INTRODUCTION

EMT (epithelial–mesenchymal transition), the programmed and differentiated processes for transition from epithelial cells to mesenchymal phenotype cells, is related with the various pathological events such as development, wound healing, tissue fibrosis, cataract, and cancer [1–4]. Among these events PCO (posterior capsule opacification), an ASC (anterior subcapsular cataract) or a common complication after cataract surgery, is developed by the induction of EMT in lens epithelial cells [5]. These cells undergo proliferation and transdifferentiation into mesenchymal-like cells such as myofibroblasts, and express the extracellular molecules, including type I collagen, α-smooth muscle actin and fibronectin [1–5]. The formation of PCO through survival, proliferation and migration of the lens epithelial cells is regulated by various growth factors: EGF (epidermal growth factor) [6], FGF (fibroblast growth factor) [7], HGF (hepatocyte growth factor) [8] and TGF-β (transforming growth factor-β) [4, 9]. During the development of anterior polar cataracts and posterior capsular cataracts, TGF-β, known as a key regulator for the development of various pathological conditions such as tissue fibrosis and scleroderma, induces pathological epithelial–mesenchymal changes and produces extracellular matrix, such as fibronectin and type I collagen, in ocular tissues for regulating the development of PCO [2, 4, 5, 9]. The TGF-β–related signal transduction initially internalizes via serine/threonine phosphorylation of TGFβR (TGF-β receptor) I after pairing TGFβRI and TGFβRII on the cell surface [10]. The phosphorylated TGFβRII directly activates the downstream mediators R-Smads (receptor-activated Smads), including Smad2 and Smad3 [10–13]. The activated Smad2/3 binds with the common mediator [Co-Smad, Sp1 (specificity protein 1) and p300] and translocates to the nucleus [14, 15]. After translocation the Smad complex induces the activation and regulation of EMT-related multifunctional genes, such as E-cadherin and vimentin [14–16]. TGF-β signalling is also found in EMT-induced HLE (human lens epithelial) cells after PCO [11, 16].

GSLs (glycosphingolipids), which are localized on the membrane surface, are involved in epithelial differentiation and maturation [17, 18]. Gangliosides, sialic acid-containing GSLs, TGF-β (transforming growth factor-β)-induced EMT (epithelial–mesenchymal transition) induces the proliferation and migration of the HLE (human lens epithelial) cells. Ganglioside GM3, simple sialic-acid-containing glycosphingolipids on mammalian cell membranes, regulates various pathological phenomena such as insulin resistance and tumour progression. However, the relationship between ganglioside GM3 and TGF-β–induced EMT in the HLE B-3 cells is poorly understood. In the present study we demonstrated that ganglioside GM3 was involved in TGF-β1-induced EMT in HLE B-3 cells. Our results indicated that the expression of ganglioside GM3 and GM3 synthase mRNA were significantly increased in TGF-β1-induced HLE B-3 cells. Reporter gene analysis also demonstrated that transcriptional activation of the GM3 synthase gene was regulated by Sp1 (specificity protein 1) in HLE B-3 cells upon TGF-β1 stimulation. Interestingly, the inhibition of ganglioside GM3 expression by d-PDMP [d-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol] and GM3 synthase shRNA (short hairpin RNA) resulted significantly in the suppression of cell migration and EMT-related signalling in HLE B-3 cells stimulated by TGF-β1. Furthermore, exogenous treatment of ganglioside GM3 rescued the expression of EMT molecules and cell migration suppressed by the depletion of ganglioside GM3 in TGF-β1-induced HLE B-3 cells. We also found that ganglioside GM3 interacted with TGFβRs (TGF-β receptors) in TGF-β1-induced HLE B-3 cells. Taken together, these results suggest that ganglioside GM3 induced by TGF-β1 regulates EMT by potential interaction with TGFβRs.

Key words: ganglioside GM3, lens epithelial cell, transforming growth factor-β1-induced epithelial–mesenchymal transition (TGF-β1-induced EMT), transcriptional regulation, transforming growth factor β1 receptor (TGFβR).

Abbreviations used: ASC, anterior subcapsular cataract; CREB, cAMP-response-element-binding protein; CBP, CREB-binding protein; d-PDMP, d-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; EGFR, EGF receptor; EMT, epithelial–mesenchymal transition; FBS, fetal bovine serum; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSL, glycosphingolipid; HLE, human lens epithelial; HPTLC, high-performance TLC; HRP, horseradish peroxidase; IP, immunoprecipitation; IR, insulin receptor; MEM, Eagle’s minimum essential medium; NF-κB, nuclear factor κB; PCO, posterior capsular opacification; R-Smad, receptor-activated Smad; RT, reverse transcription; shRNA, short hairpin RNA; Sp1, specificity protein 1; TGF-β, transforming growth factor-β; TGFβR, TGF-β receptor; VEGF, vascular endothelial growth factor.

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participate in the regulation for various biological events, such as cell proliferation, cell–cell interaction, differentiation, oncogenic transformation and signal transduction [19–21]. Gangliosides are also involved in various phenomena, such as inflammatory obesity (gangliosides GM2, GM1 and GD1a), monocyte differentiation (gangliosides GM3 and GD3) and angiogenic signalling (gangliosides GD1a and GM3) [21–25]. Ganglioside GM3, in particular, regulates some pathological phenomena and modulates their cellular internalization, such as obesity, insulin resistance and tumour progression [22,26,27]. On the cellular membrane ganglioside GM3 regulates the activation and tyrosine phosphorylation of the IR (insulin receptor), FGF receptor and platelet-derived growth factor receptor [27–30]. Also, ganglioside GM3 reduced the tyrosine phosphorylation of VEGF (vascular endothelial growth factor) receptor 2 and EGFR (EGF receptor) in VEGF- and EGF-stimulated cells [24,25].

It has been reported that GSLs and gangliosides are accumulated in larger amounts in human cataract lens tissues compared with the normal lens [31,32]. Although these sphingolipids are linked with cataract formation and development, the regulation and function between TGF-β, EMT and GSLs, including ganglioside GM3, in HLEs is still unknown. In the present study we have found that ganglioside GM3 increases the EMT in TGF-β1-exposed HLE cells and that the transcriptional activity of the GM3 synthase gene is significantly up-regulated by Sp1 in TGF-β1-induced HLE cells. The results of the present study have shown that blocking the production of ganglioside GM3 inhibits the formation of EMT and cell migrations in HLE B-3 cells induced by TGF-β1. We also found that the ganglioside GM3 associates with TGF/βRs in the cell membrane. We also, for the first time, suggest that ganglioside GM3 interacts with TGF/βRs as part of TGF-β1-induced EMT and HLE cell migration.

MATERIALS AND METHODS

Reagents

TGF-β1 was obtained from R&D Systems. Antibodies against phospho–SMAD-2 and phospho–SMAD-3 were purchased from Cell Signaling Technology. Anti-TGFβRI, anti-TGFβRII, antiphospho-serine, anti-rabbit HRP (horseradish peroxidase)-complexed anti-goat and anti-mouse IgG and anti-mouse IgM antibodies, and the Protein A/G– and Protein L–agarose complexesed anti-goat and anti-mouse IgG and anti-mouse IgM antibodies. Equal loading was confirmed using an anti-GAPDH antibody. The signals of the bound antibodies were visualized using an ECL (enhanced chemiluminescence) system (Amerham Biosciences).

Cell culture

The HLE B-3 cell line was obtained from the A.T.C.C. (Manassas, VA, U.S.A.). The cell was grown in MEM (Eagle’s minimum essential medium; PAA) with FBS (fetal bovine serum; PAA) to a final concentration of 20%, 0.1 mM sodium pyruvate, 2 mM glucose and 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C under a humidified 5% CO2 atmosphere. For the TGF-β1 treatment the cells were starved in growth medium containing 1% FBS for 18 h and in serum-free medium for 24 h. The starved cells were induced by TGF-β1 (10 ng/ml) for 24 h. For the ganglioside GM3 and TGF-β1 treatment the starved cells were pre-treated by ganglioside GM3 for 1 h and stimulated by TGF-β1 (10 ng/ml) for 24 h.

Ganglioside purification

The ganglioside isolation procedures have been described previously [33]. Briefly, total lipids from HLE B-3 cells homogenized with chloroform/methanol [1:1 (v/v)] were incubated with solvent A [30:60:8 chloroform/methanol/water (by vol.)] for 3 h. After incubation with solvent A, the lipids were separated by centrifugation (500 g for 20 min at 20°C). The lipid extracts isolated by solvent A were adjusted into a DEAE-Sephadex A-25 column. After removing the neutral lipids with solvent A, the ganglioside and other acidic lipids were eluted with solvent B [30:60:8 chloroform/methanol/0.8 M sodium acetate (by vol.)] and dried. The samples were redissolved in 0.5 ml of chloroform/methanol [1:1 (v/v)]. To alkalinize the gangliosides, the samples were incubated with 12 M ammonia solution at room temperature (20°C) overnight. After drying the alkalinized sample was desalted using a Sep-Pac C18 cartridge.

HPTLC (high-performance TLC) and immuno-TLC

The collected gangliosides were separated on plates of silica gel 60 (Merck) with chloroform/methanol/0.2% aqueous calcium chloride [55:45:10 (by vol.)] as a developing solution using HPTLC. The plates were sprayed with a resorcinol/HCl reagent and then heated at 110°C for 30 min. For immunostaining of the TLC plates, the plates were fixed by 0.1% poly(isobutyl methacrylate) in n-hexane. The plates were blocked with PBS/1% BSA for 1 h at room temperature, incubated with an anti-ganglioside GM3 antibody diluted in PBS/0.1% BSA (1:100) for 3 h at room temperature and then washed with PBS three times. The plates were incubated with a HRP-complexed anti-mouse IgM antibody diluted in PBS/0.1% BSA (1:1000) for 1 h at room temperature. After washing with PBS, the plates were visualized using an ECL (enhanced chemiluminescence) system (Amersham Biosciences).

RT (reverse transcription)–PCR analysis

Total RNA prepared from HLE B-3 cells was extracted using the Corezol reagent (Corebio). For the synthesis of cDNAs, 1 μg of total RNA was reverse transcribed by RT–PCR (Bioneer). The target genes were amplified from the cDNAs using the primers shown in Table 1 to confirm the levels of their expressions. β-Actin was used to normalize the levels of mRNA expression.

Immunoblotting analysis

Immunoblotting analysis was carried out as described previously [25]. Briefly, immune precipitates or cell lysates from HLE B-3 cells exposed to TGF-β1 or ganglioside GM3 were analysed by SDS/PAGE (10% gel) and then electrotransferred on to Protran® nitrocellulose transfer membranes (Whatman). After transfer the membranes were blocked by blocking buffer [5% non-fat dried skimmed milk in TBST (Tris-buffered saline with 0.05% Tween 20)] and incubated with anti-phospho–SMAD-2, anti-phospho–SMAD-3, anti-TGFβRI, anti-TGFβRII and anti-phospho-serine antibodies. Equal loading was confirmed using an anti-GAPDH antibody. The signals of the bound antibodies were visualized using ECL with HRP-complexed anti-rabbit and anti-mouse IgG antibodies.
Immunofluorescence microscopy

The immunostaining with fluorescence was performed as described previously [25]. Briefly, HLE B-3 cells were incubated on gelatin-coated glass coverslips in 24-well tissue culture plates. After treatment with TGF-β1 for 24 h the cells were fixed in 3.7 % formaldehyde for 30 min at room temperature and washed with PBS. The cells were blocked with 1 % BSA for 1 h and incubated with each antibody diluted in PBS/0.1 % BSA (1:200) at 4 °C overnight. After washing the incubated cells they were incubated with the FITC-conjugated anti-mouse IgG/H/A mix antibody, Alexa Fluor® 594, Alexa Fluor® 488 or Texas Red-conjugated secondary antibody (1:500 dilution) at room temperature for 1 h. After incubation, the cells were washed with PBS and observed under a fluorescence microscope (Nikon).

Construction of reporter plasmids for a luciferase assay and mutagenesis

The preparation of promoter plasmids for GM3 synthase (pGL3-1600 to pGL3-83) have been described previously [34]. Using EF-Taq PCR (Solgent) the pGL3-432-partial promoter was amplified from pGL3-432 with the primers: sense, 5'-ATGAGCTCTACGTGCAGCTGTCAGCCAAGAAA-3' (SacI site underlined) and antisense, 5'-ACAGATCTTCCTCGGGGGGCCC-C-3' (BglII site underlined). The amplified fragment was sequenced using the subcloning vector pT7Blue(R) T-vector (Promega) and inserted into the pGL3-1600 to pGL3-83) have been described previously [34]. Using EF-Taq PCR (Solgent) the pGL3-432-partial promoter was amplified from pGL3-432 with the primers: sense, 5'-ATGAGCTCTACGTGCAGCTGTCAGCCAAGAAA-3' (SacI site underlined) and antisense, 5'-ACAGATCTTCCTCGGGGGGCCC-C-3' (BglII site underlined). The amplified fragment was sequenced using the subcloning vector pT7Blue(R) T-vector (Promega) and inserted into the pGL3-Basic vector (Promega) sequenced using the subcloning vector pT7Blue(R) T-vector and antisense, 5'-GACCAGGGAGACCAAACTC-3' (BglII site underlined). The amplified fragment was sequenced using the subcloning vector pT7Blue(R) T-vector (Promega) and inserted into the pGL3-Basic vector (Promega) sequenced using the subcloning vector pT7Blue(R) T-vector (Promega) and inserted into the pGL3-Basic vector (Promega).

Table 1  Primers and expected sizes for each gene used in the present study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM3 synthase</td>
<td>5'-ATGGCGAGGCGAAGGGGGGCCC-3'</td>
<td>5'-GGAGCCATTGACGTAATTTGA-3'</td>
<td>1257</td>
</tr>
<tr>
<td>Fibronecin</td>
<td>5'-GCCATGACGTAAGCTGTCAG-3'</td>
<td>5'-ATCCCCAGCTACATGGGCCTC-3'</td>
<td>421</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>5'-TCAAAAGAGAAGGACGCTA-3'</td>
<td>5'-GACATGAGGAATCCCAAACAC-3'</td>
<td>693</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-CAAGAGATGGCGCCAGGGGT-3'</td>
<td>5'-TCGTTGCACTCCTGGGGCA-3'</td>
<td>275</td>
</tr>
</tbody>
</table>

Wound-healing assay

The wound-healing assay was performed as described previously [35]. Confluent HLE B-3 cell monolayers in 12-well plates were wounded by manually scraping the cells with a blue pipette tip. The cells were then starved and pre-treated with d-PDMP in serum-free medium for 1 h. The pre-treated cells were incubated with or without TGF-β1 (10 ng/ml). After 24 h, cell migration into the wound surface was captured by microscopy with a camera (Motic Incorporation).

Transmigration assay

The transmigration assay was performed in 24-transwell chambers of 6.5-mm diameter containing polycarbonate 8-μm pore membrane filters (Corning Incorporated). The filters of the upper well were coated with 0.1 % gelatin and the HLE B-3 cells (2×10⁴ cells/200 μl in MEM containing 1 % FBS) were added to the upper well with or without d-PDMP. To the lower well was added growth medium (2 % FBS) containing TGF-β1 (10 ng/ml). After incubation at 37 °C for 24 h in 5 % CO₂ atmosphere, the non-migrated cells were removed from the surface of upper well membrane. The cells on the filter were fixed with methanol, stained and mounted on microscope slides. The number of migrated cells was counted with microscopy from three filters.

Analysis of interaction between ganglioside GM3 and TGFβR

The interaction assay was modified and performed as described previously [25]. To analyse whether ganglioside GM3 interacts with TGFβRI or TGFβRII, HLE B-3 cells were lysed with IP (immunoprecipitation) buffer (50 mM Tris/HCl (pH 7.4),
TGF-β1 induces the expression of ganglioside GM3 during EMT in HLE B-3 cells

In human cataract lens tissues, glycolipids, including the neutral and acidic lipid fractions, are increased compared with normal lens tissues [31,32]. In addition TGF-β induces EMT for development of PCO in HLE B-3 cells [4,5]. To analyse the levels of gangliosides in TGF-β1-induced HLE B-3 cells, the acidic fraction of glycolipid was isolated and compared using TLC. As shown in Figure 1(A) the TLC data showed that the ganglioside GM3, which is lactosylceramide-containing α-2,3-linked sialic acid [36], was increased in TGF-β1-induced HLE B-3 cells compared with normal HLE B-3 cells. This increase was also confirmed by immunofluorescence staining (Figure 1B). The mRNA level of GM3 synthase after treatment with TGF-β1 was then checked in HLE B-3 cells. As shown in Figure 1(C) the transcriptional levels of GM3 synthase and the mRNA levels of fibronectin and type I collagen, well-known markers for EMT in lens epithelial cells, were significantly increased in TGF-β1-induced HLE B-3 cells. These data suggest that the expressions of GM3 synthase and ganglioside GM3 are significantly induced during TGF-β1-induced EMT of HLE B-3 cells.

Results are means ± S.D. All experiments were reconfirmed at least three times. All of the data were analysed by ANOVA followed by Student’s t test.

RESULTS

TGF-β1 induces the expression of ganglioside GM3 during EMT in HLE B-3 cells

The depletion of ganglioside GM3 suppresses TGF-β1-induced EMT and cell migration in HLE B-3 cells

During TGF-β1-induced EMT of HLE B-3 cells the levels of GM3 synthase mRNA were increased significantly (Figure 1C). In order to analyse whether transcriptional activity of GM3 synthase is regulated in TGF-β1-induced EMT of HLE B-3 cells, the transcription activity of the GM3 synthase promoter [34] using a luciferase reporter gene assay system was investigated. As shown in Figures 2(A) and 2(C) the transcriptional activities of the GM3 synthase promoter region, including between −432 and +1 and −432 and −178, were significantly increased in HLE B-3 cells treated with TGF-β1, as compared with other plasmids. Sequence analysis of the GM3 synthase promoter region between −432 and −178 was then performed. The region contained p300-, NF-κB- and two Sp1-binding elements (Figure 2B). Previously these transcriptional elements have been shown to be associated with TGF-β signalling [13–15]. To analyse which of these transcription factors are involved in TGF-β1-induced EMT, mutational inactivation analysis of the represented binding sites to each factor was performed. As shown in Figure 2(C) the transcriptional activities of each mutated plasmid to the two Sp1-binding sites were reduced in TGF-β1-treated HLE B-3 cells, as compared with mutant plasmids of p300 and NF-κB. Moreover, the double mutant plasmid of the two Sp1-binding sites reduced transcriptional activity more than single Sp1 mutant plasmids (Figure 2C). These results clearly indicate that Sp1 participates in TGF-β1-induced transcriptional activation of GM3 synthase in HLE B-3 cells.

TGF-β1-induced EMT increases the transcriptional activation of GM3 synthase mediated by Sp1 in HLE B-3 cells

During TGF-β1-induced EMT of HLE B-3 cells the levels of GM3 synthase mRNA were increased significantly (Figure 1C). In order to analyse whether transcriptional activity of GM3 synthase is regulated in TGF-β1-induced EMT of HLE B-3 cells, the transcription activity of the GM3 synthase promoter [34] using a luciferase reporter gene assay system was investigated. As shown in Figures 2(A) and 2(C) the transcriptional activities of the GM3 synthase promoter region, including between −432 and +1 and −432 and −178, were significantly increased in HLE B-3 cells treated with TGF-β1, as compared with other plasmids. Sequence analysis of the GM3 synthase promoter region between −432 and −178 was then performed. The region contained p300-, NF-κB- and two Sp1-binding elements (Figure 2B). Previously these transcriptional elements have been shown to be associated with TGF-β signalling [13–15]. To analyse which of these transcription factors are involved in TGF-β1-induced EMT, mutational inactivation analysis of the represented binding sites to each factor was performed. As shown in Figure 2(C) the transcriptional activities of each mutated plasmid to the two Sp1-binding sites were reduced in TGF-β1-treated HLE B-3 cells, as compared with mutant plasmids of p300 and NF-κB. Moreover, the double mutant plasmid of the two Sp1-binding sites reduced transcriptional activity more than single Sp1 mutant plasmids (Figure 2C). These results clearly indicate that Sp1 participates in TGF-β1-induced transcriptional activation of GM3 synthase in HLE B-3 cells.

The depletion of ganglioside GM3 suppresses TGF-β1-induced EMT and cell migration in HLE B-3 cells

During TGF-β1-induced EMT TGF-β1-related signal transduction is via phosphorylation of TGFβRs and R-Smad [10–13]. This signalling pathway induces the production of extracellular matrix constituents, such as fibronectin and type I collagen, in ocular tissues for development of PCO [2–5,9]. As shown above TGF-β1 increased the expression of ganglioside GM3 synthase mRNA and ganglioside GM3 in HLE B-3 cells. Thus we investigated whether ganglioside GM3 is related to TGF-β1-induced signalling for EMT of HLE B-3 cells. For depletion of the ganglioside GM3, three GM3 synthase shRNAs were stably transfected in HLE B-3 cells. Of these shRNAs the shRNA-3 targeting GM3 synthase mRNA (shGM3-3) highly suppressed the expression of GM3 synthase mRNA induced by TGF-β1 compared with shGM3-1 and shGM3-2 (Figure 3A).
Increase of ganglioside GM3 during EMT in lens epithelial cells

Figure 2  Transcriptional activation of GM3 synthase mediated by Sp1 in TGF-β1-induced HLE B-3 cells

(A) Schematic representation of the sequential deletion constructs of GM3 synthase promoter. HLE B-3 cells were transiently co-transfected with the deletion constructs and pCMVβ as the internal control. The transfected cells were incubated with or without TGF-β1 (10 ng/ml) for 24 h and the luciferase activity was determined. The induction of GM3 synthase promoter activity was expressed as the fold change. Results are means ± S.D. from triplicate experiments. (B) Sequence of the region between −432 and −178 in the GM3 synthase promoter including p300, NF-κB and two Sp1-binding elements involved in the response to TGF-β1-induced EMT. (C) Schematic representation of substitution mutations and these activities of p300, NF-κB, Sp1/Sp1-1 alone and the Sp1/Sp1-2 together binding elements within the GM3 synthase. The transfected cells were incubated in the presence or absence of TGF-β1 (10 ng/ml) for 24 h and luciferase activity was determined. The activity of each promoter was expressed as the fold change. Results are means ± S.D. from triplicate experiments.

Furthermore, shGM3-3 markedly diminished the generation of ganglioside GM3 in TGF-β1-induced HLE B-3 cells, as shown by immunofluorescence staining (Figure 3B). In addition the TGF-β1-induced expression of fibronectin and type I collagen was inhibited in shGM3-3-transfected HLE B-3 cells (Figure 3C). We further checked the expression of EMT-related molecules in TGF-β1-induced HLE B-3 cells in the presence of d-PDMP, an inhibitor of glucosylceramide synthase. As shown in Figure 3(C), the expression of fibronectin and type I collagen were significantly inhibited in d-PDMP-pre-treated and TGF-β1-induced HLE B-3 cells compared with only TGF-β1 treatment. This was owing to the inhibition of ganglioside GM3 production by d-PDMP; although direct inhibition of the expression of the GM3 synthase mRNA was not observed (Figure 3C). Next, we examined whether ganglioside GM3 might affect the TGF-βR signalling including phosphorylation of TGF-βR and Smad2/3, that are downstream of TGF-βR, in TGF-β1-induced HLE B-3 cells using d-PDMP or GM3 synthase shRNA. The phosphorylation of TGF-βR and Smad2/3 was increased in HLE B-3 cells induced by TGF-β1 (Figure 3D). However, the enhanced serine phosphorylation of TGF-βR induced by TGF-β1 was remarkably suppressed by d-PDMP or shGM3-3 in HLE B-3 cells (Figure 3D). Moreover, the phosphorylation of Smad2/3 was blocked by d-PDMP or shGM3 in HLE B-3 cells during TGF-β1-induced EMT (Figure 3D). These data clearly suggest that the ganglioside GM3 is associated with TGF-β1-mediated EMT in HLE B-3 cells.

Some studies have shown that EMT-induced epithelial cells undergo cell migration, moving to another area such as the anterior...
Figure 3 The roles of ganglioside GM3 for EMT in TGF-β1-induced HLE B-3 cells

The expression of ganglioside GM3 was disrupted by treatment with d-PDMP or transfection of GM3 synthase shRNA in HLE B-3 cells. After the stable transfection of shRNA for GM3 synthase or a negative control (N.C.), HLE B-3 cells were serum-starved for 6 h and incubated in the presence or absence of TGF-β1 (5 ng/ml) for 24 h. (A) RT–PCR was performed to analyse the expression levels of GM3 synthase mRNA. β-Actin was used as an internal control. (B) Ganglioside GM3 was detected by immunofluorescence staining using an anti-(ganglioside GM3) antibody. Nuclei were stained using DAPI (4′,6-diamidino-2-phenylindole). For the d-PDMP treatment, the serum-starved HLE B-3 cells were treated by d-PDMP (30 μM) for 1 h. The cells were then incubated with or without 5 ng/ml of TGF-β1 treatment for 24 h. For shGM3-3 transfection, the HLE B-3 cells transfected with shGM3-3 were serum-starved for 6 h and incubated in the presence or absence of TGF-β1 (5 ng/ml) for 24 h. (C) RT–PCR was performed to analyse the mRNA of GM3 synthase, fibronectin and collagen I. β-Actin was used as an internal control. (D) Phosphorylation of TGFβRII, TGFβRI, Smad2 and Smad3 was detected by immunoblotting using specific antibodies. β-Actin or GAPDH was included as an internal control. shGM3, shRNA for GM3 synthase.

capsular fibrosis region [2,5,37]. To check whether ganglioside GM3 is related to the EMT-induced migration in HLE B-3 cells, we performed wound healing and transmigration assays. The wound healing and transmigration abilities of HLE B-3 cells were enhanced by TGF-β1 (Figures 4C–4F). However, the wound healing and migration in TGF-β1-induced HLE B-3 cells were clearly inhibited by d-PDMP treatment (Figures 4C and 4E). shGM3-3 shRNA also repressed the TGF-β1-induced wound healing and cell migration ability of HLE B-3 cells (Figures 4D and 4F). These findings strongly indicate that ganglioside GM3 is essential for EMT and cell migration in TGF-β1-induced HLE B-3 cells.

Exogenous ganglioside GM3 recovers the suppressed EMT and cell migration in TGF-β1-induced HLE B-3 cells

We next investigated whether TGF-β receptor signalling and TGF-β1-induced EMT are affected by ganglioside GM3. As shown in Figure 4(A) the phosphorylation of Smad2/3 induced by ganglioside GM3 and TGF-β1 in HLE B-3 cells was increased compared with TGF-β1 alone (up to 10 μM of ganglioside GM3). However, the phosphorylation of Smad2/3 was not induced in HLE B-3 cells treated with ganglioside GM3 alone compared with no treatment with ganglioside GM3. This result suggests that ganglioside GM3 has an effect on the EMT under the conditions induced by TGF-β1 in HLE B-3 cells. As shown above, the expression of EMT markers, fibronectin and type I collagen, were blocked by d-PDMP or shGM3-3 in HLE B-3 cells during TGF-β1-induced EMT (Figure 3). Thus we further checked whether EMT inhibited through d-PDMP- or shGM3-suppressed generation of ganglioside GM3 in TGF-β1-induced HLE B-3 cells is recovered by exogenous ganglioside GM3. As shown in Figure 4(B) the levels of GM3 synthase mRNA expression were not changed by exogenous treatment of ganglioside GM3 in TGF-β1-induced HLE B-3 cells with d-PDMP or shGM3-3 compared with no treatment with ganglioside GM3. Nevertheless, the addition of ganglioside GM3 restored the expression of EMT markers inhibited by d-PDMP or transfection of shGM3-3 in TGF-β1-induced HLE B-3 cells. These results suggest that ganglioside GM3 is required for the activation of signalling and the expression of proteins related to EMT in TGF-β1-induced HLE B-3 cells.

On the basis of the above observations, we investigated whether the inhibition of wound healing and cell migration under suppression of ganglioside GM3 production by d-PDMP or shGM3 in TGF-β1-induced HLE B-3 cells is recovered by the exogenous ganglioside GM3. As shown in Figures 4(C) and 4(E), wound healing and cell migration suppressed by
Figure 4  Effect of exogenous ganglioside GM3 on suppressed EMT and cell migration in TGF-β1-induced HLE B-3 cells

(A) After serum-starvation for 6 h, HLE B-3 cells were incubated with or without the indicated concentrations of the ganglioside GM3 for 1 h and then treated with or without 5 ng/ml of TGF-β1 for 24 h. The phosphorylation levels of Smad2 and Smad3 was determined with the specific antibodies for phospho-(p-)Smad2 and phospho-Smad3. GAPDH was used as a control. (B) HLE B-3 cells transfected with shGM3-3 were serum-starved for 6 h and pre-incubated with or without ganglioside GM3 (10 μM) for 1 h. After incubation, the cells were incubated in the presence or absence of TGF-β1 (5 ng/ml) for 24 h. For the analysis of the effects of d-PDMP serum-starved HLE B-3 cells were treated with or without d-PDMP (30 μM) for 1 h and pre-incubated with or without ganglioside GM3 (10 μM) for 1 h. The cells were then incubated in the presence or absence of TGF-β1 (5 ng/ml) for 24 h. RT–PCR was performed to analyse the mRNA of GM3 synthase, fibronectin and collagen I. β-Actin was used as an internal control. N.C., negative control; shGM3, shRNA for GM3 synthase. (C) After serum starvation, subconfluent HLE B-3 cells on 24-well plate were treated with or without d-PDMP (30 μM) for 1 h and wounded by a blue pipette tip. The cells were pre-incubated with or without ganglioside GM3 (10 μM) for 1 h and then treated with or without 5 ng/ml of TGF-β1 for 24 h. (D) After serum starvation, subconfluent HLE B-3 cells transfected with shGM3-3 or control vector on 24-well plates were wounded by a yellow pipette tip. The cells were pre-incubated with or without commercial ganglioside GM3 (10 μM) for 1 h and then treated with or without 5 ng/ml of TGF-β1 for 24 h. Each experiment was repeated 3 times and a representative micrograph at 0 h or 24 h is shown. Initial wound areas were marked with black lines. Scale bar, 100 μm. (E and F) The transmigration assay on Transwell coated with 0.1% gelatin. (E) The serum-starved HLE B-3 cells were seeded on the upper chamber of a Transwell and then pre-incubated in the presence or absence of d-PDMP (30 μM) for 1 h. After further incubation in the presence or absence of ganglioside GM3 (10 μM) for 1 h, cells were treated with or without 5 ng/ml TGF-β1 for 24 h. (F) After serum starvation for 6 h, HLE B-3 cells transfected with shGM3-3 or control vector were seeded in the upper chamber of a Transwell, incubated in the presence or absence of ganglioside GM3 (10 μM) for 1 h and then treated with or without 5 ng/ml TGF-β1. After incubation for 24 h, the transmigrated cells under the membrane were stained with hematoxylin and eosin and counted as an average of three independent experiments. The error bars represent S.D. *P < 0.05 compared with the control.
after interaction between TGF-β and cell migration by transfection with shGM3-3 in TGF-β-induced HLE B-3 cells. Moreover, exogenous treatment with ganglioside GM3 recovered the repression of wound healing by exogenous ganglioside GM3. Furthermore, the total acidic glycolipids isolated from HLE B-3 cells in the presence or absence of TGF-β1 (5 ng/ml) for 24 h were used for immuno-TLC with an antibody against ganglioside GM3. Ganglioside GM3 (10 ng) was also loaded as a positive control.

Figure 5 Interaction of TGF-β receptors with ganglioside GM3 in EMT-induced HLE B-3 cells

To check the interaction of TGF-βRs with GM3, HLE B-3 cells were treated with or without TGF-β1 (5 ng/ml) for 24 h and then lysed by IP buffer as described in Materials and methods section. (A) IP lysate (300 μl) was immunoprecipitated using an anti-(ganglioside GM3) antibody or IgM as a negative control. The immunoprecipitates were then analysed by immunoblotting (IB) using antibodies against TGFβRI and TGFβRII. (B) IP lysate (300 μl) was immunoprecipitated with antibody against TGFβRI and TGFβRII and the immunoprecipitates were then dried, dissolved with methanol/chloroform (1:1) and analysed by immuno-TLC using an anti-(ganglioside GM3) antibody. Furthermore, the total acidic glycolipids isolated from HLE B-3 cells in the presence or absence of TGF-β1 (5 ng/ml) for 24 h were used for immuno-TLC with an antibody against ganglioside GM3. Ganglioside GM3 (10 ng) was also loaded as a positive control.

Ganglioside GM3 binds to TGF-βRs in TGF-β1-induced HLE B-3 cells

Ganglioside GM3 regulates the activation and tyrosine phosphorylation of various growth factor receptors bound to the extracellular domain of the receptor, such as EGFR, VEGF receptor 2 and IR, but not for TGFβR, in a number of cells [24–30]. The EMT signalling induced by TGF-β occurs dynamically after interaction between TGFβRI and TGFβRII localized in the lipid rafts of epithelial cells [4,10]. Ganglioside GM3 is also located on lipid rafts complexed with EGFR and caveolin-1 in human squamous carcinoma cells [38]. In order to investigate whether ganglioside GM3 is associated with TGFβRs during TGF-β1-induced EMT, we examined the interaction between TGFβRs and ganglioside GM3 in HLE B-3 cells using IP. First, the IP lysates from normal and TGF-β1-treated HLE B-3 cells were incubated with anti-(ganglioside GM3) antibody and then TGFβRI or TGFβRII was detected by immunoblotting. As shown in Figure 5(A), the level of immunoprecipitated TGFβRI or TGFβRII was increased in TGF-β1-induced HLE B-3 cells compared with the untreated HLE B-3 cells. Secondly, antibodies against TGFβRI or TGFβRII were incubated with the IP lysates and then immuno-TLC was performed using an anti-(ganglioside GM3) antibody. The ganglioside GM3, which is immunoprecipitated with TGFβRI or TGFβRII, was increased by induction of TGF-β1 in HLE B-3 cells (Figure 5B). These results suggest that the interaction of ganglioside GM3 with TGFβRI or TGFβRII is increased in TGF-β1-induced HLE B-3.

DISCUSSION

In lens development EMT induces the proliferation and transdifferentiation and develops the PCO in lens epithelial cells. It causes various eye diseases, such as cataract and loss of sight [5]. For inhibition of this event, many studies have been focused on investigating the mechanism of EMT-induced lens epithelium in vivo and in vitro [1,4,7,9]. It has been reported that TGF-β is a potent inducer of EMT [2] and induces the expression of extracellular matrix proteins, such as fibronectin and type I collagen, for fibrosis in the lens epithelial cells of anterior polar cataracts [4]. Furthermore, TGF-β enhances the expressions of the myofibroblastic/fibroblastic phenotype markers found in human ASC and known as characteristics of PCO, meaning induction of an EMT [9]. Among these studies related to EMT, the expression of GSL molecules, which are located on the outer leaflet of the plasma membrane, has been shown to be related to cataract [17,31,32]. Ogiso et al. [39,40] reported that the gangliosides GM3, GM1 and GD1a identified in human cataractous lenses resulted from aging and cataract progression. In other normal murine mammary gland mNuMG and human normal bladder HCV29 cells, gangliosides GM2 and Gg4, but not gangliosides GM3 and GM1, have an effect on TGF-β1-induced EMT [41]. Thus we investigated the function of gangliosides, sialic acid-containing GSLs, in TGF-β-induced EMT in lens epithelial cells. In the present study we explored the regulation and function of ganglioside GM3 during TGF-β1-induced EMT in human lens epithelial cells. The present study showed that the expression of ganglioside GM3 is positively regulated with cell migration and EMT-related signalling in TGF-β1-induced lens epithelial cells. The transcriptional activity of GM3 synthase was regulated by...
Sp1 in the HLE B-3 cells induced by TGF-β1. The inhibition of the activated molecules and cell migration related to EMT was recovered by exogenous ganglioside GM3 in GM3-depleted HLE B-3 cells during TGF-β1-induced EMT. Our results further showed that ganglioside GM3 binds to TGF-β1-induced TGF/βR in HLE B-3 cells. These findings suggest that ganglioside GM3 participates in TGF-β1-induced EMT in human lens epithelial cells by interaction with TGF/βRs.

Previous studies have shown that ganglioside GM3 regulates and modulates various pathological phenomena such as obesity, tumour progression and leukaemia differentiation [19–27]. Ganglioside GM3 also modulated the association of caveolin-1 with EGFR on the lipid raft and inhibited EGFR activation [38,42]. Ariga et al. [32] reported that gangliosidea GM3 and GM1 were highly detected in human cataract tissues. In the TLC and immunofluorescence analyses our data clearly showed that ganglioside GM3 was detected and increased in TGF-β1-induced HLE B-3 cells (Figures 1A and 1B). The expression of GM3 synthase mRNA was also increased, suggesting that the induction of GM3 synthase gene expression in TGF-β1-induced HLE B-3 cells would direct ganglioside GM3 biosynthesis (Figure 1C). This result also suggests that TGF-β1-responsive element(s) exists in the promoter region of the GM3 synthase gene. Reporter gene assays using transient expression systems clarified that the 5′-flanking region of the GM3 synthase exhibited TGF-β1-inducible promoter activity. Deletion analysis of the 5′-flanking region suggests that the region between −432 and −178 functions as the core promoter essential for the transcriptional activation of the GM3 synthase in TGF-β1-induced HLE B-3 cells (Figure 2). This region contains putative functional binding sites such as p300, NF-κB and Sp1, which are well-known transcriptional factors related to TGF-β signalling [15,16,43]. It is known that p300/CBP [CREB (cAMP-response-element-binding protein)-binding protein] enhances TGF-β signalling through direct modification of Smad2 [44]. Furthermore, CREB, which interacts and co-activates with CBP/p300, is involved in the up-regulation of transcriptional activity of GM3 synthase in PMA-induced HL-60 cells [21,45]. Sp1, which is a TGF-β1-responsive member of zinc-finger transcription factors, is an oncogenic Smad partner and regulates the TGF-β1-induced EMT and cell migration during cancer progression [15,46]. Therefore we have investigated further whether these factors are involved in TGF-β1-induced EMT. The promoters mutated with each Sp1-binding site were significantly reduced in TGF-β1-induced HLE B-3 cells, as compared with the wild-type plasmids between −432 and −178 (Figure 2C). However, mutant promoters of p300 and NF-κB did not affect the transcriptional activity of the GM3 synthase promoter region between −432 and −178 (Figure 2C). These results clearly indicate that Sp1 plays a role in the transcriptional activation of GM3 synthase during TGF-β1-induced EMT in HLE B-3 cells.

Studies about the interaction between gangliosides and receptors on cellular membrane have been reported [19,24,28–30]. Among these gangliosides, GM3 binds not only to EGFR, resulting in inhibition of the tyrosine phosphorylation of EGFR, but also to caveolin-1, apart from caveolar domains in the epithelial cell membrane [25,38,42,47]. In the primary lens epithelial cells caveolin-1 participates in the process of EMT [48]. It has been also demonstrated that the TGF/βRI and TGF/βRII, localized in lipid rafts, are essential for MAPK (mitogen-activated protein kinase) activation in epithelial cells during TGF-β1-induced EMT [37,49]. In TGF-β1-induced EMT of epithelial cell lines the phosphorylation of TGF/βRI and TGF/βRII after interaction with each other activates downstream molecules such as Smad2/3, and produces the extracellular matrix, such as fibronectin and collagen I, in anterior polar cataracts [5,11–13]. After activation with TGF/βR downstream TGF-β-induced epithelial cells undergo transdifferentiation and migration to target regions [5,50,51]. We demonstrated that the serine phosphorylation of TGF/βRI, TGF/βRII and Smad2/3 was blocked by the depletion of ganglioside GM3 in TGF-β1-induced HLE B-3 cells (Figure 3). Furthermore the expression of EMT-related markers and cell migration was clearly suppressed by d-PDMP and shGM3 in TGF-β1-induced HLE B-3 cells (Figures 3C and 4C–4F). Therefore these data clearly show that the ganglioside GM3 is positively regulated by TGF/βR signalling and the migration of lens epithelial cells during TGF-β1-induced EMT.

Previous studies have demonstrated that the in vitro treatment of exogenous ganglioside GM3 directly integrates and regulates growth factor receptors induced by each growth factor, such as EGF and VEGF [25,47]. On the basis of these previous studies we hypothesized that the inhibitory effect of TGF-β1-induced EMT by d-PDMP and shGM3 in TGF-β1-induced HLE B-3 cells might be recovered by treatment with exogenous ganglioside GM3. The results of the present study demonstrated that the expression of EMT-related molecules and cell migrations suppressed by treatment with d-PDMP and shGM3 are recovered by exogenous treatment with ganglioside GM3 in TGF-β1-induced HLE B-3 cells (Figure 4). These data also support the hypothesis that ganglioside GM3 might be involved in EMT under TGF-β1-induced conditions in HLE B-3 cells.

Ganglioside GM3 regulates the signalling of growth factor receptors [24,25,27,38,47]. Among these receptors, the phosphorylation of serine/threonine residues in growth factor receptors is regulated by ganglioside GM3. For example the serine phosphorylation of IR is increased by the up-regulation of endogenous ganglioside GM3 synthesis during TNF-α-induced insulin resistance in adipocytes [27]. Also ganglioside GM3 associated with caveolin-1 and CD82 on epithelial membrane triggers the Thr654 phosphorylation and internalization of EGFR [38]. In TGF-β-induced cells, the kinase domain activated by a serine/threonine residue on TGF/βRI is bound to TGF/βRII upon activation, and activated TGF/βRs induce internalization for EMT in epithelial cells [52]. On the basis of these previous studies we checked the serine phosphorylation of TGF/βRs in

Figure 6 Schematic model of the EMT-induced expression of ganglioside GM3 in lens epithelial cells

This model shows that ganglioside GM3 is produced by GM3 synthase expressed via Smad2/3-dependent Sp1 activation induced by TGF-β1 and interacts with TGF/βRs in lens epithelial cells.
TGF-β1-induced HLE-B-3 cells. Our present data indicate that ganglioside GM3 regulates the serine phosphorylation of TGFβRI, TGFβRII and Smad2/3 in TGF-β1-induced HLE-B-3 cells (Figure 3D). Also, ganglioside GM3 increased the interaction with TGFβR in TGF-β1-induced HLE-B-3 cells (Figure 5).

In conclusion, as illustrated in Figure 6, we showed for the first time that ganglioside GM3 is involved with TGF-β1-induced EMT in HLE-B-3 cells. The transcriptional activation of GM3 synthase is regulated by Sp1 activated via the Smad2/3 signal pathway in TGF-β1-induced HLE-B-3 cells, which results in the biosynthesis of ganglioside GM3 product. The ganglioside GM3 regulates the migration and EMT-related signalling in lens epithelial cells during TGF-β1-induced EMT. Ganglioside GM3 enables the interaction of TGF-β1-induced TGFβRs in HLE-B-3 cells. Our present data suggest that ganglioside GM3 may provide clues for a therapeutic target in EMT-induced cataracts for future investigation.

AUTHOR CONTRIBUTION
Seok-Jo Kim, Tae-Wook Chung, Hye-Jung Choi, Choong-Hwan Kwak, Kwon-Ho Song, Jung-Eun Cho, Hye-Won Kim, Young-Chae Chang, Young-Guk Park, Hyeun Wook Seok-Jo Kim, Tae-Wook Chung, Choong-Ho Kim and Young-Choon Lee carried out the experiments. Seok-Jo Kim, Tae-Wook Chung, Choong-Hwan Kwak, Choong-Ho Kim and Young-Choon Lee analyzed the data. Seok-Jo Kim, Tae-Wook Chung, Choong-Ho Kim and Young-Choon Lee participated in the writing of the paper.

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Increase of ganglioside GM3 during EMT in lens epithelial cells


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