

Altered Ganglioside Expression Modulates the Pathogenic Mechanism of Thyroid-Associated Ophthalmopathy by Increase in Hyaluronic Acid

Koung Hoon Kook,^{*,1,2} Youn-Hee Choi,^{2,3} Yu Rim Kim,^{1,2} Soo Jung Park,^{2,4} Ilo Jou,^{2,4} Sung Joo Kim,⁵ and Sang Yeul Lee^{*,6}

PURPOSE. The aim of this study was to determine the role of gangliosides in pathogenic mechanisms of thyroid-associated ophthalmopathy (TAO).

METHODS. The gangliosides profile and mRNA level of sialyltransferases of the orbital tissues from TAO patients ($n = 5$) and non-TAO subjects ($n = 4$) were investigated. In addition, the effect of exogenous gangliosides on the expression of hyaluronic acid was examined in orbital fibroblasts. For in vitro experiments, we used four different strains of cells obtained from non-TAO subjects with at least three replicates for each strain.

RESULTS. Trisialoganglioside 1b (GT1b) was significantly over-expressed in the orbital tissue of TAO patients compared with control tissue, whereas no significant difference was observed for either monosialoganglioside 1 (GM1) or disialoganglioside 1a (GD1a) by digital analyses of immunohistochemical images. Moreover, mRNA levels of sialyltransferase (*SAT-I* and *SAT II*) were increased in TAO patients compared with control. Exogenous GT1b strongly induced the morphologic changes related to an accumulation of sparse flocculent precipitates in lysosomes and increased the extracellular hyaluronic acid level in orbital fibroblasts with the induction of hyaluronic acid synthase, which were less by GD1a but not by GM1. The GT1b-induced morphologic changes of cells were due, at least in part, to an increase of intracellular hyaluronic acid. Co-treatment of hyaluronidase nicely attenuated the morphologic changes in orbital fibroblasts. Thy-1⁺ orbital fibroblasts were more capable of producing hyaluronic acid by exogenous GT1b.

CONCLUSIONS. The results suggest that gangliosides, particularly GT1b, may play a role in the pathologic mechanisms of TAO by

stimulating an increase in hyaluronic acid. (*Invest Ophthalmol Vis Sci.* 2011;52:264–273) DOI:10.1167/iovs.10-5276

Graves' disease is a common autoimmune disorder of the thyroid in which stimulatory antibodies bind to the thyrotropin receptor and activate glandular functions, resulting in hyperthyroidism.¹ Approximately 25% to 50% of patients with Graves' disease develop involvement of the orbit, and approximately 5% of patients suffer from severe ophthalmopathy, including intense pain, chemosis, proptosis, lid retraction, double vision, or even loss of vision.² The pathogenesis of thyroid-associated ophthalmopathy/orbitopathy (TAO) is not completely understood, but it is widely accepted that TAO is induced by the autoimmune response and not by the metabolic perturbations associated with thyroid hormone overproduction.^{3,4} Most of the clinical manifestations of TAO can be explained by the discrepancy between the increased volume of orbital tissues and the fixed volume of the bony orbit.⁵ This volumetric increase of orbital tissues is mainly due to swelling and is characterized by edematous changes of orbital fatty connective tissue⁶ and extraocular muscles without muscle cell pathology.⁷

The hallmark feature of the connective tissue remodeling encountered in TAO is the accumulation of hyaluronic acid in retroocular tissue.⁸ Hyaluronic acid is a high-molecular-weight glycosaminoglycan composed of D-glucuronic acid and N-acetyl-D-glucosamine residues. Because of its profound hydrophilic nature and extreme molecular bulk when hydrated, the accumulation of hyaluronic acid accounts, at least in part, for the expansion of orbital tissues and the expansion of the eye beyond the normal boundaries of the bony orbit.⁵ Orbital fibroblasts, which are abundant in orbital connective tissue and the orbital fatty compartment, are an important source of hyaluronic acid.⁸ A variety of inflammatory mediators, including interleukin (IL)-1 β and transforming growth factor (TGF)- β , have been shown to stimulate hyaluronic acid synthesis in cultured human orbital fibroblasts.^{9,10}

Gangliosides are sialic acid (NeuAc)-containing glycosphingolipids that have a variable sialic acid-containing oligosaccharide structure attached to an acylated ceramide core.¹¹ During ganglioside synthesis, sialyltransferases catalyze the transfer of sialic acid from cytidine monophosphosialic acid-sialic acid to the terminal position of sugar chains of glycolipids^{12,13}; ganglioside subtypes are defined by the number and site of sialic acids. Gangliosides are widely distributed in the plasma membranes of all vertebrate tissue¹⁴ and are important in cell differentiation,¹⁵ proliferation,¹⁶ and signal transduction.¹⁷ In the thyroid, gangliosides are a structurally and functionally important element of the thyrotropin receptor and affect thyrotropin binding and thyrocyte function.^{18–22} Interestingly, sialyltransferase activity is increased in the thyroid tissue of patients with Graves' disease.²³

From the Departments of ¹Ophthalmology and ⁴Pharmacology, and ²Chronic Inflammatory Disease Research Center, Ajou University School of Medicine, Suwon, Korea; ³Department of Physiology, Ewha Women's University School of Medicine, Seoul, Korea; ⁵Department of Ophthalmology, Kim's Eye Hospital, Seoul, Korea; and ⁶Department of Ophthalmology, Yonsei University College of Medicine, Seoul, Korea.

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*Each of the following is a corresponding author: Koung Hoon Kook, Department of Ophthalmology, Ajou University School of Medicine, San 5, Wonchon-dong, Yeongtong-gu, Suwon 443-721, Korea; drkook@ajou.ac.kr;

Sang Yeul Lee, Department of Ophthalmology, Yonsei University College of Medicine, 134 Sinchon-dong, Seodaemun-gu, Seoul 120-752, Korea; sylee@yuhs.ac.

In this study, we found an increased expression of trisialo-ganglioside 1b (GT1b) and an enhanced mRNA level of both *sialyltransferase (SAT-I)* and *SAT-II* in orbital tissues of patients with TAO. Orbital fibroblasts from non-TAO subjects exhibited morphologic changes on exogenous treatment with GT1b, whereas dermal fibroblasts and preadipocytes were irresponsive to it. These morphologic changes were associated with an increase in intracellular hyaluronic acid; the level of extracellular hyaluronic acid also increased in GT1b-treated orbital fibroblasts. Co-treatment of *Streptomyces* hyaluronidase with GT1b reduced the extracellular hyaluronic acid level as well as the intracellular one accompanying with the attenuation of the morphologic changes in cells. Our results demonstrate that change in gangliosides profiling modulate the pathogenic mechanism of TAO by stimulating an increase in hyaluronic acid.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and trypsin/ethylenediaminetetraacetic acid (EDTA) were purchased from HyClone (Logan, UT). Bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and *Streptomyces hyalurolyticus* hyaluronidase were obtained from Sigma-Aldrich (St. Louis, MO). Goat serum was obtained from Vector Laboratories (Burlingame, CA). Bovine brain monosialo-ganglioside 1 (GM1), disialoganglioside 1a (GD1a), and trisialo-ganglioside 1b (GT1b) were purchased from Matreya (Pleasant Gap, PA). Mouse anti-GM1 Ab (clone GMB16), mouse anti-GD1a Ab (clone GMR17), and mouse anti-GT1b Ab (clone GMR5) were purchased from Seikagaku Corporation (Tokyo, Japan). Sheep polyclonal anti-hyaluronic acid Ab, rabbit polyclonal anti-lysosome-associated membrane glycoprotein (LAMP)-2b Ab, mouse monoclonal anti-Thy-1 Ab, PE-conjugated donkey polyclonal anti-sheep Ab, and biotin-conjugated anti-mouse Ab were obtained from Abcam (Cambridge, UK). FITC-conjugated anti-mouse Ab, Alexa Fluor 350-conjugated anti-sheep Ab, and Alexa Fluor 555-conjugated anti-rabbit Ab were purchased from Molecular Probes (Eugene, OR). Streptavidin complexed with biotinylated horseradish peroxidase and 3,3'-diaminobenzidine (DAB) were obtained from Dako Cytomation (Carpinteria, CA). A hyaluronic acid-ELISA kit was obtained from Echelon Biosciences (Salt Lake City, UT). Guanidinium thiocyanate-phenol-chloroform medium was purchased from Invitrogen (TRIZol; Carlsbad, CA), and random hexamer was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). dNTP was obtained from Boehringer-Mannheim (Mannheim, Germany), and reverse transcriptase was purchased from GIBCO (Invitrogen, Carlsbad, CA). *Taq* polymerase and thermal cycler were purchased from Perkin Elmer (Norwalk, CA).

Orbital Tissue Collection and Cell Culture

Orbital fatty connective tissues were obtained as surgical waste during decompression surgery for patients with TAO ($n = 5$) and during orbital surgery for subjects without Graves' disease or other inflammatory disease ($n = 4$). Orbital fibroblasts were cultivated from the orbital tissues, and four individual cell strains of the non-TAO subjects were used for in vitro experiments. Before the decompression surgery, all TAO patients had experienced at least 6 months of inactive disease status within euthyroid condition. Patient characteristics are presented in Table 1. Dermal fibroblasts were cultivated from eyelid skin of subjects undergoing cosmetic lid surgery ($n = 3$). These activities were undertaken after informed consent was obtained from the donors according to procedures approved by the Institutional Review Board of Ajou University Hospital and following the tenets of the Declaration of Helsinki. Mouse preadipocyte cell line 3T3-L1 (ATCC CCL 92.1) was purchased from the American tissue culture collection (Manassas, VA). Cell cultures were maintained in a humidified 5% CO₂ incubator at

TABLE 1. Characteristics of TAO Patients and Non-TAO Subjects from Whom Orbital Tissues Were Obtained

	Patients with TAO ($n = 5$)	Non-TAO Subjects ($n = 4$)
Mean age, y (range)	40 (29-57)	43 (32-54)
Sex (male/female)	3/2	2/2
Smoking (yes)	0	0
Graves' disease	5	0
Radioactive iodine therapy	0	—
Surgery	0	—
Methimazole	5	—
Treatment TAO	5	0
Surgery	5	—
Prednisone	3	—
Radiation	0	—
Euthyroid	5	4
TSH-receptor antibodies	5	0
Clinical activity score (range)	1 (0-2)	—

37°C covered with DMEM containing 10% FBS and antibiotics (DMEM 10% FBS). Once a fibroblasts monolayer was obtained, cultures were serially passaged after gentle treatment using trypsin/EDTA. Liquid nitrogen was used in the long-term storage of some cultures. Medium was changed every 3 days, and cells beyond passage 6 from culture initiation were not used.

Immunohistochemistry and Measurement of Immunoreactive Areas

Orbital tissue cryostat sections (10 μ m) were prepared and fixed with acetone at room temperature for 5 minutes followed by complete air drying. Slides were kept at room temperature and incubated for 15 minutes in phosphate-buffered saline (PBS) for rehydration and 30 minutes in blocking solution containing 10% goat serum in PBS, followed by several washes in PBS. The sections were treated for 30 minutes with one of the following primary antibodies: anti-GM1 Ab (1:1000), anti-GD1a Ab (1:1000), or anti-GT1b Ab (1:1000), washed repeatedly in PBS, and incubated for 30 minutes with biotin-conjugated secondary Ab. After washing in PBS, endogenous peroxidase was blocked with 0.3% H₂O₂ diluted in methanol for 15 minutes, and streptavidin complexed with biotinylated horseradish peroxidase was applied for 20 minutes. Each slide was washed in PBS and incubated with DAB for 15 minutes; the chromogenic reaction was terminated by washing in PBS. Microscopic examinations were performed using the BX51 (Olympus Optical, Tokyo, Japan) at $\times 100$, $\times 200$, and $\times 400$ magnification. To analyze the amount of ganglioside staining, three nonperipheral, noncontinuous, random fields of view at $\times 100$, selected by one observer who had no access to the clinical details of the specimens, were exported from each slide using a microscope-mounted digital camera under the same condition of lighting throughout the experiment. Using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>), each image was converted to 8-bit gray scale, and area densities were measured from the pixels in the region of interest. To exclude background error, only pixels exceeding an intensity value >50 , based on the negative value measured from the images of specimen stained without primary antibody, were included.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was prepared from orbital fat tissue using TriZol. cDNA was synthesized from 5 μ g of total RNA using 1 μ L of random hexamer (2 μ g/ μ L), 1.25 mM dNTP, and 200 U M-MLV reverse transcriptase. PCR was performed using 3 μ L cDNA, 0.25 mM dNTP,

0.25 U of *Taq* polymerase, and 10 pmole of primer pair with a thermal cycler. PCR cycling conditions were as follows: 92°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. Primer sequences are listed in Table 2.

Morphologic Analysis of Cells

For experiments, cells were inoculated in 35 mm cell culture dishes covered with DMEM 10% FBS and allowed to attach and spread for 3 days. Then the cells were incubated overnight in DMEM supplemented with 1% charcoal-filtered FBS and antibiotics (DMEM 1% FBS). Subsequently, cells were washed and cultured in DMEM 1% FBS with or without each type of gangliosides; GM1, GD1a, and GT1b. After 48 hours of treatment, cells were fixed with 10% formalin in PBS and washed three times with PBS. Cell morphology was examined by inverted microscope (Axiovert 200M, Carl Zeiss, Baden-Württemberg, Germany). To analyze the number of cells that underwent the morphologic changes, three nonperipheral, noncontinuous, random fields of view at $\times 100$, selected by one observer who had no access to the clinical details of the specimens were exported from each slide using a microscope-mounted digital camera under the same lighting conditions throughout the experiment. Then the cells were counted manually from the obtained images. For transmission electron microscopic analyses, cells were fixed with Karnovsky's fixative solution (2% paraformaldehyde, 2% glutaraldehyde, 0.5% calcium chloride in cacodylate buffer, pH 7.2) for 30 minutes, washed with cacodylate buffer, dehydrated with a graded ethanol series, and embedded in epon. Ultrathin sections (75 nm) were obtained using a microtome (Reichert Jung Ultracut S; Leica, Vienna, Austria). Sections were mounted on grids, stained with uranyl acetate and lead citrate, and observed under an electron microscope (EM 902A; Carl Zeiss).

Cytotoxicity Assay

Cell growth and death were assessed using MTT assays. Orbital fibroblasts (1×10^5) were seeded into 24-well culture plates and incubated in DMEM 1% FBS with (4 or 40 $\mu\text{g}/\text{mL}$) or without GT1b for 48 hours. After washing cells with PBS, 50 μL of MTT solution (1 mg/mL in PBS) was added to each well, and plates were incubated for 4 hours at 37°C. The plates were centrifuged at 3000g for 10 minutes, and supernatant was carefully removed. The remaining adherent cells were then dissolved in 200 μL dimethyl sulfoxide (DMSO), and optical density was measured at 570 nm using a microplate reader.

Alcian Blue Staining

Orbital fibroblasts cultured on poly-D-lysine-coated slide were treated with GT1b in DMEM 1% FBS for 48 hours. Cells were then washed twice with PBS and fixed in 4% paraformaldehyde for 20 minutes. After three washings with PBS, cells were stained with alcian blue solution (1% w/v alcian blue in 3% acetic acid, pH 2.5) for 30 minutes. After four washings with distilled water and dehydration with 95% ethanol, the slide was mounted and examined.

Quantitative Assay of Hyaluronic Acid by ELISA

Orbital fibroblasts were plated in 6-well plastic culture plates and incubated for the indicated time periods with or without gangliosides (GM1, GD1a, or GT1b), in DMEM 1% FBS. Supernatants from the cell cultures were collected, and hyaluronic acid concentrations were determined using a competitive binding hyaluronic acid-ELISA kit according to the manufacturer's instructions. Briefly, after adding of 100 μL of standards and samples into corresponding wells, 150 and 100 μL of diluents were added to blank and zero hyaluronic acid control wells, respectively. Fifty microliters of working detector was added to all wells except the blank and mixing, and the plate was covered and incubated for 1 hour at 37°C. Then 100 μL of controls and samples were transferred to the corresponding wells of the hyaluronic acid-ELISA plate. After incubation for 30 minutes at 4°C, the solution was discarded, and the wells were washed and 100 μL of working enzyme suspension was added to each well. After incubation for 30 minutes 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 minutes. Finally, absorbance was spectrophotometrically measured at 570 nm. The concentration of hyaluronic acid in the sample was determined using a standard curve generated with known amounts of hyaluronic acid.

Immunofluorescence Assay

Orbital fibroblasts cultured on poly-D-lysine-coated slide were treated with GT1b in DMEM 1% FBS for 48 hours. After washing with PBS and fixing with 4% paraformaldehyde, the cells were then washed in PBS and incubated for 10 minutes at room temperature with permeabilization buffer (PBS containing 0.1% Triton X-100). After washing several times with PBS, the cells were blocked with 5% BSA in PBS for 30 minutes at room temperature and then incubated overnight at 4°C with the following primary antibodies: anti-GT1b Ab (1:1000), anti-hyaluronic acid Ab (1:1000), and anti-LAMP2b Ab (1:1000). Cells were washed three times and incubated for 2 hours at room temperature with the following secondary antibodies: FITC-conjugated anti-mouse Ab, Alexa Fluor 350-conjugated anti-sheep Ab, and Alexa Fluor 555-conjugated anti-rabbit Ab. To examine the relevance of Thy-1 expression with the GT1b-induced hyaluronic acid increase in orbital fibroblasts, anti-Thy-1 Ab (1:1000) and anti-hyaluronic acid Ab (1:1000) were used for primary Ab, followed by the use of PE-conjugated anti-sheep Ab for the secondary one. After mounting, the cells were observed under a confocal microscope (Carl Zeiss).

Statistical Analysis

Student's *t*-tests were used in analyzing the difference expression of gangliosides and *SAT* in orbital tissues of patients with TAO and normal subjects. The effect of gangliosides on the hyaluronic acid increase and the attenuative effect of hyaluronidase on the ganglioside-induced morphologic changes were analyzed using the same tests. Statistical software (SPSS 15.0; SPSS Inc., Chicago, IL) was used for analysis, and $P < 0.05$ was considered statistically significant.

TABLE 2. Sequences of Primers Used for RT-PCR

Genes	Sense 5'-3'	Antisense 5'-3'
<i>SAT I</i>	GACCCCTCTGGAACCTCTTGCC	CCAAAGTACTTCATCGCACA
<i>SAT II</i>	ATCCCAGCATAAATCGGC	AGAAGGGCCAGAAGCCAT
<i>SAT IV</i>	GGACCCCTGAAAGTGCTCA	TCTCCAGCATAGGGTCCA
<i>HAS1</i>	TGTGTATCCTGCATCAGCGGT	CTGGAGGTGACTTGGTAGCATAACC
<i>HAS2</i>	GTGTTATACATGTCGAGTTACTTCC	GTCCATATTGTTGCCCTTCTCCGC
<i>HAS3</i>	GGTACCATCAGAAGTTCCTAGGCAGC	GAGGAGAATGTTCCAGATGCG
<i>Actin</i>	GCCATCTCCTGCTCGAAGTCTAG	CATGTTTGAGACCTTCAACACCCC

HAS, hyaluronic acid synthase; *SAT I*, β -galactoside α -2,3-sialyltransferase 5; *SAT II*, β -galactoside α -2,8-sialyltransferase 1; *SAT IV*, β -galactoside α -2,3-sialyltransferase 1.

RESULTS

GT1b Overexpressed in Orbital Tissues of TAO Patients

To determine whether gangliosides are associated with the pathogenesis of TAO, we examined the level of gangliosides in orbital tissue by immunohistochemical staining. Orbital fat tissues were obtained during orbital decompression surgery of TAO patients ($n = 5$) and during orbital surgery of subjects without Graves' disease or other inflammatory diseases as a control ($n = 4$). Monoclonal antibodies specific for each ganglioside subtype (GM1, GD1a, and GT1b) were used for staining. As shown in Figure 1A, GM1 and GD1a were abundantly expressed in TAO and control tissues. The mean GM1-stained area (%) in TAO sections was 7.8 ± 2.9 and in control sections was 6.6 ± 0.2 , showing no statistical difference ($P = 0.507$). The area stained for GD1a was 1.7 ± 1.2 in control sections and 2.7 ± 1.4 in sections from TAO patients, with no significant difference ($P = 0.320$). However, GT1b expressed cells were found only in TAO, not in control tissues. GT1b expression was significantly increased in orbital tissues of TAO patients compared with control tissues ($3.6 \pm 1.4\%$ and $0.03 \pm 0.06\%$, respectively; $P = 0.004$; Fig. 1B).

Increased mRNA Level of Sialyltransferases in Orbital Tissues from Patients with TAO

Cellular ganglioside composition relies on the balance between the activities of several sialyltransferases acting at the branching points of ganglioside biosynthesis.²⁴ RT-PCR analysis of orbital fatty connective tissue was used to detect the mRNA level of: sialyltransferase I (*SAT I*, ST3 β -galactoside α -2,3-sialyltransferase 5), which converts lactosylceramide to the a-series monosialoganglioside GM3; sialyltransferase II (*SAT II*,

ST8 α -N-acetyl-neuraminidase α -2,8-sialyltransferase 1), which converts GM3 to b-series disialoganglioside GD3; and sialyltransferase IV (*SAT IV*, ST3 β -galactoside α -2,3-sialyltransferase 1), which converts GM1 to GD1a (a-series) and GD1b to GT1b (b-series). As shown in Figure 2, the mRNA levels of *SAT I* and *SAT II* were significantly increased in TAO tissue compared with control tissue ($P = 0.001 = 0.049$, respectively), corresponding to the increased expression of GT1b shown in Fig 1. Although the difference was not statistically significant, the mRNA level of *SAT IV* in the patient group was slightly higher than the control ($P = 0.160$). These results suggest that increased expression of GT1b in the orbital tissue of TAO patients may be due to the enhanced activities of sialyltransferases.

Effect of Gangliosides GM1, GD1a, and GT1b on Orbital Fibroblast Morphology

Exogenous gangliosides interact with plasma membranes and modulate transmembrane signaling pathways.¹⁶ Because the orbital tissues of TAO patients showed increased expression of GT1b and sialyltransferase, we tested the impact of added gangliosides on orbital fibroblasts. The three types of gangliosides (GM1, GD1a, GT1b; $40 \mu\text{g}/\text{mL}$ each) were applied individually to the orbital fibroblasts obtained from non-TAO subjects ($n = 4$), dermal fibroblasts ($n = 3$), and preadipocytes. As shown in Figure 3A, GM1 induced no noticeable morphologic changes in orbital fibroblasts at 48 hours compared with control, whereas GD1a yielded a few cells with morphologic changes including more rounding and brightening of cells. The morphologic changes were most prominent in GT1b-treated cells. None of the gangliosides induced morphologic changes of the dermal fibroblasts or preadipocytes up to 48 hours, indicating that the effect of GT1b on orbital fibroblasts is cell

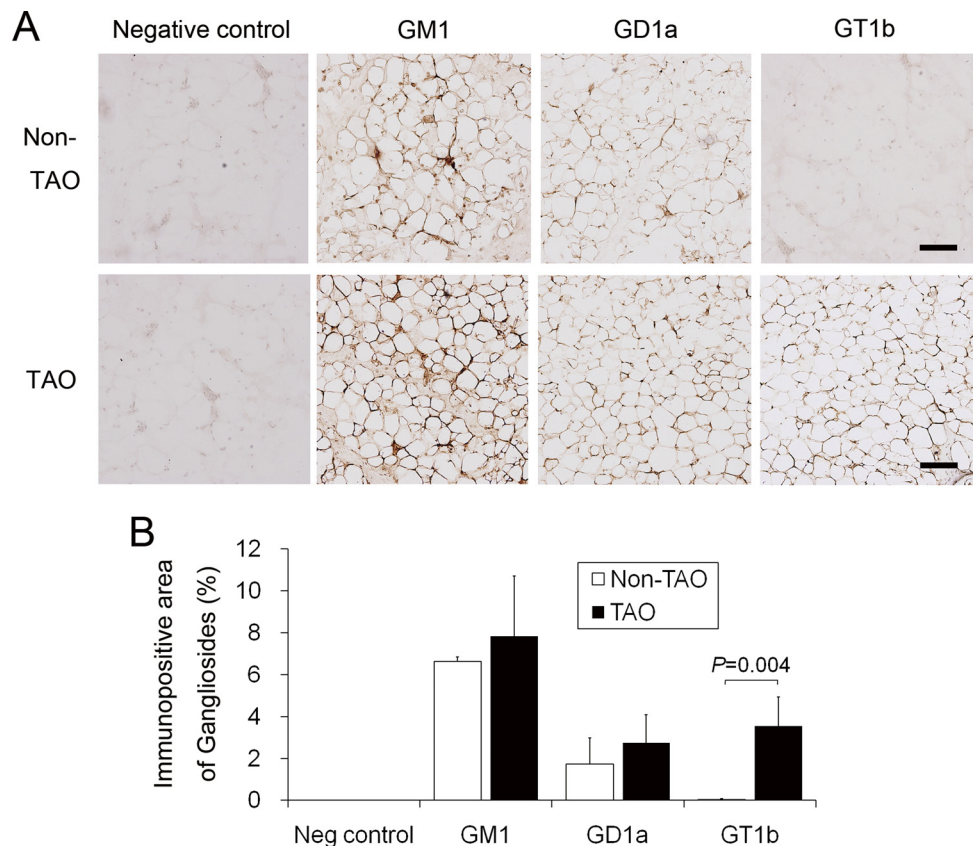


FIGURE 1. Increased GT1b expression in orbital tissues of TAO patients. **(A)** Orbital fatty connective tissues were obtained during decompression surgery of patients with TAO ($n = 5$), and from non-TAO subjects who underwent orbital surgery for noninflammatory reasons as control ($n = 4$). Cryosections ($10 \mu\text{m}$) were stained for GM1, GD1a, and GT1b, respectively. Representative experiment showing increased GT1b expression in orbital tissue of TAO patient compared with control. Scale bars, $200 \mu\text{m}$; negative control, no primary Ab. **(B)** Quantitative analysis of immunoreactivity. Positively stained areas were measured using Image J software program (bar graph, mean \pm SD).

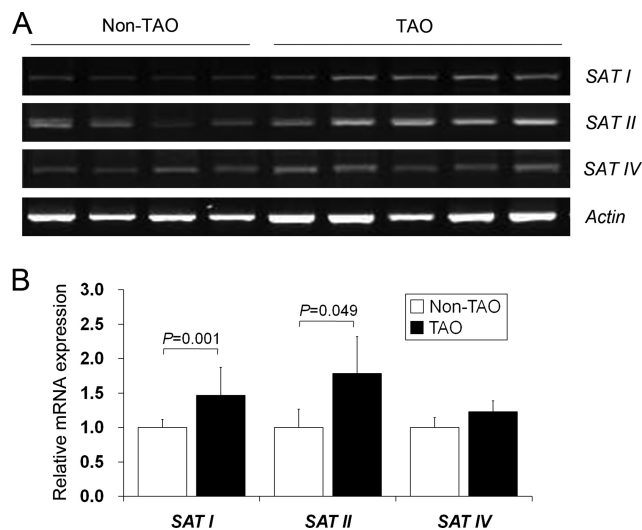


FIGURE 2. Enhanced mRNA expression of sialyltransferases in orbital tissues of TAO patients. (A) RT-PCR analysis of three subtypes of sialyltransferases (*SAT I*, *SAT II*, and *SAT IV*) in orbital tissues obtained from TAO patients ($n = 5$) and from control subjects ($n = 4$). Five micrograms of total RNA was used from each orbital tissue, and RT-PCR was performed. The experiment shown is representative of the three studies performed. (B) Relative signal intensities of sialyltransferases. Densitometric measurements were performed with the bands from (A). The measured intensities of sialyltransferases were corrected by the level of *actin* in each lane and analyzed (bar graph, mean \pm SD).

specific. Ultra-structural studies showed that GT1b-treated orbital fibroblasts were filled with numerous single membrane-bound bodies containing flocculent, medium-dense materials and particulate precipitates (Fig. 3B). To clarify whether these morphologic changes of orbital fibroblasts by GT1b were resulted from its impact on the cell viability, MTT assays were performed with the range of dose used in this study. As shown in Figure 3C, the viability of orbital fibroblasts from non-TAO subjects was not affected by the incubation of GT1b at concentrations of up to 40 $\mu\text{g}/\text{mL}$ for 48 hours.

Increase of Hyaluronic Acid in Orbital Fibroblasts by GT1b

TAO is associated with an accumulation of hyaluronic acid in the extracellular space of orbital connective tissue,⁸ and orbital fibroblasts are believed to be the source of hyaluronic acid. Interestingly, the electromicroscopic findings observed in Figure 3B are similar to the ones of histiocytes from a patient with a lysosomal storage disease, which is caused by a genetic deficiency of hyaluronidase resulting in the intracellular accumulation of hyaluronic acid.²⁵ To determine whether the intravesicular precipitate observed in GT1b-treated orbital fibroblasts was hyaluronic acid, we performed IFA of GT1b-treated orbital fibroblasts using antibodies for hyaluronic acid as well as the lysosomal marker LAMP2b. Compared with untreated control, GT1b-treated cells had numerous deposits of hyaluronic acid, especially along cellular membranes, and some of the deposited hyaluronic acid was co-localized with lysosomes (Fig. 4A). Moreover, corresponding to the differential effect of gangliosides on the morphologic changes in orbital fibroblasts shown in Figure 3A, GT1b-treated cells were most strongly stained by alcian blue, indicating the intracellular increase of hyaluronic acid (Fig. 4B). In addition, the extracellular concentrations of hyaluronic acid were measured after treatment with each subtype of gangliosides: GM1, GD1a, or GT1b, using competitive ELISA. Orbital fibroblasts were treated with 40 $\mu\text{g}/\text{mL}$ of each ganglioside, and the concentration of hyal-

uronic acid in supernatant was measured at various time points up to 48 hours. As the data in Figure 4C demonstrate, the level of hyaluronic acid increased threefold after 48 hours of treatment with GT1b compared with control cells (197.8 ng/10⁵ cells and 61.0 ng/10⁵ cells, respectively). GD1a induced the increase of hyaluronic acid level at 24 hours after treatment (68.2 ng/10⁵ cells; 28.4 ng/10⁵ cells in untreated control), but the level fell into the insignificant range at 48 hours (83.9 ng/10⁵ cells). GM1 showed no significant effect on the increase of hyaluronic acid in orbital fibroblasts throughout the observed periods. RT-PCR analysis was performed to examine the effect of GT1b on hyaluronic acid synthase (HAS) expression. As shown in Figure 4D, mRNA levels of three isoforms of HAS (HAS1, 2, and 3) were increased by GT1b treatment at 12 and 24 hours. Collectively, our results indicate that exogenous GT1b treatment increases both extracellular and intracellular levels of hyaluronic acid in orbital fibroblasts with the induction of synthesis.

Attenuation of GT1b-Induced Morphologic Changes in Orbital Fibroblasts by Hyaluronidase

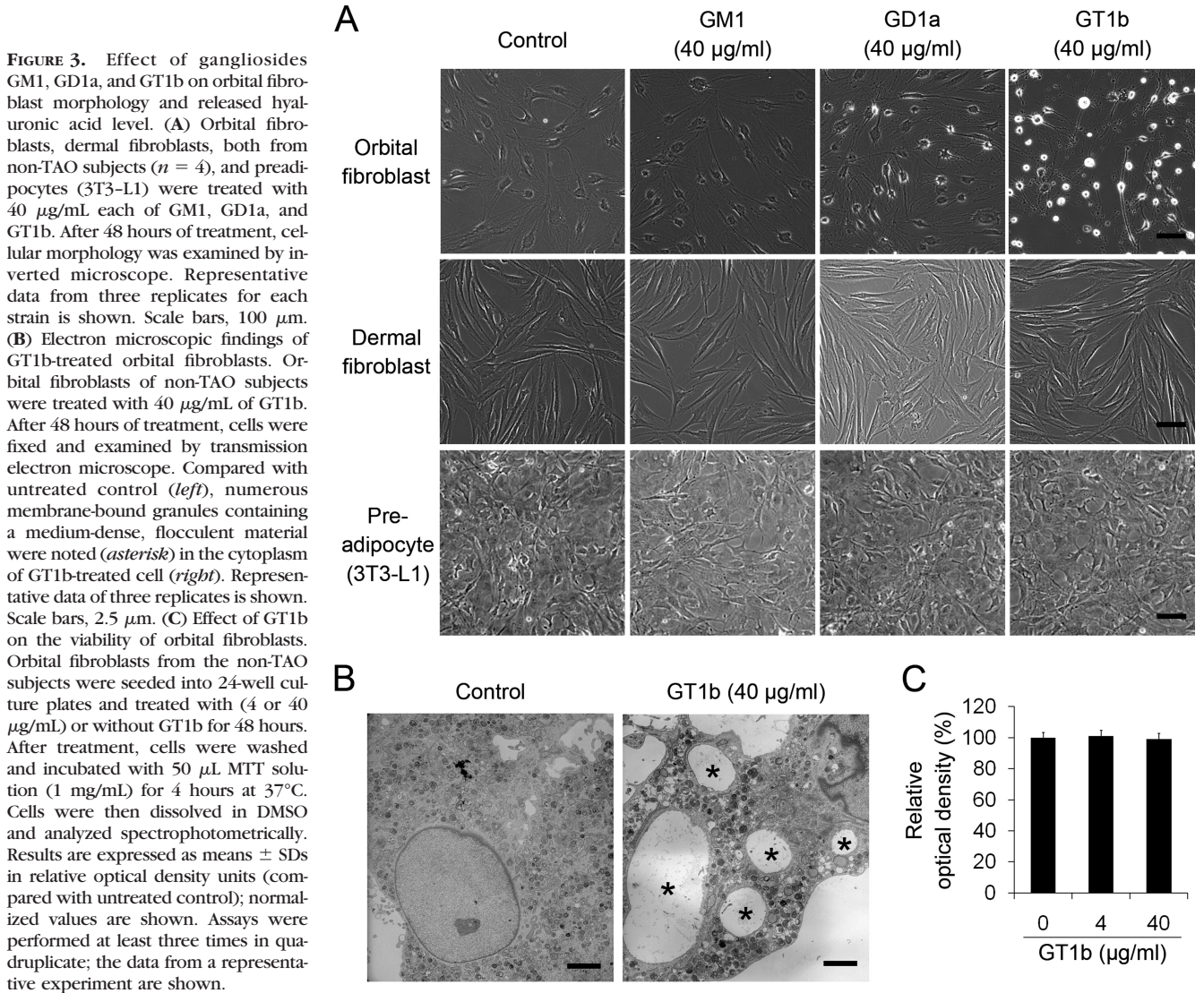
To examine the relevance of GT1b-induced morphologic changes with the increase of hyaluronic acid in orbital fibroblasts, 5 units/mL of *Streptomyces* hyaluronidase was added with GT1b (40 $\mu\text{g}/\text{mL}$). After 48 hours of incubation, cells that underwent morphologic changes were counted. The level of hyaluronic acid in the culture media was reduced after co-treatment of orbital fibroblasts with hyaluronidase and GT1b (data not shown). As shown in Figure 5A, the GT1b-induced morphologic changes of cells were attenuated by co-treatment with hyaluronidase. And correspondingly, cells co-treated with hyaluronidase stained for alcian blue with decreased intensity compared with the ones treated with GT1b only. The percentage of morphologically changed cells those were co-treated with GT1b and hyaluronidase was significantly less than that of cells treated with GT1b alone ($10.3 \pm 2.6\%$ and $41.1 \pm 7.9\%$, respectively; $P = 0.001$; Fig. 5B). Hyaluronidase alone had no detectable effect on cellular morphology. Taken together, our data suggest that GT1b causes morphologic changes of orbital fibroblasts by increasing hyaluronic acid, and hyaluronidase can attenuate this effect.

Different Capability of Orbital Fibroblasts to Increase Hyaluronic Acid by GT1b Depending on Thy-1 Expression

Orbital fibroblasts are believed to make up two populations according to the expression of Thy-1.²⁶ Interestingly, these cells in each subpopulation exhibit distinct phenotypes.²⁷ To investigate whether Thy-1⁺ and/or Thy-1⁻ fibroblasts were capable of increasing hyaluronic acid by GT1b treatment, we performed IFA of GT1b-treated orbital fibroblasts using antibodies for Thy-1 and hyaluronic acid. As shown in Figure 6, cells showing the increase in staining for hyaluronic acid by GT1b were also stained strongly for Thy-1 (left lower inset), but Thy-1⁻ cells were not stained for hyaluronic acid (right upper inset).

DISCUSSION

Our results demonstrate for the first time the increased expression of GT1b in orbital tissues from TAO patients. Exogenous GT1b strongly induced the morphologic change and the increase of hyaluronic acid in orbital fibroblasts from non-TAO subjects, which were less by GD1a and not by GM1. The effect of GT1b was shown specifically in orbital fibroblasts, but not in dermal fibroblasts or preadipocytes. The appearance of new gangliosides and changes in the cellular ganglioside profile are



important aspects of cellular metabolism,^{28–30} and several sialyltransferases catalyze the conversion of one ganglioside to another.³¹ In certain pathologic states, the upregulation of one sialyltransferase results in the overproduction of a particular ganglioside and an increase in the overall ganglioside concentration.³² Kiljanski et al. found that mRNA levels of *SAT I* and *SAT IV* were significantly increased in thyroid tissues from patients with Graves' disease.²³ Here we show that mRNA levels of *SAT I*, which catalyzes the conversion of lactoceramide into GM3 (an a-series monosialoganglioside), and *SAT II*, which catalyzes the conversion of GM3 into GD3 (a b-series substrate for GT1b), were significantly increased in the orbital tissues of TAO patients, suggesting that the increased expression of GT1b in TAO orbital tissue was resulted from the increased activities of these enzymes. Increased GT1b expression could be either a cause or an effect of TAO. In our system, exogenous GT1b induced morphologic changes and overproduction of hyaluronic acid in orbital fibroblasts obtained from non-TAO patients. Therefore, we may propose that the increased expression of GT1b and the enhanced activity of sialyltransferases in the orbital tissues of TAO patients are involved in the cause of disease pathogenesis.

From the insight of a changed ganglioside profile in certain pathologic states, several studies have been conducted to elucidate the differential effect of gangliosides depending on their

subtypes. In our study the morphologic changes and the increase of hyaluronic acid in orbital fibroblasts were most strikingly induced by GT1b, less by GD1a, and not by GM1. Similar to our observations, gangliosides have relative potency in inhibition of the binding of fibroblast growth factor (FGF)-2 to FGF receptors in transformed fetal bovine aortic endothelial cells (GM7373); GT1b > GD1b > GM1.³³ However, the reason for the differential effect of gangliosides by their subtypes is not fully understood. The oligosaccharide chain of gangliosides contains a variable number of sialic acid (*N-acetyl*-neuraminic acid, NeuAc) residues. Compared with GM1 and GD1a, which are in the a-series characterized by the linkage of one sialic acid to the nonreducing end of the galactose part, GT1b belongs to the b-series, which contains the structure NeuAc(2→8)NeuAc attached to the internal galactose.³⁴ Moreover, GT1b has three sialic acids: two linked to the nonreducing end of the galactose part and one terminal sialic acid residue (two in GD1a and one in GM1).³⁴ Thus, it may be speculated that this structural difference of gangliosides played a role in their ability to induce the morphologic changes and increase hyaluronic acid in orbital fibroblasts.

Hyaluronic acid, a high-molecular-weight glycosaminoglycan, is believed to be an important mediator of TAO because of its profound hydrophilic nature and its extreme molecular bulk when hydrated. Orbital fibroblasts synthesize a high level of

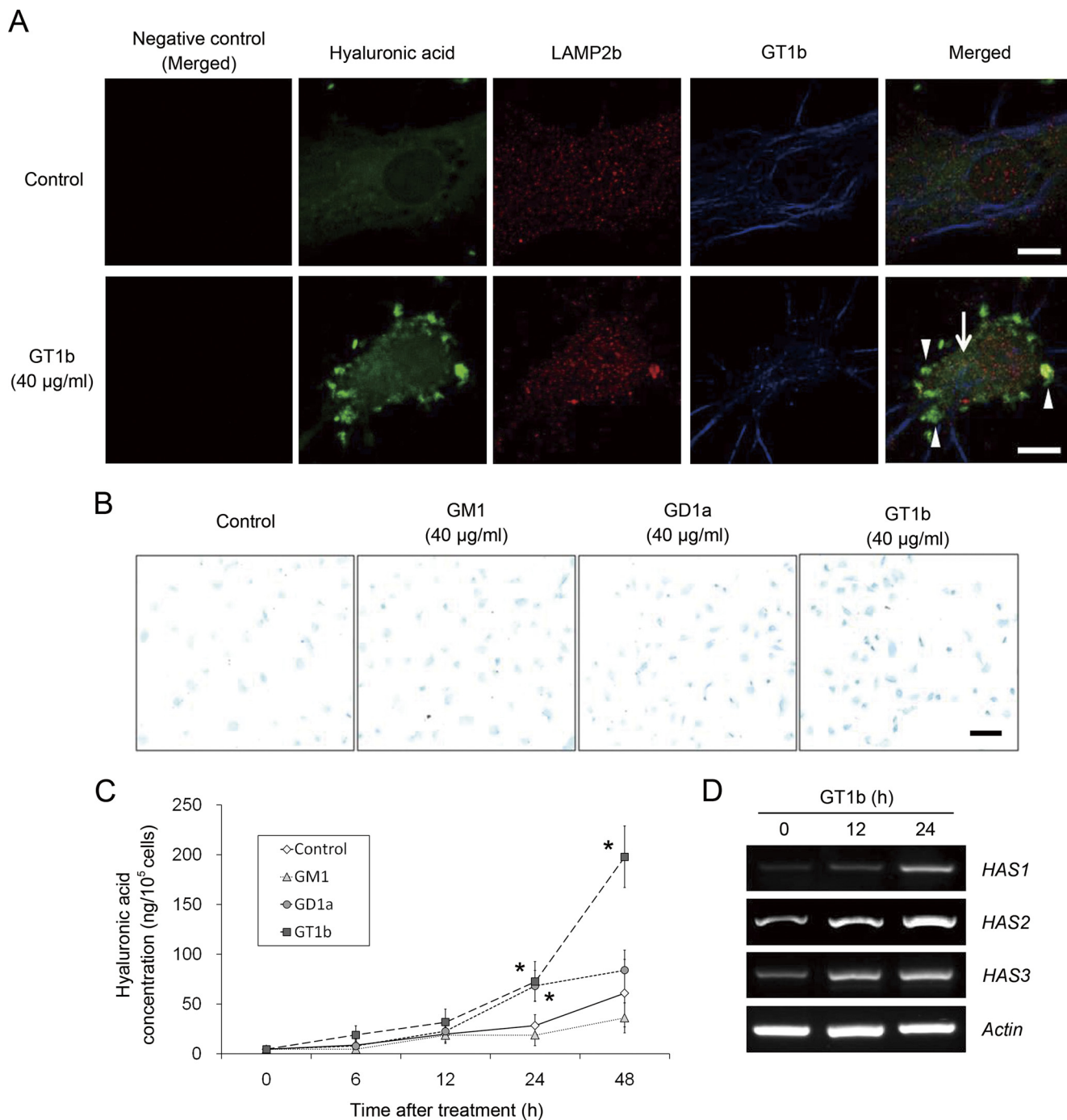


FIGURE 4. Induction of hyaluronic acid synthesis by GT1b in orbital fibroblasts. **(A)** Orbital fibroblasts obtained from non-TAO subjects ($n = 4$) were treated with 40 µg/mL of GT1b for 48 hours. After fixing, immunofluorescence staining was performed with primary antibodies for anti-hyaluronic acid (green), anti-LAMP2b (red), and anti-GT1b (blue); multiple spotty depositions of hyaluronic acid in cell cytoplasm, especially along the cell membrane (arrowheads), which partly co-localized with lysosomes (arrow). Representative example of immunofluorescence staining examined by confocal microscope from three independent experiments. Scale bars, 10 µm. Negative control, no primary Ab. **(B)** Alcian blue-stained images of gangliosides-treated orbital fibroblasts. Orbital fibroblasts from the non-TAO subjects were treated with 40 µg/mL of each ganglioside: GM1, GD1a, and GT1b, and cells were stained with alcian blue (pH 2.5). Light blue stain indicates hyaluronic acid. Representative from three independent experiments. Scale bars, 100 µm. **(C)** Concentration of released hyaluronic acid in gangliosides-treated orbital fibroblasts measured by ELISA. Orbital fibroblasts from non-TAO subjects were treated as described in **(B)**. Culture media was collected at each indicated time point and subjected to analysis with a specific ELISA for hyaluronic acid. Adherent cells were trypsinized and counted for standardization. Graph shows mean ± SD of three independent replicates (* $P < 0.05$). **(D)** RT-PCR analysis of *HAS1*, *HAS2*, and *HAS3* mRNA levels in GT1b-treated orbital fibroblasts of non-TAO subjects. Cultures were treated with GT1b (40 µg/mL) for 12 or 24 hours. RNA was extracted and subjected to RT-PCR using isoform-specific primers. The experiment shown is representative of three independent studies performed.

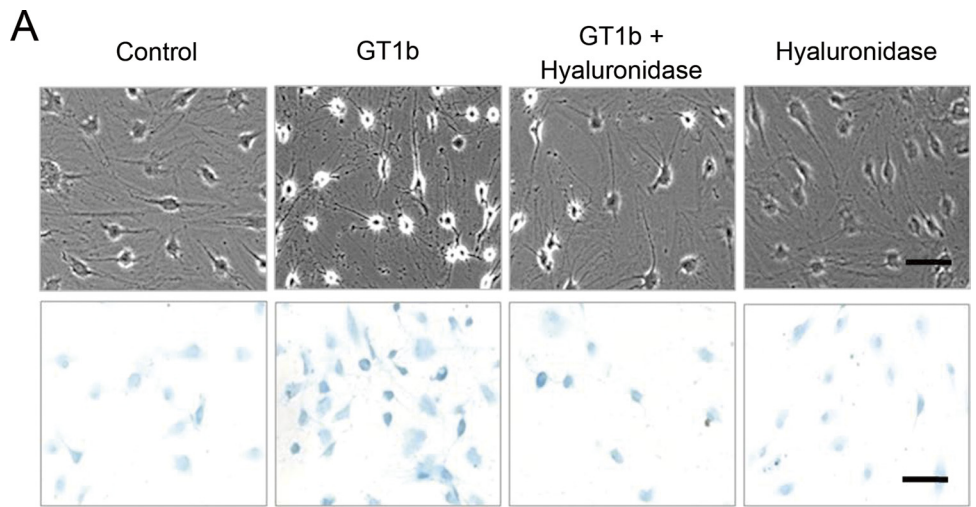
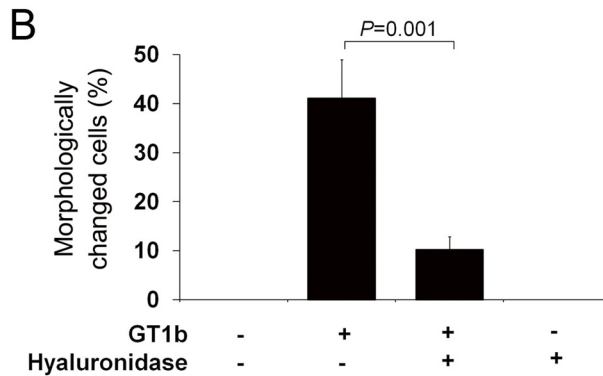


FIGURE 5. The attenuation of the GT1b-induced morphologic changes and increase of hyaluronic acid by hyaluronidase in orbital fibroblasts. **(A)** Cultivated orbital fibroblasts from non-TAO subjects ($n = 4$) were treated with $40 \mu\text{g/mL}$ of GT1b and with or without 5 units/mL of *Streptomyces* hyaluronidase. After 48 hours of incubation, cells were examined by inverted microscope (*upper*) or by alcian blue staining (pH 2.5) (*lower*). Light blue stain indicates hyaluronic acid. Representative from three independent experiments. Scale bars, $100 \mu\text{m}$. **(B)** Number of cells showing the morphologic changes in **(A)** was counted and presented as a percentage of the total counted cells (bar graph, mean \pm SD).



hyaluronic acid, and the accumulation of hyaluronic acid leads to the expansion of orbital tissues, resulting in the expansion of the eye beyond the normal boundaries of the bony orbit.⁵ Hyaluronic acid is synthesized by a class of integral membrane proteins called hyaluronic acid synthase (HAS), of which vertebrates have three types (HAS1, HAS2, and HAS3), and ex-

truded through the plasma membrane into the extracellular matrix or onto the cell surface.³⁵ Inflammatory mediators such as IL-1 β and TGF- β , which are highly expressed in the orbital tissues of TAO patients, have been reported to increase hyaluronic acid synthesis in cultured human orbital fibroblasts by the induction of HAS.^{10,36} In our study, GT1b increased mRNA

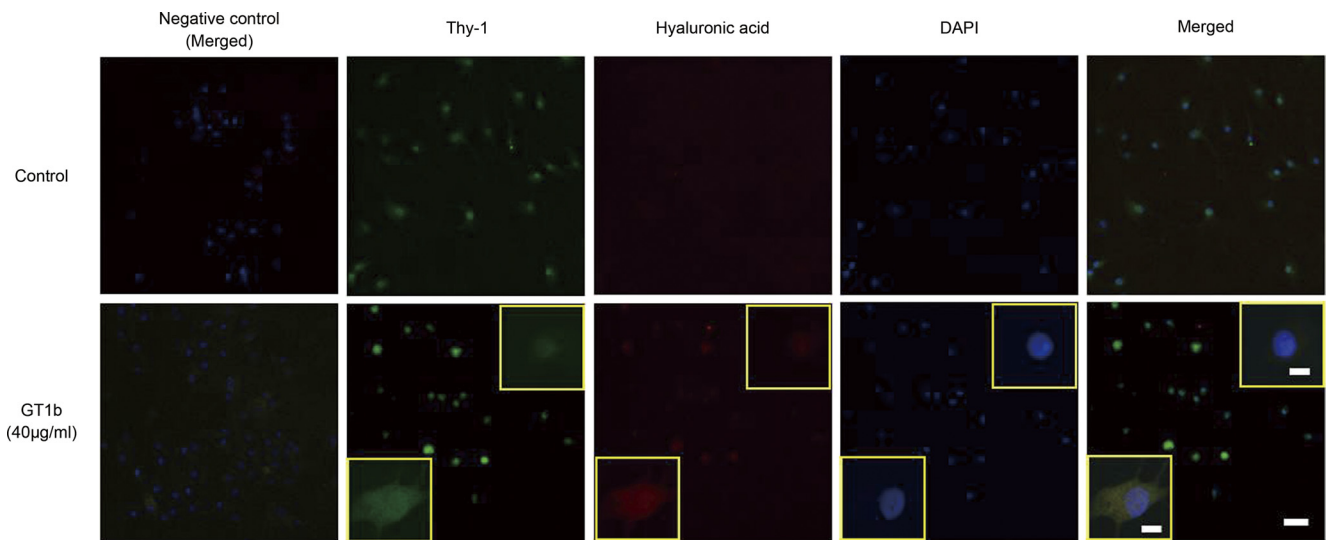


FIGURE 6. Different capability of orbital fibroblasts to increase hyaluronic acid by GT1b depending on Thy-1 expression. Orbital fibroblasts from non-TAO subjects ($n = 4$) were treated with $40 \mu\text{g/mL}$ of GT1b for 48 hours. After fixing, immunofluorescence staining was performed with primary antibodies for Thy-1 (*green*) and hyaluronic acid (*red*), and DAPI (4',6-diamidino-2-phenylindole) was used for nuclear stain (*blue*); positively stained cells for hyaluronic acid and Thy-1 (*left lower insets*), and double negative cells (*right upper insets*). Representative example of immunofluorescence staining examined by confocal microscope from three independent experiments. Scale bars: $50 \mu\text{m}$; $10 \mu\text{m}$ in *insets*.

levels of these enzymes, suggesting that GT1b induced the synthesis of hyaluronic acid in orbital fibroblasts. Interestingly, a potential role of GT1b in modulating the immunologic process has been suggested. GT1b suppresses the immunoglobulin production in human peripheral blood mononuclear cells without affecting the proliferation or viability of cells.³⁷ GD1b, GT1b, and GQ1b, the b-series of gangliosides, enhance IL-2 and IFN- γ production in phytohemagglutinin-stimulated human T cells, whereas GM1, GM2, GM3, GD1a, GD2, and GD3 do not alter the secretion of these cytokines.³⁸ Thus, it could be suggested that GT1b is one of the potent enhancers of HAS expression in orbital fibroblasts, as well as the inflammatory mediators such as IL-1 β and TGF- β .

Here we have shown that GT1b induces the dramatic enhancement of intracellular hyaluronic acid as well as extracellular ones in orbital fibroblasts. The increase of extracellular hyaluronic acid was initiated 6 hours after GT1b treatment, but the morphologic changes were not noticeable until at least 24 hours after treatment (Y-HC and KHK, unpublished observation, 2009), indicating that the morphologic changes followed the increase of hyaluronic acid. Although the detailed mechanism is not elucidated, we have provided evidence for a function of GT1b as a strong inducer of the accumulation of hyaluronic acid and subsequent morphologic changes in orbital fibroblasts. Hyaluronic acid is mainly a pericellular matrix component; however, intracellular hyaluronic acid has been localized in cytoplasm and the nuclei of proliferating cells.^{39,40} When hyaluronic acid is taken up from the pericellular matrix, it is destined for eventual degradation in the lysosomes.⁴¹ In our study, the increase of intracellular hyaluronic acid induced by GT1b treatment partly co-localized with the lysosomes. In addition, human orbit possesses no organized lymphatic net,⁴² into which most extracellular hyaluronic acid eventually diffuses for subsequent catabolizing.⁴³ The specific characteristics of orbital fibroblasts, resident in orbit where no lymphatic outflow system exist, may explain the abundance of hyaluronic acid in the lysosomes of orbital fibroblasts observed in our system. In terms of TAO, it will be interesting to determine whether gangliosides, including GT1b, affect the expression and activity of hyaluronidase in orbital fibroblasts.

In this study, GT1b induced the morphologic changes in orbital fibroblasts but failed to do so in dermal ones or preadipocytes. Unlike other fibroblasts derived mostly from the mesoderm, orbital fibroblasts are known to originate from the neuroectoderm.⁴⁴ Orbital fibroblasts have unique site-specific characteristics compared with other types of fibroblasts.¹ Proinflammatory cytokines, such as leukoregulin and IL-1 β , potentiate the induction of orbital fibroblast genes that play important roles in the orbital inflammatory responses but do not stimulate inflammatory response in dermal fibroblasts.^{45,46} Such site-specific characteristics of orbital fibroblasts may be relevant to our results. In addition, orbital fibroblasts comprise two subsets based on the surface expression of Thy-1, a surface glycoprotein.²⁶ Each subset can respond to different extracellular stimuli and differentiate into distinct cell types. Thy-1⁺ fibroblasts can differentiate into myofibroblasts when treated with TGF- β , whereas Thy-1⁻ ones can differentiate into mature adipocyte by peroxisome proliferator-activated receptor γ agonists as well as with c-AMP-enhancing agents.^{47,48} Thy-1 is overexpressed in orbital tissues in TAO,⁴⁹ and Thy-1⁺ cells are capable of cytokine-induced production of prostaglandin E2, interleukin-8, and hyaluronic acid.⁴⁸ In accordance with these previous reports, our data demonstrated that Thy-1⁺ orbital fibroblasts increased hyaluronic acid by exogenous GT1b, whereas Thy-1⁻ ones failed to do so.

In summary, our results provide initial evidence that gangliosides, especially GT1b, have a possible role in the pathogenesis of TAO, and this suggests that further investigations are

warranted for new possible therapeutic targets against sialyltransferases and gangliosides. Moreover, considering that hyaluronidase successfully attenuated the effect of GT1b on orbital fibroblasts, this also suggests the possibility that hyaluronidase may be a candidate for new localized therapeutic or preventive modality.

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