Distribution of Glycosaminoglycans in an Early Osteoarthritis-like Lesion in the Mandibular Condylar Cartilage of Senescence-Accelerated Mice

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The purpose of this study was to determine the immunolocalization of glycosaminoglycans (GAGs) in the mandibular condylar cartilage of senescence-accelerated mice (SAM) with early osteoarthritis (OA). The cartilages in the mandibular condyles and in the proximal tibial epiphyses from two 10-month-old SAM strains, SAMP8 and SAMR1 (a control), were examined for the immunolocalization of chondroitin-4-sulphate (C4S) and keratan sulphate (KS). Morphologically, the mandibular condylar cartilage was disrupted with clefts in SAMP8 but not in SAMR1. C4S was distributed throughout the two cartilages. In contrast, KS was located in extracellular regions in the fibrous and proliferative cell layers of mandibular cartilage and in the lower maturative cell layer of epiphyseal cartilage. The two GAGs, particularly KS, were intensely immunostained around the clefts. These results suggest that C4S and KS could play differential roles in the structural function of articular cartilage and their functions might be involved in early mandibular OA lesions.

Key words: Glycosaminoglycan, Mandibular condylar cartilage, Osteoarthritis, Senescence-accelerated mouse

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Introduction
Degenerative joint diseases, including osteoarthritis (OA), are common, particularly in the elderly. OA shows two principal morphologic changes: progressive softening, ulceration, and focal disintegration of the articular cartilage and the formation of bone and cartilage excrescences at the joint margins (1). The study of early OA before severe disintegration of the cartilage is crucial for understanding how OA begins, but insufficient opportunities are available in human subjects. Surgical animal models have shown matrix changes of the articular cartilage in early OA. The very fast alteration appears to be an increase in the water content, associated with thickening of the articular cartilage (2, 3). Very shortly after the increase in cartilage water, proteoglycans among matrix proteins show a number of changes; their glycosaminoglycan (GAG) composition changes (2), they show impaired aggregation (3), and they show increase of the concentration and the content (4). The GAGs, constituents of proteoglycans, have a high affinity against water due to their high charge density. In addition, they provide resiliency to mechanical loading (5, 6, 7), and thereby the action of GAGs could be involved in the progression of early OA with the water increase in and the thickening of the articular cartilage.

The major proteoglycan in cartilage is aggrecan, a large proteoglycan, which represents some 5-10 % of the tissue wet weight (8). Cartilage also contains collagen-binding small leucine-rich proteoglycans: decorin, a chondroitin-4-sulphate (C4S) proteoglycan, and fibromodulin, a keratan sulphate (KS) proteoglycan (8, 9). These small proteoglycans represent only 1-2 % of the total mass of proteoglycans in cartilage, but their molar amounts are of the same magnitude as that of aggrecan (8). These proteoglycans strongly bind to type I and type II collagens and most likely regulate the collagen fibril formation (10). Thus, their actions may be involved in the process of OA.
The temporomandibular joint (TMJ) is a diarthrodial joint like other load-bearing articular joints. Unlike most diarthrodial joints in which the articular surfaces are covered by hyaline cartilage, the articular surfaces of mandibular condyles are covered by fibrocartilage. It is unknown whether the molecular basis of OA processes in TMJ is the same as that in the other joints. Although a number of studies using surgically experimental animal models, simulating an early stage of mandibular OA, have been performed (11, 12, 13), there is no information available regarding the changes of GAGs or small-leucine rich proteoglycans in OA lesion at TMJ. The senescence-accelerated mouse (SAM) has been developed at Kyoto University in Japan by selective sister-brother matings of mice of the AKR/J strain (14, 15). Most strains have a unique bone phenotype with OA-like morphology similar to the TMJs different from other joints (16). To investigate the role of GAGs in an early stage of OA in the TMJ, we clarified the immunolocalization of the two GAGs, C4S and KS, in the cartilages of mandibular condyles and tibial epiphyses of two SAM strains, a senescence-prone inbred strain SAMP8 and a senescence-resistant strain SAMR1 as a control.

Materials and methods

Animals and tissue preparation

Five SAMP8 and SAMR1 (aged 10 months) each were used in this study. Under pentobarbital anesthesia, after the animals were perfused from the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), mandibular condyles and proximal tibial epiphyses were dissected and further fixed at 4°C overnight in the same fixatives. The specimens were thoroughly rinsed with 0.01 M PBS and then decalcified with 10% ethylenediamine tetraacetic acid in 0.01 M PBS (pH 7.4) at 4°C for 2 weeks. After dehydration with a graded series of ethanol, specimens were passed through xylene and embedded in paraffin. Sagittal sections, each 5 μm thick, were cut and were deparaffinized. Some sections were stained with hematoxylin and eosin for routine microscopic examination. The experimental protocol was approved by the Ethical Committee for Animal Experiments at our institute.

Immunohistochemistry

Immunostaining procedures for C4S and KS were basically the same as described in the previous study (17). Sections were treated with 0.25 units/ml chondroitinase ABC (Seikagaku Co., Tokyo, Japan) in 40 mM Tris-HCl buffer (pH 8.0) containing 40 mM sodium acetate and 0.01% bovine serum albumin for 1 h at 37°C to either generate or better expose specific epitopes (18). The endogenous peroxidase in the sections was treated using 3% hydrogen peroxide in methanol. After a brief rinse with PBS, non-specific binding sites in the sections were blocked with 5% skim milk for 30 min. The monoclonal anti-C4S and anti-KS antibodies 2-B-6 and 5-D-4 (1:250 dilution; Seikagaku Co.) were used as primary antibodies (18). These antibodies have been commonly used for immunohistochemical analysis of the two GAGs in several animal tissues, in particular, mineralized tissues, i.e., rat TMJ (19), bovine and rat teeth (17, 20, 21), pig and mouse tooth germs (22, 23). The sections were incubated with the primary antibodies for 2 h at room temperature. After a brief rinse with PBS, the sections were then incubated with biotinylated goat anti-mouse IgG as secondary antibody for 30 min. After rinsing for 5 min with PBS three times, the sections were exposed to the streptavidin-peroxidase conjugate in Vectastain® Elite kit (Vector Laboratories, Burlingame, CA, USA) for 10 min and then washed again for 5 min with PBS. Color was developed using peroxidase-substrate solution in the same kit, and then dehydrated with a graded series of ethanol. After passing through xylene, coverslips were mounted with malinin (Muto Pure Chemicals Co., Tokyo, Japan).

Results

The mandibular condylar cartilages of 10 month-old mice in SAMP8 and SAMR1 were non-growing articular cartilage without hypertrophic cell layer and erosion layer, but they exhibited morphology different from each other, particularly in the central area on the sagittal plane in the cartilage, i.e. the load-bearing region (Figs. 1a and 1b). Compared with SAMR1, the upper and lower maturative cell layers were thicker and the fibrous and proliferative cell layers were irregularly organized and thickened in SAMP8. Clefts were readily found at the irregularly organized layers, reaching from the articular surface to the proliferative cell layer or the upper maturative cell layer. The tibial epiphysis in SAMP8 was histologically different from that in SAMR1 in the growth plate (Figs. 2a and 2b). The apparent structure of growth plate had disappeared in SAMP8 but it remained in SAMR1. The maturative cell layer of the articular limb cartilage in SAMR1 was present in some (as seen at the proximal tibial epiphysis in Fig. 2) but not all samples (as seen in the distal femoral epiphysis in Fig. 2), thicker than that in SAMP8.

The immunostaining for C4S was distributed in the extracellular matrix and pericellular area throughout the condylar cartilage in both strains (Figs. 3a and 3b), while that for KS was distributed only in the extracellular matrix of the fibrous and proliferative cell layers but not in the pericellular area (Figs. 3c and 3d). C4S and KS were located in the irregularly thickened fibrous and proliferative cell layers around the cleft area in SAMP8 (Figs. 3a and 3c). In particular, the intensity of immunostaining for KS looked higher in the irregularly organized layers than in the normal organized layers in
SAMR1 (Figs. 3c and 3d). At the tibial epiphysis, C4S was also distributed in the extracellular matrix and pericellular area throughout the articular and growth plate cartilages in both strains, and was immunostained even in the matrix regions where the growth plate disappeared in SAMP8 (Figs. 4a and 4b). In contrast, KS was immunostained exclusively in the lower maturative cell layer in the articular cartilage but not in the growth plate cartilage (Figs. 4c and 4d).

Discussion
This study showed that 10 month-old SAMP8 spontaneously exhibited an early OA-like lesion in the TMJ, consistent with the previous report by Chen and his coworkers (16). Previous reports using surgical animal models have shown that the content and the distribution of GAGs were altered in an early stage of simulated OA in the TMJ. Unilateral disk perforation in rabbit TMJ leads temporomandibular condyle into developing an early OA-like lesion such as ulceration of the condylar surface and thickening of the cartilaginous layer (11, 12). These earlier studies revealed that the synthesis of GAG but not of collagen increased in the degenerative condylar cartilage with surgical treatment, and they suggested that the turnover of small and large proteoglycans and the proportion of small proteoglycans increased, while the population of the largest proteoglycan decreased. The present study shows for the first time the distribution of GAGs in an early OA-like lesion of TMJ. C4S was naturally distributed in the extracellular matrix and pericellular area throughout the normal condylar cartilage, and it remained even in the thickened maturative layer and in the irregularly thickened fibrous and proliferative layers around clefts. KS was intensely distributed in the irregularly organized area around clefts. The distribution of these two GAGs in the thickened layers likely supports the increased content of GAGs in simulated early OA of TMJ shown in the previous study (11).

The proteoglycans that have been found in mandibular condylar cartilage and possess C4S chains are...
aggrecan and decorin. Aggrecan is distributed in the maturative layer but not in the fibrous and proliferative layers (24). Unilateral bite raise in rat induces hyperplasia of the cartilaginous maturative zone and aggrecan appears to be preferably expressed in this zone (13). In contrast, decorin is distributed primarily in the superficial articular layer in rat young adult mandibular condyle (25). Although it is only speculative, C4S that was distributed in the fibrous and proliferative cell layers around the cleft region in this study may be GAG chains of decorin but not of aggrecan. KS that was intensely located in the same region around clefts may be GAG chains of fibromodulin. Fibromodulin was first isolated from articular cartilage (26), although it has not been isolated from TMJ. This molecule, distributed in the cartilaginous maturative cell layer and calcified cartilage in old rat knee joints (27), is well associated with the distribution of KS in tibial epiphysis in this study (Figs. 4c and 4d). If decorin and fibromodulin, members of small leucine-rich proteoglycans, were distributed, this might explain why the proportion of small proteoglycans was enhanced in rabbit mandibular condylar cartilage with unilateral disk perforation surgery (12).

The roles of C4S and KS play in the early degenerative changes of mandibular condyle are unknown. The GAGs could compensate for resilience to the mechanical loading in disorganized collagen networks in the early degenerative TMJ. If small leucine-rich proteoglycans, such as decorin and fibromodulin, were distributed, the two proteoglycans might be able to play different roles in the disorganized articular cartilage. Type I collagen but not type II collagen is composed of the fibrous and proliferative cell layers of mandibular condylar cartilage as a major matrix protein (28). Decorin and fibromodulin bind to type I collagen fibrils, but their binding sites are different in, or very close to, a ‘gap’ zone on the collagen...
fibrils (29, 30). They regulate collagen fibrillogenesis and their actions seem to be tissue-specific (31). Fibromodulin and lumican, other small leucine-rich proteoglycans, have different development stages in the regulation of collagen fibrillogenesis (32), although the stage that decorin has in the function is unknown. It is just speculative but possible that decorin and fibromodulin could be involved in or might play different role in compensation for resilience to the mechanical loading and/or in regulation of fibril formation of newly synthesized collagens in disorganized collagen networks in the early degenerative TMJ.

The distribution of C4S and KS in normal mandibular condylar cartilage in SAMR1 is not completely consistent with the previous study using rat mature TMJ (19). The previous study has shown that the immunoreactivity of C4S is positive in the transitional and maturative cell layers but negative in the fibrous and proliferative cell layers of the central area of mandibular condylar cartilage, while that of KS is positive in the transitional cell layer, weakly positive in the maturative cell layer but negative in the fibrous and the proliferative cell layers. The discrepancy between the results of the present and the previous studies may be due to differences between animal species, mouse and rat, or may be due to potential senescence of SAMR1. SAMR1 did not apparently exhibit degenerative changes of mandibular condyle and tibial epiphysis in this study, and this strain is a control strain against senescence-prone strains among SAM, such as SAMP8 (14, 15). Compared with senescence-prone strains, SAMR1 does not exhibit many pathologic phenotypes, but it shows non-thymic lymphoma and histiocytic neoplasia.

It is currently unclear whether OA-like degenerative change of mandibular condyle in SAMP8 is a consequence of senile changes and/or is due to other factors. Chen and co-workers have shown that feeding of soft foods reduces degenerative alterations of TMJ of SAM strains (33), and this finding suggests that, at least, mechanical overloading may play a role in the pathogenesis of OA in the TMJ. In other joints, there seems to be an inverse relationship between OA and osteoporosis epidemiologically (34, 35). Also among SAM strains, only SAMP6 exhibits osteoporosis-like phenotype (14, 15) but does not show OA-like lesion in TMJ as SAMR1 (16). It is unclear but possible that mechanical loading and bone quality (and/or cartilage quality) is involved in the onset of OA in TMJ. Overloadings by abnormal occlusion or changes of bone quality according to senescence may affect degenerative changes of the TMJ.

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