Depletion of Gangliosides Enhances Cartilage Degradation through the Upregulation of Chondrocyte Apoptosis and Matrix Degrading Enzyme Expression

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Introduction: Osteoarthritis (OA) is the most common degenerative joint disorder of the elderly. The pathology of OA is characterized by a progressive degradation of articular cartilage. However, the actual mechanisms of cartilage degradation remains unclear, despite a large number of gene and protein based studies designed to address the process. The development of OA therapies requires 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 [1]. These molecules form clusters on cell membranes, where the GSLs modulate transmembrane signaling. GSLs are known to be critical for the maintenance of chondrocyte homeostasis [2]. Although Ugcg (the gene encoding UDP-glucose ceramide glucosyltransferase, the first committed step in GSL synthesis) can be the therapeutic target molecule of OA, mice that are homozygous null for Ugcg exhibit an embryonic lethal phenotype. This fact indicates that Ugcg-targeted OA therapy may cause serious systemic effects. Nonetheless, specific subclasses of GSLs that influence individual systems and conditions are considered ideal targets for clinical applications. GSLs contain diverse types of glycolipids, and are classified into several groups depending on their structural features. Gangliosides constitute one of the most abundant molecules in mammalian cells. Previous studies showed that the total ganglioside content of OA cartilage is decreased by 40% [3]. These results suggest that gangliosides may play a crucial role in OA pathogenesis. To test this hypothesis, we employed a strain of mice genetically engineered to lack GM3 synthase (GM3S) [4]. GM3 serves as a precursor molecule for most of the more complex ganglioside species; mice lacking GM3S are deficient in almost all of the gangliosides synthesized from GM3. The aim of this study was to analyze the functional roles of gangliosides on OA pathogenesis to verify the GM3S as a clinical target molecule of OA.

Methods: Methods: Animals: We adopted GM3S null-KO mice (GM3S−/−), and wild-type C57BL/6 mice as controls. Instability-induced OA model: The right knee joint of 8-week-old mice was destabilized to induce OA [5]. The mice were euthanized 8 weeks after surgery. Age-associated OA model: Mice were followed for the spontaneous development of OA up to 15 months of age. Culture of cartilage explants: The femoral head cartilage was harvested from 4-week-old mice and cultured for 72 hours with 10 ng/ml IL-1α. The proteoglycan content in the medium and digested cartilage (%PG) was measured using a dimethyl-methylene blue assay. The concentration of MMP-13 and nitric oxide (NO) in the cultured medium were measured with ELISA and Griess Reagent System. Quantitative real-time reverse transcriptase-PCR (RT-PCR): Primary chondrocytes isolated from 6-day-old mice were cultured for 6, 12, 24, 48 hours with 10 ng/ml IL-1α. Total RNA was extracted from the chondrocytes and the relative messenger RNA (mRNA) expression of MMP-13 and ADAMTS-5 was measured by ΔΔCt method. Transient transfection of GM3S: The expression vector for GM3S was St3gal5 Mouse cDNA Clone and the mock control vector was pCMV6-Entry (OriGene). The chondrocytes were transfected using Lipofectamine LTX (Invitrogen). Quantification of GSLs by mass spectrometry (MS): GSLs were recovered from mouse chondrocyte pellets (cultured for 6, 12, 24, 48 hours) by chloroform-methanol extraction. Samples were subjected to matrix-assisted laser desorption ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) MS analysis. Statistical analysis: Two-way factorial analysis of variance (ANOVA) with subsequent use of Tukey-Kramer multiple comparison tests were performed to determine significant differences between groups.

Results: Enhanced development of instability-induced OA in GM3S−/− mice: Histological findings of instability-induced OA were more progressed in the knees of GM3S−/− mice (n = 6, Mankin score: 9.5 ± 1.1 vs 7.5 ± 0.8, P < 0.05, Fig. 1A). Enhanced development of age-associated OA in GM3S−/− mice: Histological findings of age-associated OA were more progressed in GM3S−/− mice at 15 months of age (n = 6, Mankin score: 6.1 ± 1.2 vs 4.1 ± 0.9, P < 0.05, Fig. 1B). Enhanced cartilage degradation in GM3S−/− mice during in vitro culture: IL-1α stimulation remarkably enhanced cartilage degradation in GM3S−/− mice through the overexpression of MMP-13 and chondrocyte apoptosis. Depletion of gangliosides upregulates expression of genes encoding matrix degrading enzymes: MMP-13 and ADAMTS-5 mRNA levels gradually increased, peaking at 12 hours before subsequently falling. At 12 hours, mRNA levels in chondrocytes from GM3S−/− mice were significantly elevated (Fig 2). Overexpression of GM3S suppresses the levels of MMP-13 and ADAMTS-5 expression: The transfection of chondrocytes with a GM3S plasmid vector yielded an approximately 70-fold increase in the quantity of GM3S mRNA. The combination of GM3S transfection and 12 hours
of stimulation with IL-1α yielded significant decreases in the levels of MMP-13 (n = 3, 5.40 ± 0.4 vs 3.02 ± 0.40, P < 0.01, Fig 3B) and ADAMTS-5-encoding mRNA (n = 3, 0.051 ± 0.0062 vs 0.026 ± 0.0018, P < 0.05, Fig 3C). Gangliosides are the dominant species of GSLs: Among all the GSL species, gangliosides, globo-series, and (neo)lacto-series were detected at measurable quantities by the MALDI-TOF/TOF MS analysis. The amount of gangliosides was significantly higher than that of globo- and (neo)lacto-series at almost all time points (Fig 4).

**Discussion:** As with our previous study with human OA cartilage, the MS data in this study showed that gangliosides were the most abundant in all GSLs. These results strongly suggest the significance of gangliosides in OA pathogenesis. Our results revealed that GM3S null-KO mice enhanced OA development due to upregulation of chondrocyte apoptosis and matrix degrading enzyme expression. On the other hand, overexpression of GM3S suppressed the expression of MMP-13 and ADAMTS-5. These results indicate that gangliosides exert chondroprotective effects by suppressing the expression of MMP-13- and ADAMTS-5-encoding genes. The phenotypes of GM3S-null mice were very close to those of cartilage-specific Ugcg-/- mice. However, GM3S null-KO mice enhanced OA development without growth deficiency despite knowing Ugcg-/- null-KO to be embryonic lethal. Based on our results, GM3S is the candidate for a therapeutic molecule which exerts less influence on systemic conditions than Ugcg. Further studies are required to clarify the mechanisms and to discover the target molecule in all gangliosides.

**Significance:** This is the first attempt to show the functional role of gangliosides in the development of OA. Although further studies are required to confirm our speculation, gangliosides may be target molecules for a novel and effective strategy for the treatment of OA.

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**Figure 1.** Histologic findings of instability-induced OA models (A) and age-associated OA models (B).
Figure 2. Relative gene expression for genes encoding MMP-13 (A) and ADAMTS-5 (B) at the indicated time points after stimulation with interleukin-1α (IL-1α).

Figure 3. Association of the overexpression of the gene encoding GM3 synthase (GM3S) with down-regulated expression of the genes encoding MMP-13 and ADAMTS-5.
Figure 4. Quantification by mass spectrometry (MS) of glycosphingolipids (GSLs) in cultured chondrocyte pellets.