Depletion of gangliosides enhances cartilage degradation in mice

F. Sasazawa, T. Onodera, T. Yamashita, N. Seito, Y. Tsukuda, N. Fujitani, Y. Shinohara, N. Iwasaki

Objective: Glycosphingolipids (GSLs) are ubiquitous membrane components that play a functional role in maintaining chondrocyte homeostasis. We investigated the potential role of gangliosides, one of the major components of GSLs, in osteoarthritis (OA) pathogenesis.

Design: Both age-associated and instability-induced OA models were generated using GM3 synthase knockout (GM3S−/−) mice. A cartilage degradation model and transiently GM3S−/−transfected chondrocytes were analyzed to evaluate the function of gangliosides in OA development. The amount of each series of GSLs in chondrocytes after IL-1β stimulation was profiled using mass spectrometry (MS).

Results: OA changes in GM3S−/−mice were dramatically enhanced with aging compared to those in wild-type (WT) mice. GM3S−/−mice showed more severe instability-induced pathologic OA in vivo. Ganglioside deficiency also led to the induction of matrix metalloproteinase (MMP)-13 and ADAMTS-5 secretion and chondrocyte apoptosis in vitro. In contrast, transient GM3S transfection of chondrocytes suppressed MMP-13 and ADAMTS-5 expression after interleukin (IL)-1β stimulation. GSL profiling revealed the presence of abundant gangliosides in chondrocytes after IL-1β stimulation.

Conclusion: Gangliosides play a critical role in OA pathogenesis by regulating the expression of MMP-13 and ADAMTS-5 and chondrocyte apoptosis. Based on the obtained results, we propose that gangliosides are potential target molecules for the development of novel OA treatments.

© 2013 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

A R T I C L E  I N F O
Article history:
Received 19 August 2013
Accepted 30 November 2013

Keywords:
Osteoarthritis
Mice
Gangliosides
Glycosphingolipids
Interleukin-1β

S U M M A R Y

Introduction

The pathology of osteoarthritis (OA) is characterized by a progressive degeneration of articular cartilage. In healthy individuals, the homeostasis of articular cartilage is maintained primarily by chondrocytes that are responsible for the synthesis and degradation of the extracellular matrix (ECM). However, the actual mechanism(s) of cartilage degradation remain unclear, despite a large number of gene- and protein-based studies designed to address the process. The development of OA therapies will require the identification of new molecular targets involved in the degradation mechanism.

Glycosphingolipids (GSLs) are a group of glycolipids that are widely distributed on vertebrate plasma membranes. These molecules form clusters on cell membranes, where the GSLs modulate transmembrane signaling and mediate cell-to-cell and cell-to-matrix interactions. GSLs are known to be critical for the maintenance of chondrocyte homeostasis. However, mice that are homozygous null for Ugcg (the gene encoding UDP-glucose ceramide glucosyltransferase (Glccer synthase), the first committed step in GSL synthesis) also exhibit an embryonic lethal phenotype. Nonetheless, specific subclasses of GSLs that influence individual systems and conditions are considered ideal targets for clinical applications.

GSLs comprise diverse types of glycolipids, and are classified into several groups depending on their structural features, including lacto-series, neo lacto-series, globo-series, isoglobo-series, and ganglio-series (gangliosides). In the context of OA, previous studies have shown that the total ganglioside content of OA cartilage is decreased by 40%.
Animals and generation of gene deletions

GM3 synthase knockout (GM3S−/−) mice were generated as described previously. Male adult (4-week-old and 8-week-old) and 6-day-old C57BL/6 mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Mice were utilized after 7-day-acclimatization following transportation. All the purchased mice recovered normal behavior within 24 h after transportation. Mice were housed in a temperature- and humidity-controlled environment under 12-h light/12-h dark conditions and fed a standard rodent diet. All experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee of the Hokkaido University Graduate School of Medicine (Sapporo, Japan).

Age-associated OA model

As a model of age-associated OA, joints were compared between wild-type (WT; GM3S+/+) and mutant (GM3S−/−) mice sacrificed at 4 or 15 months of age. For each animal, the entire knee joint was dissected and evaluated for the spontaneous development of OA.

Instability-induced OA model

An instability-induced OA model was created in 8-week-old WT or mutant mice as previously described. Animals were anesthetized and the right knee joint was destabilized by transection of the medial collateral ligament and removal of the cranial half of the medial meniscus using a microsurgical technique (OA side). A sham operation was performed on the left knee joint using the same approach without ligament transection and meniscectomy (sham side). Mice were recovered for 8 weeks and then sacrificed for histologic assessment.

Histologic analysis

Samples were fixed in 10% buffered formalin, decalcified in 10% ethylenediaminetetraacetate (pH 7.5), dehydrated, embedded in paraffin, and sectioned at 5-μm thicknesses. Sections then were subjected to staining with hematoxylin and eosin (H&E) and Safranin O. OA severity in each mouse was quantified using the Mankin scoring system. In addition, we evaluated the changes in synovium and subchondral bones using a scoring system with low power images. We used the same scoring system previously described. Each sample was evaluated independently by three observers who were blinded with regard to experimental group, and the resulting scores were averaged to provide a mean score.

Culture of femoral head cartilage explant

In vitro cartilage catabolism was analyzed by culturing mouse femoral head cartilage with IL-1β (Sigma, St. Louis, MO). The femoral head cartilage was harvested from 4-week-old mice and pre cultured for 48 h at 37°C in a humidified atmosphere of 5% CO2 and 95% air, using Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing 1% antibiotic solution (penicillin-streptomycin, Gibco-BRL, Grand Island, NY), 2 mM glutamine (Wako, Osaka, Japan), 10 mM 4-(2-HydroxyEthyl)-1-PiperazineEthaneSulfonic acid (HEPES) (Gibco-BRL), 50 μg/ml ascorbate (Sigma), and 10% fetal bovine serum (FBS; Nichirei Biosciences Inc., Tokyo, Japan). The explants then were washed three times with serum-free DMEM and cultured for an additional 72 h in serum-free DMEM containing 10 ng/ml mouse IL-1β.

Immunohistochemistry

Processed but unstained sections of mouse femoral head cartilage were deparaffinized, and endogenous peroxidase activity was quenched. After treatment with chondroitinase ABC (0.25 units/ml, Sigma–Aldrich, Tokyo, Japan), the sections were incubated overnight at 4°C with polyclonal antibody against the carboxyl-terminus of matrix metalloproteinase (MMP)-13 (1:200 dilution; Chemicon, Temecula, CA). Samples then were washed three times with phosphate-buffered saline (PBS) and incubated with a biotinylated secondary antibody, the primary antibody was omitted for the negative control. For semi-quantitative data, at 400× magnification, the positive cells were counted from three different fields of observation; the number of counted cells was then averaged. Positive cell ratio was calculated with the total chondrocyte number of each field. Each sample was evaluated independently by three observers who were blinded with regard to the experimental groups they were observing.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

To investigate chondrocyte apoptosis, the TUNEL assay was performed using an in situ Apoptosis Detection Kit according to the

![Fig. 1. Schematic of the GSL synthetic pathway in mammalian cells. GSLs from ceramide through glucosylceramide (GlCer) are synthesized by GlCer synthase, encoded by the Ugcg locus. Lactosylceramide (LacCer) is the key branching point of GSL biosynthesis, with four alternate pathways (lacto-series, neo lacto-series, globo-series, and isoglobo-series) branching off from LacCer. Virtually all the ganglio-series GSLs (gangliosides) are detected as a-series and b-series gangliosides, both of which are synthesized from the shared precursor molecule GM3 itself the product of GM3 synthase (GM3S). GM3S is deficient in almost all of the gangliosides synthesized from GM3. The aim of the present study was to analyze the functional roles of gangliosides in OA pathogenesis, thereby validating the GM3S as a potential clinical target molecule in the treatment of OA.](image-url)
recommendations of the manufacturer (Takara Bio Inc., Otsu, Japan). For a negative control, terminal transferase in the TUNEL reaction mixture was omitted. TUNEL-positive cell ratio was calculated as described above.

Proteoglycan release from cultured mouse cartilage explants

To quantify the proteoglycan release from mouse cartilage explants with cartilage degradation, the proteoglycan content in the medium and digested cartilage was measured as sulfated glycosaminoglycan using a dimethylmethylen blue assay as previously described16. The amount of proteoglycan released from a mouse cartilage explant into the medium was quantitatively expressed as the percentage release of proteoglycan (proteoglycan amount in the medium divided by the total amount of proteoglycan in the serum and cartilage explants × 100).

Enzyme-linked immunosorbent assay (ELISA)

MMP-13 in the mouse femoral head culture supernatant was measured using an ELISA kit for mouse MMP-13 according to the recommendations of the manufacturer (USCN Life Science, Houston, TX).

Quantification of nitric oxide (NO)

NO in the mouse femoral head culture supernatant was quantified based on the amount of nitrite, the product generated upon degradation of NO23. A Griess reagent system was used for this assay according to the recommendations of the manufacturer (Promega, Tokyo, Japan).

Isolation of chondrocytes

Immature mouse chondrocytes were obtained from the hip and knee joints of 6-day-old mice as previously described24,25. Isolated primary mouse chondrocytes were cultured for 24 h in serum-free DMEM plus 10 ng/ml mouse IL-1α for further analysis.

Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the samples using the RNeasy Mini kit (Qiagen, Hilden, Germany). For complementary DNA synthesis, 1.0 μg of RNA was reverse-transcribed using random hexamer primers (Promega) and ImProm II reverse transcriptase (Promega). Real-time RT-PCR was performed using an Opticon II system (Bio-Rad, Tokyo, Japan). Signals were detected using an SYBR Green qPCR Kit (Finnzymes, Yokohama, Japan) with the gene-specific primers (Supplementary Table I). The relative messenger RNA (mRNA) expression of each targeted gene was expressed as the Ct value of each gene normalized to the Ct value of the GAPDH-encoding gene by using the ΔΔCt method42.

Plasmid preparation and transient transfection

Plasmid vectors were designed by and purchased from OriGene (Rockville, MD). The expression vector for GM3S was St3gal5 (NM_0010535228) Mouse cDNA Clone (OriGene MC209401) and the mock control vector was pCMV6-Entry (OriGene PS100001). Mouse chondrocytes were seeded in 24-well tissue culture plates at 6 × 10⁴ cells/well. After 24 h, the chondrocytes were transfected using Lipofectamine LTX (Invitrogen, Carlsbad, CA). For each well of a Lipofectamine LTX transfection, 500 ng DNA was combined with 1.25 μl Plus Reagent (Invitrogen) in 100 μl Opti-MEM I reduced serum medium (Gibco/Invitrogen, Carlsbad, CA) for 5 min. Lipofectamine LTX (5 μl) was added to the wells, and the mixture was incubated for 30 min. An aliquot of 100 μl DNA/Lipofectamine LTX complex was added to each well and mixed gently. After 24 h incubation, the cells were rinsed twice with PBS and placed in DMEM containing 1% antibiotic solution and 10% FBS for 24 h27. Finally, cells were rinsed twice with PBS and incubated in serum-free DMEM with or without 10 ng/ml IL-1α for 12 h.

Quantification of GSIs in the cultured mouse chondrocytes

GSIs were recovered from mouse chondrocyte pellets, cultured with IL-1α for 0, 6, 12, 24, and 48 h, by chloroform–methanol extraction43. To quantify the GSI content, the glycan component of the GSIs was released by enzymatic digestion with GSL-specific endo-glycosidase I and II (EGCase I and II) derived from Rhodococcus species29 (EGCase II; Takara Bio Inc.). The EGCase-digested solutions were subjected to glycoblotting using a protocol reported previously29. Samples were subjected to matrix-assisted laser desorption ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry (MS) analysis on an Ultraflex II TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a reflector, and controlled by the FlexControl 3.0 software package (Bruker Daltonics). Peaks were detected as proton-added ions. Masses were annotated using the FlexAnalysis 3.0 software package with reference to the GlycoSuiteDB (http://glycosuitedb.expasy.org/glycosuite/glycdb) and SphinGOMAP (http://www.sphingomap.org/) databases for the structural identification of glycans.

Statistical analysis

Data are expressed as means and 95% confidence intervals (95% CI). All data were checked for homogeneity of variance using Bartlett’s test. Continuous variables were evaluated using two-way factorial analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison tests. When variances were significantly different, non-parametric Wilcoxon tests with Bonferroni correction were used. Comparison of data from two groups of samples was performed using Student’s t test. Mankin scores were compared using non-parametric Wilcoxon test. P values of less than 0.05 were considered significant. Data analysis was carried out using statistical software JMP Pro 10.0 (SAS Institute, Cary, NC).

Results

Depletion of gangliosides in mice enhances the development of OA with aging

Mice were followed to 15 months of age to assess the spontaneous development of OA with aging. At 4 months of age, there were no significant OA changes in the knee joints when comparing WT and GM3S−/− mice [Fig. 2(A)]. Mankin scores showed no significant difference between the mouse genotypes (WT: 0.67 (95% CI: 0.12–1.21), GM3S−/−: 0.83 (95% CI: 0.04–1.62) [P = 0.78]; n = 6). At 15 months of age, slight OA changes were detected in the knee joints of the WT mice, where mild, superficial cartilage erosion, and a slight reduction in Safranin O staining were observed [Fig. 2(A)]. On the other hand, the joints of 15-month-old GM3S−/− mice exhibited greater OA changes, including joint deterioration, compared to those of WT mice [Fig. 2(A)]. These histologic findings were quantitatively confirmed by Mankin scores (WT: 4.17 (95% CI: 3.13–5.20), GM3S−/−: 6.17 (95% CI: 4.77–7.56) [P = 0.023]; n = 6). To confirm the hypocellularity in more deteriorated OA cartilage,
chondrocyte numbers in the tibial side of the knee were manually counted; a significant decrease was observed in GM3S/C0/C0 mice compared to WT mice (WT: 551.83 (95% CI: 490.47–613.20), GM3S/C0/C0: 472.0 (95% CI: 424.55–519.45) \(P = 0.026\); \(n = 6\)).

Depletion of gangliosides in mice enhances the development of instability-induced OA

We analyzed the development of instability-induced OA changes in WT and GM3S/C0/C0 mice. In the sham-operated knees, no significant OA changes were observed in either mouse genotype. At 8 weeks after instability-inducing surgery, the joints of WT mice showed moderate OA changes with cartilage erosion and reduction in Safranin O staining [Fig. 2(B)]. The depletion of gangliosides resulted in more severe OA changes. In the joints of GM3S/C0/C0 mice at 8 weeks after surgery, the non-calciﬁed zone was almost completely lost, and the cartilage erosion extended into the calcified zone beneath the tidemark. Quantitative assessment using the Mankin scale supported these histologic ﬁndings (WT: 7.50 (95% CI: 6.62–8.38), GM3S/C0/C0: 9.50 (95% CI: 8.21–10.79), \(P = 0.013\); \(n = 6\)). Other OA ﬁndings, such as inﬁltration of inﬂammatory cells, osteophyte formation, or subchondral bone accrual, were not observed in their whole knee joints [Fig. 2(C), Supplementary Fig. 1].

Depletion of gangliosides in mice enhances IL-1α-induced cartilage degradation

Histologic examination of the explants without IL-1α stimulation revealed no signiﬁcant difference in Safranin O or TUNEL staining between the mouse genotypes [Fig. 3(A)]. Under IL-1α stimulation, Safranin O staining for proteoglycans was reduced in GM3S/C0 mouse cartilage compared to WT mouse cartilage [Fig. 3(A)]. TUNEL staining was enhanced in GM3S/C0 mouse cartilage compared to WT mouse cartilage [Fig. 3(A)]. To measure the occurrence of apoptosis, the TUNEL-positive cells were counted to permit calculation of the TUNEL-positive cell ratio [Fig. 3(B)]. The ratio was signiﬁcantly elevated in chondrocytes from GM3S/C0 mice compared to those from WT mice (WT: 17.42% (95% CI: 14.68–20.16%), GM3S/C0: 31.34% (95% CI: 27.19–35.49%) \(P < 0.0001\); \(n = 6\)). Immunostaining for MMP-13 also was signiﬁcantly enhanced in GM3S/C0 mouse cartilage compared to WT mouse cartilage [Fig. 3(A)]. MMP-13-positive cell ratio in chondrocytes from GM3S/C0 mice was also signiﬁcantly elevated compared to
those from WT mice (WT: 51.79% (95% CI: 48.61–54.96%), GM3S<sup>-/-</sup>: 40.04% (95% CI: 35.20–44.88%) [P = 0.0042]; n = 6) [Fig. 3(C)]. Under in vitro conditions, IL-1α stimulation caused a significant elevation in the percentage release of proteoglycans in GM3S<sup>-/-</sup> mouse cartilage compared to that in WT mouse cartilage (WT: 48.33% (95% CI: 45.20–51.47%), GM3S<sup>-/-</sup>: 57.45% (95% CI: 49.37–65.53%) [P = 0.0071]) [Fig. 3(D)]. In this analysis, we made four femoral heads (two mice) as one sample. We utilized four samples (eight mice) per group.

We found significant elevations in MMP-13 protein concentration in the IL-1α-containing medium used to culture cartilage explants from GM3S<sup>-/-</sup> mice compared to that from WT mice (WT: 344.04 pg/ml (95% CI: 275.90–412.18 pg/ml), GM3S<sup>-/-</sup>: 580.11 pg/ml (95% CI: 499.56–660.65 pg/ml) [P < 0.0001]) [Fig. 3(E)]. In addition, elevations in NO concentration were found in the medium used to culture cartilage explants from GM3S<sup>-/-</sup> mice compared to that from WT mice, but they were not statistically significant (WT: 10.43 μM (95% CI: 9.26–11.60 μM), GM3S<sup>-/-</sup>: 12.88 μM (95% CI: 11.04–14.72 μM)).

Fig. 3. Cartilage degradation induced by interleukin-1α (IL-1α) stimulation. A, Histologic findings in cartilage explants from WT and GM3S<sup>-/-</sup> mice, cultured with IL-1α and subjected to Safranin O (Saf-O) staining, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining, and MMP-13 immunostaining. Bars = 10 μm. B, TUNEL-positive cell ratio in cultured cartilage explants from WT and GM3S<sup>-/-</sup> mice (n = 6). C, MMP-13-positive cell ratio in cultured cartilage explants from WT and GM3S<sup>-/-</sup> mice (n = 6). D, Proteoglycan release in cultured cartilage explants from WT and GM3S<sup>-/-</sup> mice (n = 4 samples (eight mice) per group). E, Quantification of MMP-13 release in mouse femoral head culture supernatant, as determined by ELISA (GM3S<sup>-/-</sup>: n = 5 samples (10 mice) and WT: n = 8 samples (16 mice) per group). B–E, Values are the means and 95% CI.

**P < 0.01 vs WT mice.
11.66–14.11 μM \([P = 0.0942]\) [Supplementary Fig. 2]. In these analyses, we made four femoral heads (two mice) as one sample. We utilized five samples (10 mice) of GM3S\(^{-/-}\) mice and eight samples (16 mice) of WT mice per group.

Depletion of gangliosides upregulates expression of genes encoding matrix-degrading enzymes in chondrocytes cultured with IL-1α

To confirm the in vivo findings under in vitro conditions, mRNA expression levels of genes encoding two major matrix-degrading enzymes, MMP-13 and ADAMTS-5, were measured in IL-1α-cultured chondrocytes from mice of both genotypes (\(n = 3\)). Cultured chondrocytes were collected after 0, 6, 12, 24, and 48 h of stimulation with IL-1α, and transcript levels were plotted over time [Fig. 4(A) and (B)]. Similar trends were observed for the transcripts of both genes: mRNA levels gradually increased, peaking at 12 h before subsequently falling. At 12 h, mRNA levels in chondrocytes from GM3S\(^{-/-}\) mice were significantly elevated compared to those from WT mice (MMP-13: WT: 0.91 (95% CI: 0.58–1.24), GM3S\(^{-/-}\): 1.57 (95% CI: 1.41–1.73) \(P < 0.0001\); A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS)-5: WT: 0.028 (95% CI: 0.021–0.035), GM3S\(^{-/-}\): 0.051 (95% CI: 0.044–0.058) \(P < 0.0001\); \(n = 3\)). The Ct values of GAPDH were stable under these experimental conditions.

Overexpression of GM3S suppresses the levels of MMP-13- and ADAMTS-5-encoding transcripts in chondrocytes cultured with IL-1α

As a control, we demonstrated that transfection of chondrocytes with a GM3S plasmid vector yielded an approximately 70-fold increase in the quantity of GM3S mRNA compared to cells transfected with a mock vector (mock vector: 0.015 (95% CI: 0.0017–0.023), GM3S vector: 1.06 (95% CI: 0.16–1.96) \(P = 0.038\); \(n = 3\)) [Fig. 5(A)]. The combination of GM3S transfection and 12 h of stimulation with IL-1α yielded significant (compared to control samples) decreases in the levels of MMP-13 (mock vector: 5.40 (95% CI: 4.37–6.43), GM3S vector: 3.02 (95% CI: 2.02–4.02) \(P = 0.002\); \(n = 3\)) [Fig. 5(B)] and ADAMTS-5-encoding mRNA (mock vector: 0.051 (95% CI: 0.036–0.067), GM3S vector: 0.026 (95% CI: 0.022–0.031) \(P = 0.014\); \(n = 3\)) [Fig. 5(C)]. Notably, in the absence of IL-1α stimulation, chondrocytes with enhanced GM3S expression exhibited no apparent changes in the levels of MMP-13 or ADAMTS-5-encoding mRNAs (data not shown). The Ct values of GAPDH were stable under these experimental conditions.

Gangliosides are the dominant species of GSLs

Among all the GSL species, gangliosides (ganglio-series), globo-series, and (neo)lacto-series were detected at measurable quantities; others were detected only at trace amounts. The amount of gangliosides was significantly higher than that of globo-series and (neo)lacto-series at almost all time points [Fig. 6, Table I]. This analysis also showed that the amount of gangliosides gradually increased in a time-dependent manner until 48 h. The amounts at 24 and 48 h were significantly higher than the original amount (0 h): ganglioside concentrations (expressed as pmol per 1 \(\times 10^6\) cells) were 10.90 (95% CI: 0.063–21.74) at 0 h and 34.66 (95% CI: 20.81–48.51) at 48 h \(P < 0.0001\); \(n = 3\)).

Discussion

GSLs have been shown to play suppressive roles in a mouse model of arthritis\(^6\). Among GSLs, gangliosides, including GM3 and its derivatives, are known to be related to arthritis diseases\(^{10–12}\). Therefore, we hypothesized that gangliosides have a critical role in OA pathogenesis. To test our hypothesis, three different OA models, age-associated, instability-induced, and IL-1α-induced models, were assessed in GM3S deletion mice. The current study showed that the depletion of gangliosides accelerated OA development in all three models. Interestingly, younger GM3S\(^{-/-}\) mice exhibited a phenotype similar to that of WT mice; with age, signs of age-associated arthritis were more severe in the mutant mice compared to the WT animals. These observations suggest that ganglioside deficiency has age-associated effects on chondrocyte activities such as maintaining homeostasis or matrix fragility. These findings are strongly reminiscent of the phenotype of Ugcg\(^{-/-}\) mice, which harbor a chondrocyte-specific deletion of GSLs\(^5\).

Inflammation is one of the important factors in OA pathogenesis\(^{30,31}\). Among GSLs, gangliosides are known to be associated with inflammatory reaction. However, the relationship between gangliosides and inflammation is still controversial\(^{12–26}\). We have previously shown that accelerated immunoreactions were observed in rheumatoid arthritis model with GM3S\(^{-/-}\) mice\(^12\), suggesting that the acceleration of inflammatory reactions was the most suspicious reason for the enhancement of OA in GM3S\(^{-/-}\) mice. We intensively examined the histologic findings of instability-induced model mice to assess the inflammation to discover if the invasion of the instability-inducing procedure would have triggered inflammatory reactions. However, both genotypes of
mice showed only mild inflammation without obvious differences. We consider two possible reasons for this result. First, the induced inflammation was merely so mild that we couldn’t detect the difference. Second, the instability-inducing procedure didn’t stimulate Th-17 cells which produce IL-17, because the accelerated immunoreactions which we observed in our previous study were triggered by IL-17 instead of IL-1. It is generally accepted that the overexpression of the gene encoding GM3 synthase (GM3S) with down-regulated expression of the genes encoding MMP-13 and ADAMTS-5 in response to stimulation with interleukin-1α (IL-1α) in chondrocytes from WT mice. A, GM3S mRNA expression in the chondrocytes was up-regulated at 48 h after transfection. B, C, Chondrocytes overexpressing GM3S and stimulated with IL-1α had significantly suppressed levels of MMP-13 – (B) and ADAMTS-5 – (C) encoding mRNA, as compared with those in chondrocytes without overexpression and without IL-1α stimulation. The mRNA expression values of each target gene are normalized to the cycle threshold of the GAPDH-encoding transcript. Values are the means and 95% CI (n = 3). *: \( P < 0.05 \), and **: \( P < 0.01 \).

The pathophysiology of OA consists primarily of the enhancement of chondrocyte apoptosis and the increased production of matrix-degrading enzymes. There-fore, we hypothesized that, in chondrocytes, gangliosides exert chondroprotective effects by suppressing the expression of MMP-13- and ADAMTS-5-encoding genes.

GSLs are a complex of diverse molecules; each series of GSLs is known to be related to several biological phenomena.\(^{3,45-47}\) Notably, gangliosides, which are synthesized by GM3S, have a relationship with OA pathogenesis, as demonstrated in previous analyses using human cartilage.\(^{10,31}\) As a first step in testing our hypothesis, we examined mice depleted for ganglio-series GM3 and its derivatives as a result of mutation of the GM3S gene. The depletion of GM3 and its derivatives yielded clinical signs of OA with severity equivalent to that seen in mice depleted for a whole range of GSLs.\(^{3}\) This result suggests that GM3 and its derivatives are the (functionally) dominant GSL species in chondrocytes. To address the relative abundance of different species in this cell type, we profiled all GSLs by MS after IL-1α stimulation. This analysis revealed that ganglio-series (a- and b-series) gangliosides are the most abundant series of GSLs.\(^{3}\) These results indicate that, among various series of GSLs, gangliosides play a dominant role in OA pathogenesis.

The ultimate purpose of OA research is to clarify the pathogenesis of OA and to develop treatments for this illness, such as disease-modifying osteoarthritis drugs (DMOADs).\(^{38}\) To facilitate...
clinical application, more efficacious drugs with reduced side-effects will be needed. Although cartilage-specific Ugcg−/− mice showed an enhancement of OA, uridine diphosphate (UDP)-glucose ceramide glucosyltransferase is not an ideal therapeutic target because the phenotype of Ugcg null-KO mice is known to be embryonic lethal[8–9] [Fig. 7(A) and (B)]. This fact indicates that Ugcg-targeted OA therapy might cause serious systemic effects. Our results revealed that GM3S null-KO mice enhanced OA development without growth deficiency[13] [Fig. 7(C)], suggesting that GM3S and its enzymatic products are candidate targets for therapies that may exert less influence on systemic conditions than the Ugcg gene product.

There are several limitations to this study. First, we note the possible effects on OA pathogenesis of compensatory increases in the levels of o-series gangliosides. A recent study profiling GSLs in GM3S−/− mice revealed elevated production of o-series gangliosides in GM3S−/− mice [Fig. 1B]. Further elucidation of the dynamics of GSLs in GM3S−/− mice will contribute to better understanding of GSL-dependent processes. Second, the precise mechanism of OA development in ganglioside-depleted animals is not fully understood. Although our data suggests that gangliosides may exert chondro-protective effects by suppressing chondrocyte apoptosis and the expression of matrix degrading enzymes, the mechanisms by which gangliosides regulate these processes are still unknown. Despite these limitations, the current study supports a role for GSLs in OA pathogenesis, emphasizing the importance of the GSL synthetic pathway distinct from the steps extending from GlcCer synthase [Fig. 1] to GM3 synthase. Further research will be needed to clarify the possible involvement in OA pathogenesis of other (downstream) components of the GM3 synthetic pathway.

In conclusion, the depletion of gangliosides enhanced OA development, elevating MMP-13 and ADAMTS-5 expression and accelerating chondrocyte apoptosis. Moreover, the over-expression of GM3S in vitro suppressed MMP-13 and ADAMTS-5 expression. These results suggest that gangliosides play a suppressive role in OA development and may provide a target for the design of novel anti-OA therapeutics. Further work will be required to identify the specific ganglioside(s) responsible for OA pathogenesis.

Author contributions

All authors have made substantial contributions to (1), (2), and (3) below:

(1) The conception and design of the study, or acquisition of data, or analysis and interpretation of data;
(2) The drafting of the article or revising it critically for important intellectual content;
(3) Final approval of the version to be submitted.

Role of the funding source

This work was supported by a Grant-in-Aid for Challenging Exploratory Research from the Japan Society for the Promotion of Science and the Uehara Memorial Foundation. The sponsor had no involvement in the study design; collection, analysis and interpretation of data; the writing of the manuscript; or in the decision to submit the manuscript for publication.
Conflict of interest
The authors have no conflicts of interest to disclose.

Acknowledgments
The authors thank Dr. Monma, Dr. Matsuoka, Ms. Ueguchi, and Ms. Suyama for supporting our experiments.

Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2013.11.015.

References


