingolipids that have a variable sialic acid-containing oligosaccharide structure attached to an acylated ceramide core.[19] The subtypes of gangliosides are determined by the number and the site of sialic acid attached. In the process of gangliosides synthesis, sialyltransferases catalyze the transfer of sialic acid from cytidine monophosphosialic acid (CMP)-sialic acid to terminal position of sugar chains of glycolipids.[20, 21] Gangliosides are widely distributed in the plasma membranes of all vertebrate tissue,[22] having roles in cell differentiation,[23] proliferation[24] and signal transduction.[25] Furthermore, the appearance of new gangliosides and changes in cellular ganglioside profiles following the switch in synthetic pathway are generally considered to be important aspects of cellular metabolism.[26-28] The upregulation of individual sialyltransferase results in an overproduction of individual ganglioside and overall increase of plasma concentration of gangliosides in certain pathologic states.[29]

In thyroid, gangliosides are considered as a structurally and functionally important element of thyrotropin receptor affecting thyrotropin binding and thyrocyte function in physiologic status.[30-33] In Graves' disease, autoantibodies to Forssman glycolipid

(Gb5) have been found in serum of patients.[34] Moreover, altered pattern of ganglioside profile in thyroid membranes[35] and increased activity of sialyltransferase as well as increased GD1a, one of disialogangliosides, in thyroid tissue from patients with Graves' disease[36] have been reported. Therefore, it appears to be highly possible that gangliosides play a role in pathogenesis of Graves' ophthalmopathy, especially related to orbital fibroblast. However, few studies have determined the role of ganglioside in Graves' ophthalmopathy. The purpose of this study was to investigate the pattern of expression of gangliosides in orbital tissue and orbital fibroblast cultured from patients with Graves' ophthalmopathy including the expression of sialyltransferase, and the effect of ganglioside on orbital fibroblast in resting state from normal subjects to determine the possible role of ganglioside in pathogenesis of Graves' ophthalmopathy.

II. MATERIALS AND METHODS

1. Reagents and antibodies

Bovine brain gangliosides mixture (Table 1), monosialoganglioside 1 (GM1), disialoganglioside 1a (GD1a) and trisialoganglioside 1b (GT1b) were purchased from Matreya (Pleasant Gap, PA. USA). Hyaluronan ELISA kit was from Echelon Biosciences Inc. (Salt Lake City, UT. USA). Monoclonal antibodies specific for GM1, GD1a, and GT1b were purchased from Seikagaku Corporation (Tokyo, Japan). Biotin-conjugated goat anti-mouse antibodies were purchased from Abcam (Cambridge, UK).

Table 1. Constitutions of gangliosides mixture

Type	Percent
GM1	18%
GD1a	55%
GD1b	15%
GT1b	10%
GQ1b, GM2, GD2, GD3	2%

GM1: Monosialoganglioside 1; GD1a: Disialoganglioside 1a; GD1b: Disialoganglioside 1b; GT1b: Trisialoganglioside 1b; GQ1b: Quadrisialoganglioside 1b; GM2: Monosialoganglioside 2; GD2: Disialoganglioside 2; GD3: Disialoganglioside 3;

2. Cell culture

Orbital fibroblasts were cultured as previously reported. Briefly, orbital fat tissues obtained from patients with Graves' ophthalmopathy during surgical procedure of orbital decompression were triturated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone, Logan, UT. USA), 100 μg/ml of penicillin (Hyclone, Logan, UT, USA) and 100 unit/ml of streptomycin (Hyclone, Logan, UT. USA). The obtained orbital tissue was exposed in collagenase (Sigma, St. Louis, MO. USA) to yield a single cell suspension, which was plated in 75-cm^2 T-flask (BD Falcon, San Jose, CA. USA). With the same manner, orbital fibroblasts and dermal fibroblasts were obtained from patients undergoing orbital surgery without any inflammatory reason or cosmetic eyelid surgery. Mouse preadipocyte cell line 3T3-L1 (ATCC CCL 92.1) was obtained from American tissue culture collection (Manassas, VA. USA). Once cells were obtained, the cultures were maintained using DMEM containing 10% fetal bovine serum in a humidified 5% CO_2 incubator at 37˚C and serially passaged using trypsin/EDTA. Liquid N_2 was used in the long-term storage of some cultures. Medium was changed every 3 days, and cells beyond passage 8 from culture initiation were not used. For experiments, cells were inoculated in 35mm cell culture dish and allowed to attach and spread for 2-3 days prior to each experiment.

3. Immunohistochemistry

Orbital tissue cryostat sections were fixed on gelatin-coated glass slides, dried in air for 2 hours and fixed with acetone at room temperature for 5 min followed by complete air drying. They were then incubated for 15 min at room temperature in PBS. After incubation with 10% goat serum for 10 min and washing with PBS, primary antibody was applied for 30 min at room temperature. Subsequently, they were washed 3 times with PBS and incubated with biotin-conjugated secondary antibody for 30 min. After endogenous peroxidase was blocked with 0.3% H_2O_2, they were diluted in methanol for 15 min, and biotinylated horseradish peroxidase - streptoavidin complex was applied for 20 min. DAB (3,3'-diaminobenzinidine) (DakoCytomation, Carpinteria, CA. USA) was used for chromogen and methyl green was used for counterstaining. Microscopic examinations were performed using the BX51 (Olympus Optical, Tokyo, Japan) at ×100, ×200, and ×400 magnification.

4. Analysis of immunoreactivity

After microscopic examination of each immunohistochemically stained slide, photographs were taken with microscope-mounted digital camera for representing area at ×200 magnification. The photographs were converted using Gradient map function in Adode photoshop personnel software program (Adobe Systems Incorporated, San Jose, CA. USA) applying red color to area showing high immunoreactivity and blue color to area showing no immunoreactivity with color gradient spectrum. The area of positively stained area was measured according to color gradient using image analysis personnel computer software program (Image J 1.38X, NIH, USA).

5. Phase contrast microscopic examination

After treatment under each experimental setting, cells were fixed in 10% formalin in phosphate-buffered saline (PBS) and then washed three times with PBS. Microscopic examination was performed using the Axiovert 200M (Carl Zeiss AG, Baden-Württemberg, Germany) and photographs were taken with microscope-mounted digital camera.

6. Adipocyte staining with Oil Red O

Cells were stained with oil red O as described by Green and Kehinde (1975). A stock solution of oil red O (0.5 g in 100 ml of isopropanol) was prepared and passed through a 0.2 μm filter. To prepare the working solution, 6 ml of the stock solution was mixed with 4 ml of distilled water, left for 1 hour at room temperature, and filtered through a 0.2 μm filter. Cells in 35 mm dish were fixed with 10% formalin in PBS for 1 hour at 4°C, and stained with 300 μl of the oil red O working solution for 15 min at room temperature. After washing the dishes with distilled water, retained dye was eluted by isopropanol. The cells in dish were observed under the phase contrast microscope.

7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared using TriZol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 5 ug of total RNA using 1 μl of random hexamer (2 μg/μl) (Amersham Pharmacia Biotech Inc., Uppsala, Sweden), 1.25 mM dNTP (Boehringer-Mannheim, Mannheim, Germany) and 200 U M-MLV reverse transcriptase (Gibco BRL, NY. USA). PCR was performed using 0.25 mM dNTP, 0.25 U of Taq polymerase (Perkin Elmer, Norwalk, CA. USA), 10 pmole of primer pair and 3 μl of cDNA with a thermal cycler (PerkinElmer, NY. USA). PCR cycling conditions were as follows: 92°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute.

Table 2. Sequences of primers used for RT-PCR

Genes	Sense	Antisense
ST3Gal I	5'-GGACCCTGAAAGTGCTCA-3'	5'-TCTCCAGCATAGGGTCCA-3'
ST3Gal V	5'-GACCCTCTTGGAACTCTTGCC-3'	5'-CCAAACTGACTTCATCGCACA-3'
ST8Sial I	5'-ATCCCAGCATAATTCGGC-3'	5'-AGAAGGGCCAGAAGCCAT-3'
C/EBPβ	5'-AACTTTGGCACTGGGG-3'	5'-GGCCCGGCTGACAGTT-3'
PPARγ1	5'-AAAGAAGCCGACACTAAACC-3'	5'-CTTCCATTACGGAGAGATCC-3'
COX-2	5'-GTTCCACCCGCAGTACAG-3'	5'-GGAGCGGGAAGAACTTGC-3'
IL-16	5'-ATGCCCGACCTCAACTCCTC-3'	5'-CTCCTGATGACAATCGTGAC-3'
IL-6	5'-TCAATGAGGAGACTTGCCTG-3'	5'-GATGAGTTGTCATGTCCTGC-3'
MCP-1	5'-TTCTCAAACTGAAGCTCGACATCTCGCC-3'	5'-TGTGGAGTGAGTGTTCAAGTCTTCGGAGTT-3'
IL-8	5'-TTGGCAGCCTTCCTGATTTC-3'	5'-AACTTCTCCACAACCCTCTG-3'
Actin	5'-GCCATCTCCTGCTCGAAGTCTAG-3'	5'-CATGTTTGAGAC-3'

ST3Gal I : β-galactoside α-2,3-sialyltransferase 1; *ST3Gal V* : β-galactoside α-2,3- sialyltransferase 5; *ST8Sial I* : β-galactoside α-2,8-sialyltransferase 1; *C/EBPβ* : CCAAT/enhancer binding protein beta; *PPARγ* : Peroxisome proliferator-activated receptor gamma; *COX-2* : Cyclooxygenase-2;

IL: Interleukin; *MCP-1* : Monocyte chemoattractant protein-1

8. Quantitative assay of hyaluronic acid by enzyme-linked immunosorbent assay (ELISA)

Orbital fibroblasts were plated in 6 -well plastic culture plates and incubated for the indicated time periods with or without Gmix treatment. Supernatants from the cell cultures were then collected, and hyaluronic acid (HA) concentrations were determined using a competitive binding HA-ELISA kit (Echelon Biosciences, Salt Lake City, UT, USA), according to the manufacturer's instructions. Briefly, after 100µl of standards and samples were added into corresponding wells, 150 µl and 100 µl of diluent were added to blank control and zero HA control wells, respectively. After adding 50 µl of working detector to all wells except the blank and mixing, the plate was covered and incubated for one hour at 37°C. Then, 100 µl of controls and samples were transferred to the corresponding wells of the HA-ELISA plate. After incubation for 30 min at 4°C, the solution was discarded, and the wells were washed and 100 µl of working enzyme suspension was added to each well. Following incubation for 30 min at 37°C and washing, 100 µl of working substrate solution was added to each well and the plate was incubated in the dark at room temperature for 30 min. Finally, absorbance was spectrophotometrically measured at 570nm. The concentration of HA in the sample was determined using a standard curve generated by using known amounts of hyaluronic acid.

9. Statistical analysis

Statistical analysis was performed using SPSS ver. 11.5 (SPSS Inc. Chicago, IL. USA), and significance was determined when P value < 0.05.

III. RESULTS

1. Trisialoganglioside GT1b is expressed in orbital tissue from patient with Graves' ophthalmopathy

To examine the expression of different subtypes of gangliosides in orbital tissue from patients with Graves' ophthalmopathy, immunohistochemical staining was performed on orbital fat tissue, obtained during orbital decompression surgery and other orbital or lid surgery, from patients without any inflammatory disease including Graves' disease for control. Monoclonal antibodies specific for gangliosides (GM1, GD1a, and GT1b) were used for immunohistochemical staining and methyl green was used for counterstaining. As shown in Fig 1A, hematoxylin-eosin stain showed characteristic feature of orbital connective tissue which was primarily composed of adipocytes both in control and Graves' ophthalmopathy, and no noticeable differences between two groups. In immunohistochemical staining, GM1 and GD1a were abundantly found in the cytoplasm and cellular membrane of adipocytes in both control and Graves' ophthalmopathy. However, sections from patients with Graves' ophthalmopathy revealed GT1b stained with prominently strong intensity and quantity, compared with control which showed no detectable GT1b positive cells (Fig. 1A). In analysis by measuring immunoreactive area from slides of five patients with Graves' ophthalmopathy including three normal subjects, GT1b positively stained area of group with Graves' ophthalmopathy was significantly greater than that of normal subjects ($P<0.05$), while no difference was noticed in analysis for GM1 and GD1a (Fig. 1B).

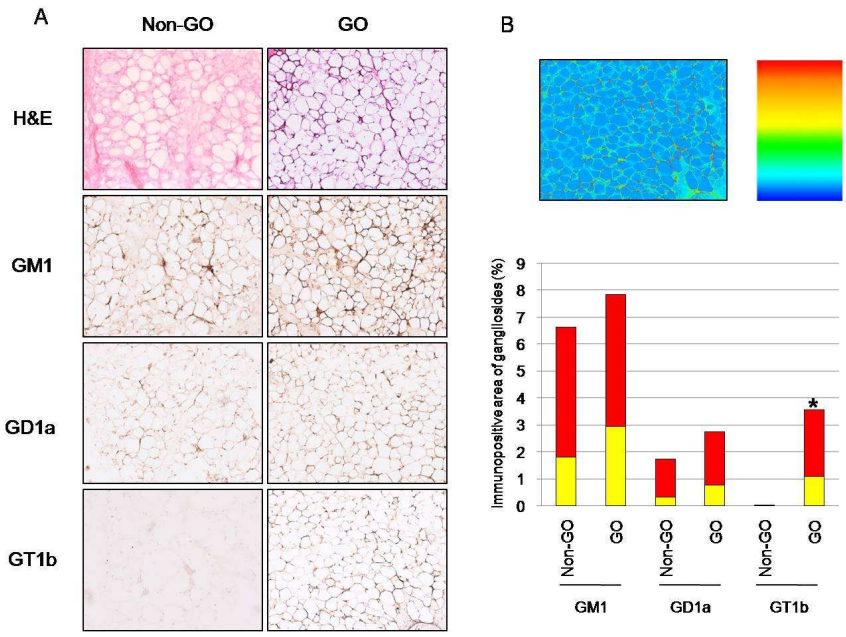

Figure 1. Expression of gangliosides in orbital tissue. A. Immunostaining was performed for orbital tissue. There was no distinguishable feature which shows positive stain for GM1 and GD1a along cytoplasm and cellular membrane of adipocytes between sections from patient with Graves' ophthalmopathy (GO, right panel) and from subjects without Graves' disease or any other inflammatory disease (Non-GO, left panel). Although GT1b-positively stained cells were not detected in the section from Non-GO subject, GT1b-expressed adipocytes were abundantly found in the section from patient with Graves' ophthalmopathy. (×100) B. Immunoreactive area was measured and analyzed for slides from five Graves' ophthalmopathy patients and three normal subjects. Immunopositive areas are red and yellow zones (upper panel, left). Red showed higher immunoreactivity than yellow (upper panel, right). Red bar in graph indicates red area of immunoreactivity and yellow bar is yellow area in slides. GT1b positively stained area of group with Graves' ophthalmopathy was significantly greater than that of normal subjects (*, $P<0.05$), while no difference was noticed in analysis for GM1 and GD1a.

2. Sialyltransferase IV (ST3GalI) mRNA is increased in orbital fibroblasts from patients with Graves' ophthalmopathy

To verify the above finding that showed expression of GT1b in orbital tissue from Graves' ophthalmopathy, which was not found in control, RT-PCR was performed to elucidate the expression pattern of sialyltransferases in cultured orbital fibroblasts: Three subtypes of sialyltransferase [sialyltransferase I (ST3Gal V), sialyltransferase II (ST8Sial I), and sialyltransferase IV (ST3Gal)] are known to participate; the conversion of lactosylceramide to monosialoganglioside (GM3), GM3 to GD3, and GD1b to GT1b, respectively. As shown in Fig.2, the expressions of ST3Gal V and ST8Sial I showed no noticeable difference between control and patients with Graves' ophthalmopathy. However, the expressions of ST3Gal I were strikingly increased in two (Pt4 and Pt5) of five Graves' ophthalmopathy patients compared to the control, while one patient (Pt3) showed slight increase of the expression and the remaining two patients (Pt1 and Pt2) showed no significant difference from the control.

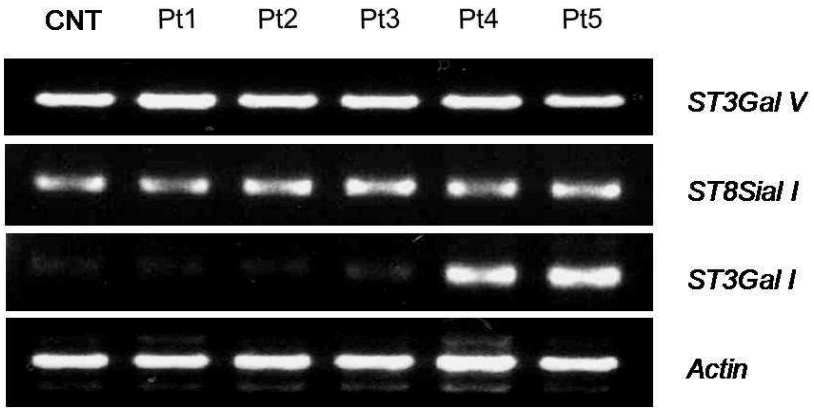

Figure 2. Expression of sialyltransferases gene in orbital fibroblasts. Orbital fibroblasts were obtained from orbital tissue of patients with Graves' ophthalmopathy, subjects without Graves' ophthalmopathy or any other inflammatory disease for control. RT-PCR was performed for three subtypes of sialyltransferases (*ST3Gal V*, *ST8Sial I*, and *ST3Gal I*). Although no noticeable increases of the expressions of *ST3Gal V* and *ST8Sial I* were noted in Graves' ophthalmopathy patients compared to control (CNT), the expressions of *ST3Gal I* were strikingly increased in two of five Graves' ophthalmopathy patients (Pt4 and Pt5), while one patient (Pt3) showed slight increase of the expression and remaining two patients (Pt1 and Pt2) showed no clear difference from control.

3. Gangliosides treatment induce morphological change of cultured orbital fibroblast from normal subject

Since the above results showed altered pattern of ganglioside expression depending on different subtypes of ganglioside and increased expression of sialyltransferase, the different subtypes of gangliosides were applied to orbital fibroblasts isolated from subjects without Graves' disease or any other inflammatory disease, to test the effect of ganglioside on orbital fibroblast in resting state. As shown in Fig. 3, the orbital fibroblasts treated with GM1 showed no clear morphological changes in both 24 hours and 48 hours compared with control, however, GD1a induced morphological change of the cells with more round and bright cytoplasm in 24 hours. These morphological changes in 24 hours were more prominent in GT1b-treated cells than GD1a. Besides, although the effect of GD1a was slightly decreased in 48 hours, the cells treated with GT1b showed not only sustained effects, but also increased population with morphological changes. These changes were most drastic in orbital fibroblasts treated with gangliosides mixture, which contains several subtypes of gangliosides including GT1b (Fig. 3).

Next, gangliosides mixture, which showed the most potent effect on morphological changes of orbital fibroblasts, was applied to dermal fibroblasts and preadipocyte cell line (3T3-L1) to check whether the gangliosides were still effective to other types of cells besides orbital fibroblasts. Interestingly, however, treatment of these cells with gangliosides mixture by the same protocol as in orbital fibroblasts showed no detectable morphological changes up to 48 hours (Fig. 4).

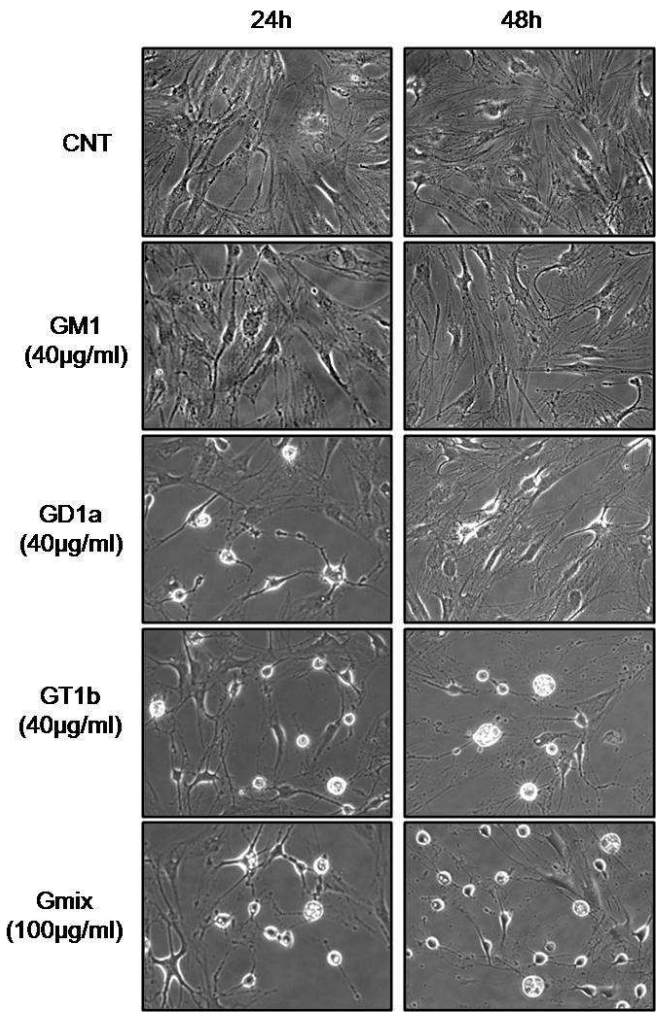

Figure 3. The effect of different subtypes of gangliosides treatment on morphological changes of orbital fibroblasts. Phase contrast microscopic examination of orbital fibroblasts after treatment with different subtypes of gangliosides. While GM1 (40μg/ml) had no significant effect on orbital fibroblasts in both 24 and 48 hours after treatment compared to untreated control (CNT), GD1a (40μg/ml) induced morphological changes, showing more round and bright cytoplasm in 24 hours after treatment. These changes were more prominent when treated with GT1b (40μg/ml), and most drastic when treated with gangliosides mixture (Gmix) (100μg/ml). (×400)

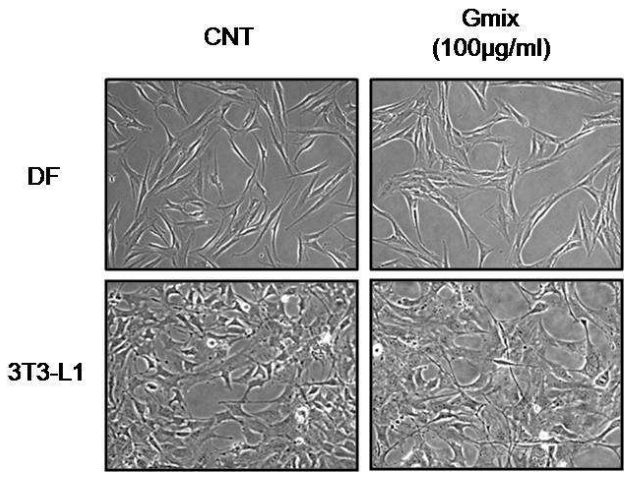

Figure 4. The effect of gangliosides mixture treatment on morphological changes of dermal fibroblast and preadipocyte. Phase contrast microscopic examination of dermal fibroblasts (DF) and preadipocyte cell line (3T3-L1) after treatment with 100μg/ml of gangliosides mixture (Gmix) was performed. No detectable morphological changes were noticed up to 48 hours compared to untreated control (CNT). (×400)

To verify the effect of gangliosides mixture on orbital fibroblasts, cells were treated with various concentrations of gangliosides mixture (up to 200 μg/ml) for 48 hours. Fig. 5 shows that the number of cells with morphological changes was increased, and more prominent changes in individual cells were observed, showing more round and bright cytoplasm when treated with higher concentration of gangliosides mixture. This suggests a dose-dependent effect of gangliosides mixture on orbital fibroblasts.

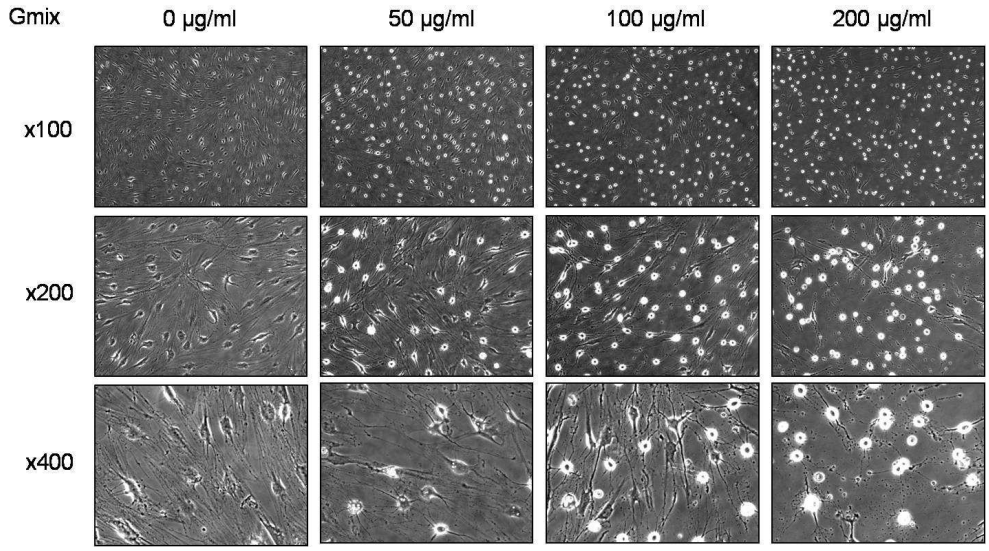

Figure 5. The effect of gangliosides treatment on morphological changes of orbital fibroblasts. On phase contrast microscopic examination, gangliosides mixture (Gmix) induced morphological changes of orbital fibroblasts, showing more round and brighter perinuclear cytoplasm than untreated control, in dose-dependent manner up to 200 μg/ml of concentration.

4. Gangliosides treatment induce differentiation of cultured orbital fibroblast from normal subject into adipocyte

To examine whether this morphological changes of orbital fibroblasts after treatment with gangliosides mixture were related to adipogenesis, which is known as one of the major pathologic findings in Graves' ophthalmopathy, Oil red O staining as well as RT-PCR for CCAAT/enhancer binding protein beta (*C/EBPβ*) and Peroxisome proliferator-activated receptor gamma 1 (*PPARγ1*) were performed. After 48 hours of treatment with 100 μg/ml of gangliosides mixture, orbital fibroblasts showed multiple vacuoles in cytoplasm, and they were positively stained with Oil red O, indicating lipid droplets (Fig. 6A). After treatment with 100 μg/ml of gangliosides mixture, the induction of *C/EBPβ* was increased at 6 hours, the increased level was still kept compared to untreated control. The induction of *C/EBPβ* reached a peak level at 24 hours after treatment. The increase of *PPARγ1* induction was less drastic than *C/EBPβ*, but the pattern at various time points was similar, showing peak point at 24 hours after treatment (Fig. 6B).

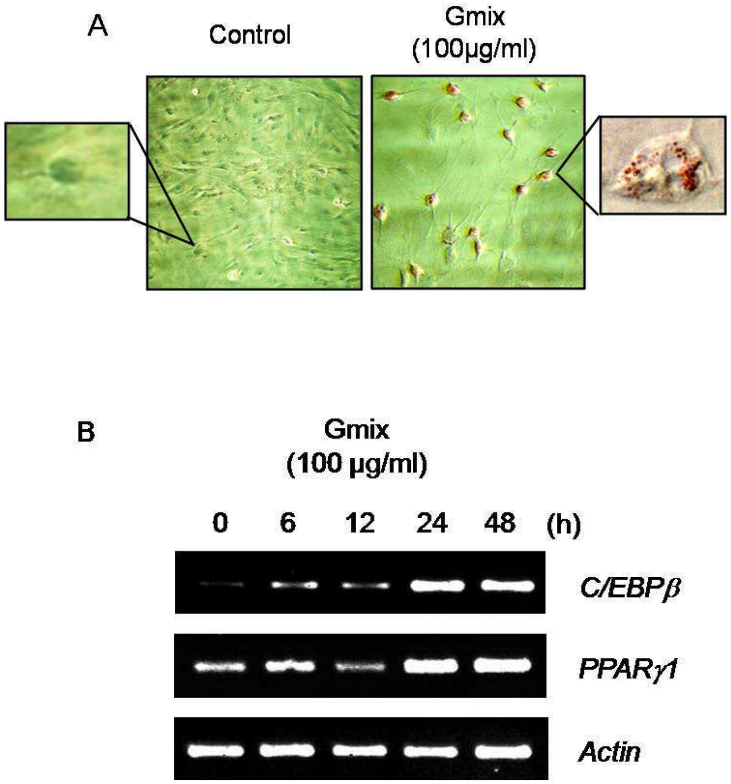

Figure 6. Differentiation of orbital fibroblast into adipocyte by gangliosides treatment. A. Oil red O staining of orbital fibroblast after treatment with gangliosides mixture. Oil red O stain was performed at 48 hours after treatment with 100 μg/ml of gangliosides mixture (Gmix). More and larger droplets were noticed in gangliosides mixture-treated orbital fibroblast than untreated control, and they were stained with red color indicating lipid. B. RT-PCR for *C/EBPβ* and *PPARγ1* from OF following treatment with gangliosides mixture at various time points. Increased induction of *C/EBPβ* as well as *PPARγ1* expression was noted at 24 and 48 hours after gangliosides mixture treatment.

5. Gangliosides treatment promote hyaluronic acid production in cultured orbital fibroblast from normal subject

It has been reported that orbital fibroblast is the source for synthesizing high levels of hyaluronic acid, which is believed as one of the key substances related to orbital edema, leading to proptosis in patients with Graves' ophthalmopathy. Therefore, the effect of gangliosides on hyaluronic acid production in orbital fibroblast was examined using competitive ELISA kit. Thus, orbital fibroblasts were obtained from patients undergoing lid or orbital surgery with non-inflammatory issue, and were treated with 100 μg/ml of gangliosides mixture, and the concentration of hyaluronic acid released was then measured at different time points up to 48 hours. Although no noticeable changes up to 6 hours after treatment, change of concentration of released hyaluronic acid was then noticed. After reaching a peak level at 24 hours (more than seven times the control), the concentration of hyaluronic acid released was decreased at 48 hours. However, even at 48 hours, the concentration of released hyaluronic acid in gangliosides mixture-treated orbital fibroblasts was still more than four times the untreated control (Fig. 7).

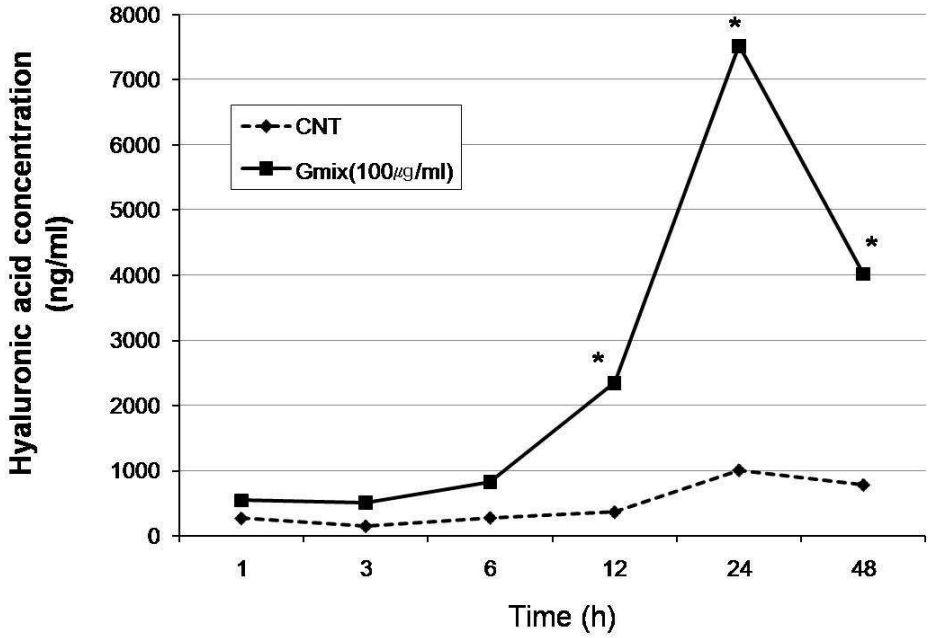

Figure 7. Increased hyaluronic acid in orbital fibroblasts by gangliosides treatment. After treatment of orbital fibroblasts with 100 μg/ml of gangliosides mixture (Gmix), the amount of hyaluronic acid released was measured using competitive ELISA kit. The concentration of hyaluronic acid released was increased with time elapse up to 24 hours compared to untreated control (CNT). At 48 hours after treatment, concentration of released hyaluronic acid was decreased somewhat, however, still kept much higher than control. (*, $P<0.05$)

6. Gangliosides treatment enhance mRNA expression of inflammatory cytokines and chemokines in orbital fibroblast cultured from normal subject

As described above, orbital fibroblasts have close relations with immune cells such as T, B lymphocyte and mast cell, producing inflammatory cytokines in the pathogenesis of Graves' ophthalmopathy. Therefore, RT-PCR for genes of inflammatory cytokines and chemokines (interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-16 (IL-16), monocyte chemoattractant protein-1 (MCP-1) including cyclo-oxygenase-2 (COX-2) was performed to examine the effect of gangliosides on orbital fibroblasts in regard with inflammation. When orbital fibroblasts from normal subjects were treatment with 100 μg/ml of gangliosides mixture, induction of all the genes examined was noticeably increased from 24 hours and kept up to 48 hours (Fig. 8).

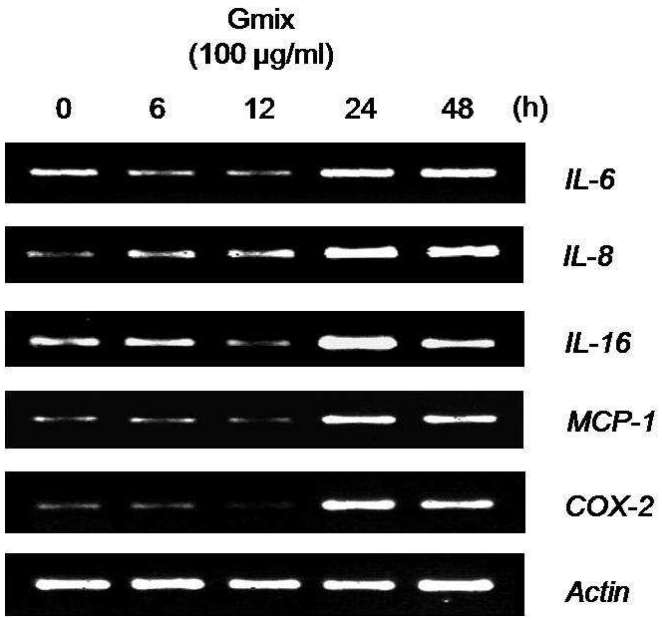

Figure 8. Enhanced mRNA expression of inflammatory cytokines and chemokines by gangliosides treatment. After treatment of orbital fibroblasts with 100 μg/ml of gangliosides mixture (Gmix), RT-PCR was performed. Increased gene expression levels of IL-6, IL-8, IL-16, MCP-1, and COX2 were noted from 24 hours up to 48 hours.

IV. DISCUSSION

Graves' disease is a common autoimmune disorder of thyroid in which stimulatory antibodies bind to the thyrotropin receptor and activate glandular function, resulting in hyperthyroidism. Clinically, Graves' disease is characterized by diffuse goiter, thyrotoxicosis, infiltrative orbitopathy and ophthalmopathy, and occasionally infiltrative dermopathy. About 25% to 50% of patients with Graves' disease develop the clinical involvement of the eye,[2] and approximately 5% of patients suffer from severe ophthalmopathy, including intense pain, chemosis, proptosis, lid retraction, double vision or even loss of vision.[3]

Most of the clinical symptoms and signs of Graves' ophthalmopathy can be explained on a discrepancy between the increased volume of swollen orbital tissues, including extraocular muscles, as well as expansion of orbital fatty connective tissues and the fixed volume of bony orbital space. Although individual fibers of involved extraocular muscle are intact, they are widely separated by edematous connective tissues,[37] and these perimysial tissues contain excess amount of glycosaminoglycans which are predominantly composed of hyaluronan and chondroitin sulfate.[38] A similar accumulation of glycosaminoglycans is apparent in the fatty connective tissue compartments of the posterior orbit,[6] as well as the emergence of a population of newly differentiated fat cells.[39] In addition, histological examination revealed the infiltration of bone-marrow derived cells, including T and B lymphocytes and mast cells, into orbital tissues of patients with Graves' ophthalmopathy, suggesting that the inflammatory process is closely related to pathology.[6] Although the pathogenic mechanism of Graves' ophthalmopathy is not yet clearly understood, orbital

fibroblasts, which reside abundantly in orbital connective tissues, have been suggested to have a primary role in pathogenic mechanisms.

In this study, GT1b was found to be expressed in orbital fatty connective tissue from patient with Graves' ophthalmopathy, but not in the tissue from non-Graves' subjects. Gangliosides are a particular glycolipid class characterized by containing a variable number of sialic acid residues, which are anchored to extracytosolic leaflet of cell membranes through the ceramide moiety and play a variety of biological functions, including cellular recognition and adhesion as well as signaling. Several subtypes of gangliosides have been identified, depending on the number and site of sialic acid bound to lactoceramide backbone. Depending on the number of sialic acid residues bound, GM, GD, GT, and GQ have been characterized from human, and increased concentration of specific type of gangliosides is generally considered to be important aspects of cellular metabolism.[26-28] Each subtype of gangliosides can be substrate for others, and several types of sialyltransferases are known to catalyze this biosynthetic pathway of gangliosides.[40] This study revealed that the mRNA level of sialyltransferase-4a (STGal I), which converts GM1 into GD1a and GD1b into GT1b, was increased in orbital fibroblast from patients with Graves' ophthalmopathy, compared to non-Graves' subject, in support of previous studies that total sialyltransferase activity as well as STGal I mRNA level were increased in thyroid tissue from patients with Graves' disease,[36] and that the portion of GM was decreased in orbital fibroblast from patient with Graves' ophthalmopathy, whereas the portion of GD was increased, when evaluated by thin layer chromatography.[41]

This study revealed that gangliosides induced differentiation of orbital fibroblasts which were in resting state from non-Graves' subjects into mature adipocyte. This

effect was more remarkable when treated with GT1b compared with GD1a, and indistinguishable from GM1 compared to untreated control, however, gangliosides were not effective to dermal fibroblast and preadipocyte. It has been well known that the expansion of orbital adipose tissue is one of the pathologic characteristics of Graves' ophthalmopathy.[6] Since the capacity of orbital fibroblasts to undergo adipocyte differentiation under specific stimulation was reported in 1996,[11] in which differentiation medium was composed of insulin, triiodothyronine, carbaprostacyclin, thyrotropin, dexamethasone, and isobutylmethylxanthine, orbital fibroblasts have been believed to be the source of expanded orbital adipose tissue in patients with Graves' ophthalmopathy. Although exact mechanisms and effect of these particular agents are not clearly elucidated, the enhanced cAMP level is thought to be one of possible mechanisms of differentiation of orbital fibroblast into adipocyte.[42, 43] Agonist for PPARγ such as rosiglitazone has also been reported to have adipogenic effect on orbital fibroblast,[44] and recent a study revealed that activated T lymphocyte drives adipogenesis of orbital fibroblast through generating prostaglandins, which act as PPARγ ligand.[45] Although molecular mechanisms by which ganglioside induces adipogenesis of orbital fibroblast has not yet been established, increased level of PPARγ mRNA was observed from 24 hours after gangliosides treatment and this time course was compatible with morphological changes (Fig. 5). Compared to previous reports, the effect of gangliosides, especially GT1b and Gmix, on inducing adipogenesis of orbital fibroblast in the present study was very rapid and strong. Moreover, this was highly specific to orbital fibroblast, showing no effect on dermal fibroblast and preadipocyte.

In addition to the expansion of orbital fatty connective tissue through differentiation of orbital fibroblast into adipocyte, edema of orbital tissues, including fatty

connective tissue and extraocular muscles, is also one of typical pathologic characteristics of Graves' ophthalmopathy, and hyaluronic acid is abundantly accumulated in this edematous tissue.[46] Hyaluronic acid is a hydrophilic glycosaminoglycan, which attracts water into surrounding tissue, resulting in edema. When treated with IFN-γ, orbital fibroblast increases hyaluronic acid by 50%, whereas no effect on dermal fibroblast.[8] Leukoregulin is an another stimulator for synthesis of hyaluronic acid in orbital fibroblast, stimulating by up to 15-fold.[9] In this study, gangliosides were found to increase hyaluronic acid in orbital fibroblasts. Although the mechanism involved in the increase of hyaluronic acid remains largely unclear, it might be caused by the increase of synthesis or release because orbital fibroblasts do not express hyaluronidase which degrades hyaluronic acid.[47]

Another characteristic of Graves' ophthalmopathy is the inflammation, especially in active stage, and this study revealed that gangliosides induced some inflammatory cytokines and chemokines in orbital fibroblast. In a previous study which examined the cytokine profile in orbital tissue of active versus inactive Graves' ophthalmopathy patients, mRNA levels of IL-1β, IL-2, IL-6, IL-8, and IL-10 were significantly high in orbital tissues from active Graves' ophthalmopathy patients.[48] Furthermore, orbital fibroblast is known to express CD40 through which intercellular communication with inflammatory cells such as T lymphocyte and mast cells occur.[49, 50] In this study, the striking increases of IL-8, MCP-1, and COX2 mRNA expression were noted. Primary function of IL-8 is the induction of chemotaxis in its target cells such as neutrophil and granulocytes, and MCP-1 is known as a chemoattractant for monocytes and lymphocyte.[51] COX2, also known as prostaglandin-endoperoxide synthase-2 (PTGS-2) or prostaglandin-endoperoxide H synthase-2 (PGHS-2), is the key enzyme in biosynthesis of prostanoids, and is involved in inflammation. Leukoregulin[17, 52] and

IL-1β[16] are reported to induce PGHS-2 in orbital fibroblast, resulting in increased synthesis of prostaglandin E_2. IL-6 is also thought as one of major pro-inflammatory cytokines related to pathogenesis of Graves' ophthalmopathy. The expression of thyrotropin receptor in orbital fibroblast is stimulated by IL-6,[53] and the expression of IL-6 in orbital fibroblast is induced by IL-1β.[54] Although the degree of increase was not remarkable compared to that by IL8, MCP1, and COX2, ganglioside enhanced IL-6 after 24 hours of treatment in this study. IL-16 is a cytokine characterized as a chemoattractant for certain immune cells which express the cell surface molecule CD4. The induction of IL-16 by treatment of orbital fibroblasts with immunoglobulins from patients with Graves' disease was reported,[55] and this was thought to be mediated through the insulin-like growth factor I receptor pathway.[56] In this study, ganglioside slightly increased the mRNA level of IL-16 at 24 hours after treatment, followed by decrease to basal level. Although the exact mechanisms of time dependent inductions of examined cytokines and chemokines are not clear, these results suggest possible relationship between ganglioside and inflammatory process in orbital tissue.

V. CONCLUSION

This study showed that GT1b was expressed in orbital tissue from patient with Graves' ophthalmopathy, which was not found in orbital tissue from normal subject, and that ST3GalI mRNA was increased in orbital fibroblasts from patients with Graves' ophthalmopathy compared to normal subject. Gangliosides treatment induced the differentiation of cultured orbital fibroblast from normal subjects into adipocyte. In addition, gangliosides treatment promoted hyaluronic acid production, and enhance mRNA expression of inflammatory cytokines and chemokines in orbital fibroblast cultured from normal subjects. In conclusion, the results described herein suggest possible role of gangliosides in pathogenesis of Graves' ophthalmopathy.

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<ABSTRACT (IN KOREAN)>

그레이브스 눈병증의 병리 기전에서 ganglioside 의 역할

<지도교수 이 상 렬>

연세대학교 대학원 의학과

국 경 훈

그레이브스 눈병증은 자가면역 갑상샘 질환인 그레이브스씨 병에 연관되어 발생하는 안와질환으로, 그 병리 기전은 명확히 밝혀지지 않았으나 안와 결체 조직 내 존재하는 안와섬유모세포가 주요한 역할을 하는 것으로 알려져 있다. Ganglioside는 sialic acid를 포함하고 있는 glycosphingolipid로서 모든 조직의 형질막에 광범위하게 존재하며, 세포의 분화와 증식, 그리고 신호전달의 기능을 하는 것으로 알려져 있다. 그레이브스씨 병에서는 갑상샘 세포막의 ganglioside의 발현 형태가 변화됨이 보고되었으며, 갑상샘 조직에서 GD1a의 증가와 sialyltransferase의 활성도가 증가되어 있음이 보고되었다. 그러므로 ganglioside가 그레이브스 눈병증의 병리기전에도 관여할 것임이 예상되나 현재까지 연구된 바

없다. 본 연구의 목적은 그레이브스 눈병증의 병리 기전에 있어 ganglioside의 역할을 밝히고자 하였으며, 그레이브스 눈병증 환자의 수술 시 얻어진 안와 조직과 이로부터 배양한 안와섬유모세포를 재료로 하여 연구를 시행하였다. 본 연구 결과, 그레이브스 눈병증 환자의 안와 지방 조직 내에 정상 안와 지방 조직 내에서는 발현되지 않는 GT1b가 발현되어 있음을 관찰할 수 있었으며, 그레이브스 눈병증 환자로부터 얻은 안와섬유모세포에서 sialyltransferase-4a의 mRNA 발현량이 증가되어 있음을 관찰할 수 있었다. 또한 정상 안와 조직으로부터 얻은 안와섬유모세포에 ganglioside를 처리한 결과, GM1은 안와섬유모세포의 분화를 유도하지 않았으나 GD1a와 GT1b는 안와섬유모세포의 지방세포로의 분화를 유도하였고, GT1b 처리 후의 분화 정도가 더 확연하였으며, ganglioside mixture의 처리가 분화에 대한 최대의 효과를 보임을 관찰할 수 있었다. 그리고 이러한 ganglioside처리 후 지방세포로의 분화 유도는 dermal fibroblast와 preadipocyte에서는 관찰되지 않았다. 또한 안와섬유모세포에 대한 ganglioside 처리는 유리된 hyaluronic acid의 양을 증가시킴을 관찰하였으며, 염증관련 cytokine과 chemokine 및 COX-2의 mRNA의 발현량의 증가를 유도 시킴을 관찰할 수 있었다. 이상의 결과로 ganglioside는 안와섬유모세포에 대하여 그레이브스 눈병증의 주요 병리 변화를 유도함을 밝힐 수 있었으며, 그러므로

ganglioside및 연관 효소들이 그레이브스 눈병증의 병리 기전에 관여할 것으로 사료된다.

핵심 되는 말 : 그레이브스 눈병증, 안와섬유모세포, 염증, 지방세포 분화, ganglioside, hyaluronic acid