Pivotal role of Sirt6 in the crosstalk among ageing, metabolic syndrome and osteoarthritis

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1. Introduction

Metabolic syndrome is a common phenotype associated with an increased risk of cardiovascular disease or diabetes. It is well known that individuals suffering from metabolic syndrome display a characteristic imbalance in their adipokine profile, which leads to profound changes in insulin sensitivity and other biochemical alterations of metabolites.

Osteoarthritis (OA) is a chronic degenerative joint disorder characterized by articular cartilage destruction and osteophyte formation. Obesity and high body mass index are associated with a higher incidence of OA [1,2]. Recent studies have revealed that a metabolic syndrome rather than obesity itself is implicated in the etiology of OA [3]. However, the reasons why there is such a correlation between metabolic syndrome and OA remains elusive.

Adipose tissue has been characterized as an active endocrine organ. The infrapatellar fat pad (IPFP) is a unique fat depot that is located intracapsularly and extrasynovially in the joint and is in close contact with articular cartilage. Recently, the endocrine function of the IPFP has been implicated in the initiation and progression of OA [4]. We previously disclosed that the murine IPFP secretes inflammatory cytokines within twelve weeks of exposure to HFD [5]. This model reveals that adipocyte hypertrophy closely links OA progression through secretion of inflammatory cytokines [5].

Ageing is also a pivotal risk factor for osteoarthritis. The stress-response and chromatin-silencing factor Sir2, a yeast sirtuin, is a NAD+-dependent histone deacetylase and is involved in controlling ageing [6]. In mammals the sirtuin family contains seven genes (SIRT1–SIRT7) encoding sirtuin proteins that differ in tissue specificity, subcellular localization, enzymatic activity and targets. Among them, SIRT6 is localized to the nucleus and is involved in transcriptional silencing, genome stability, and longevity [7]. Sirt6 is implicated in the regulation of life span and ageing through the regulation of NFκB function [7]. We previously disclosed that Sirt6 is expressed in chondrocytes and controls proliferation and differentiation of chondrocytes through the regulation of Indian hedgehog (Ihh) expression [8]. These facts suggest possible roles of Sirt6 in the maintenance of the articular cartilage. However, the precise role of Sir76 in OA pathogenesis is poorly understood. This study sought to investigate the involvement of Sirt6 in the ageing of articular tissues and in the development of HFD-induced OA by using a murine HFD-induced osteoarthritis model. Furthermore, we compared the phenotype of the articular tissues between Sirt6
decicient mice revealed both attenuated chondrocyte hypertrophy and proteoglycan synthesis, although chondrocyte senescence was enhanced as shown in the aged WT mice. Thus Sirt6 has key roles in the relationship among ageing, metabolic syndrome, and OA.

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global haploinsufficient mice and mesenchyme-specific Sirt6 deficient mice to evaluate the critical roles of Sirt6 in the chondrocytes.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Animal Care and Use Committee of Tokyo Medical and Dental University and were carried out in accordance with the approval guidelines. Sirt6± mice on a 129X1/Sv background were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Prx1-Cre (B6.Cg-Tg[Prx1-Cre]1Cjt/J) mice were kindly supplied by Dr. Hiroshi Asahara. Sirt6+/f mice (FVB.129S6(Cg)-Sirt6tm1.1Cxd/J) and Prx1-Cre mice were crossed to generate Prx1-Cre; Sirt6+/f mice, and their progeny were intercrossed to obtain Prx1-Cre; Sirt6+/f mice. The mice were sacrificed and analyzed at six months of age. Sirt6 heterozygous mice (Sirt6± mice) or littermate mice (WT mice) were fed a diet containing 32% fat for the high fat diet group (HFD) or 48% fat for the normal diet group (ND) (HFD32 and CE-2; CLEA Japan, Inc. Tokyo, Japan) [23] from the age of six months. All of the animals were allowed unrestricted activity and were provided food and water ad libitum. None of the mice died during the experimental period.

2.2. Cell culture conditions

Primary epiphyseal chondrocytes were isolated from 5-day-old mice as previously reported [24]. Briefly, cartilage tissues, including the femoral heads, femoral condyles and tibial plateau, were cut into small pieces and digested twice for 45 min each with 3 mg/ml type I collagenase. The cartilage pieces were then incubated in 0.5 mg/ml type I collagenase. The cartilage pieces were then digested into small pieces and digested twice for 45 min each with 3 mg/ml type I collagenase. The cartilage pieces were then incubated in 0.5 mg/ml type I collagenase at 37°C in a thermal incubator with 5% CO2 overnight. The next day, cell aggregates were dispersed by pipetting. The cells were cultured in 12-well plates seeded with 5×10^4 cells per well in DMEM/F12 medium containing 10% FBS and antibiotics. The cells were transfected with 50 nM Sirt6 siRNA (Ambion, Austin, TX, USA) in a Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and maintained in medium supplemented with 10% FBS for 24 h. The target sequence of Sirt6 siRNA was 5’GAAGCUCUAAAUGCAUAAU3’ (forward) and 5’UUAUUUGCAUUGGACGUUCG3’ (reverse).

2.3. Assessment of OA severity

Sirt6± mice and the littermates were sacrificed at twelve weeks after initiating the diet (n = 7). Prx1-Cre+Sirt6+/f mice (n = 6) were sacrificed at 6 months of age. Whole knee joints were removed by dissection, fixed in 4% paraformaldehyde, and decalcified in EDTA. After dehydration and paraffin embedding, serial 5-μm sagittal sections were made from the whole medial compartment of the joint as mentioned previously [5]. Quantitative osteophyte determination was made as mentioned previously [5]. Briefly, the medial part of the sections, in which the central region of the medial meniscus was continuous, were selected and the protruded region, which stained green by Safranin-O staining, at the anterior edge of the tibial plateau was defined as bony osteophyte and quantified using ImageJ software (Media Cybernetics, Carlsbad, CA). For the evaluation of OA severity, representative sections were selected from the medial tibial plateau and medial femoral plateau, and scored with osteoarthritis research society international (OARSI) scoring [25]. Two representative sections from each mouse were blindly evaluated by three different readers.

2.4. Glucose tolerance test (GTT)

Mice were fasted overnight (18 h) before GTT was performed, by an intraperitoneal injection of glucose (0.5 g/kg; Sigma–Aldrich, St. Louis, MO) to assess glucose clearance. Blood samples were taken from the tail vein before and 30, 60, 90 and 120 min following the injection. Blood glucose level was measured using Glutest neo alpha (Sanwa kagaku kenkyusho CO., LTD, Nagoya, Japan).

2.5. Immunohistochemistry

The protein expression of type X collagen (ColX), Matrix Metalloprotease-13 (MMP-13), Sirt6 and Plasminogen activator inhibitor-1 (PAI-1) was determined by immunohistochemistry using anti-ColX antibody (LSL Japan), anti-MMP-13 (Abcam Biochemicals, Cambridge, UK), anti-Sirt6 (Abcam Biochemicals, Cambridge, UK) and anti-PAI-1 (Abcam Biochemicals, Cambridge, UK) respectively, according to the manufacturer’s instructions. The signal was visualized using peroxidase-conjugated avidin and diaminobenzidine from a Vectastain kit, according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA, USA).

2.6. TUNEL assay

Apoptotic cells in the articular cartilage were detected by a TUNEL detection kit according to the manufacturer’s instructions (Takara Shuzo, Kyoto, Japan).

2.7. Statistical analysis

Data are expressed as the mean ± 1 SD. Statistical analysis was performed with the Mann–Whitney U test or Tukey–Kramer test. P values less than 0.05 were considered significant.

3. Results

3.1. Impact of Sirt6 haploinsufficiency with or without HFD on the insulin tolerance

Body weights of Sirt6± mice in 129X1/SvJ strains were comparable to WT littermates at six and nine months of age (Fig. 1a). To investigate the role of Sirt6 in obesity-induced OA, Sirt6± mice and the littermates were fed HFD or normal diet (ND) for three months from 6 months of age. Both WT and Sirt6± mice fed the HFD weighed 20% more after twelve weeks on the diet (Fig. 1a, p > 0.05). To evaluate the limb mesenchymal cell-specific function of Sirt6, we developed the conditional Sirt6 deletion mice carrying floxed Sirt6 alleles bred with Prx1-Cre transgenic mice (Prx1-Cre;Sirt6+/f mice). Body weights of Prx1-Cre;Sirt6+/f mice were comparable to the controls at six months of age (Fig. 1a). To explore the role of Sirt6 insufficiency in glucose homeostasis and insulin sensitivity, glucose tolerance tests (GTTs) was performed for WT and Sirt6± mice (Fig. 1b). In ND group, fasting blood glucose level was lower in Sirt6± mice at 90 and 120 min following glucose injection than in WT mice (Fig. 1b,a). However, after being fed HFD for 12 weeks, Sirt6± mice showed significantly higher blood glucose levels at 30 and 60 min following glucose injection as compared to those in the other littermates (Fig. 1b,b). These data suggest that Sirt6± mice fed an HFD were more susceptible to metabolic syndrome than WT mice.

3.2. mRNA profiles in the IPFP

Although Sirt6 overexpression has been reported to have protective roles against diet-induced metabolic diseases [9], the roles of Sirt6 in the fat tissue depot response to HFD have yet to be elucidated. Thus we monitored the mRNA expression levels of inflammatory cytokines/adipokines of the IPFP at sacrifice. Real-time
RT-PCR analysis revealed the expression of inflammatory cytokines, such as TNF-α and IL-6, were spontaneously enhanced in the IPFP in Sirt6 ± mice whereas this effect was not observed in Prx-1Cre;Sirt6f/f mice (Fig. 1c). When Sirt6 ± and the littermate mice were fed HFD, leptin mRNA was similarly increased in the IPFP (Fig. 1c). The expression of TNF-α and IL-6 in the IPFP was the
highest in the Sirt6±HFD group (Fig. 1c). Sirt6 mRNA was deleted in the chondrocytes of Prx-1Cre;Sirt6f/f mice, whereas the expression level of Sirt6 in the IPPF was comparable in these mice (Fig. 1d).

3.3. Histological analysis of the femorotibial joints

Next, the knee joints were histologically investigated. Histological investigation of the femorotibial joints of nine-month-old mice frequently revealed vertical clefts down to the layer immediately below the superficial layer in association with moderate reduction of Safranin-O intensity only in Sirt6± mice (Fig. 2a). When HFD was applied to these mice, the histological features of OA were observed both in WT and Sirt6± mice, although the histological alteration was more apparent in Sirt6± mice (Fig. 2a). The OA severity based on OARSI score indicated Sirt6± mice scored higher compared to WT littermates (Fig. 2b). Safranin-O staining revealed glycosaminoglycan (GAG) concentration was markedly reduced in Prx-1Cre;Sirt6f/f mice (Fig. 2a). When Sirt6 was knocked down in the epiphyseal primary chondrocyte by Sirt6 siRNA, aggrecan transcript was significantly suppressed (Fig. 2c). These results indicated Sirt6 was required for the expression of glycosaminoglycan in the articular cartilage.

3.4. Immunohistological analysis for OA markers

We next evaluated the expression of the markers of OA in articular cartilage by immunohistochemistry (Fig. 2d, f). Staining for type X collagen (ColX), an extracellular matrix marker for chondrocyte hypertrophy, was enhanced at the deep layer of the articular cartilage in WT HFD, Sirt6± ND, and Sirt6± HFD groups (Fig. 2d). The expression of MMP-13, a matrix protease implicated in the OA process, was also significantly increased at the superficial layer of articular cartilage in Sirt6± ND and Sirt6± HFD groups compared to WT or control, respectively (Fig. 2f, g). On the other hand, CoIX expression and the number of hypertrophic chondrocytes were reduced in Prx-1Cre;Sirt6f/f mice (Fig. 2d, e). Furthermore, the expression of MMP-13 in Prx-1Cre;Sirt6f/f mice was similar compared to the control mice (Fig. 2f). These findings indicated endogenous Sirt6 deletion in the chondrocytes did not accelerate the expression of MMP-13 and CoIX.

Osteophyte formation is among the characteristic features of OA. Sirt6 haploinsufficiency was associated with enhanced osteophyte formation at the anterior edge of the sagittal section of the tibial plateau, whereas osteophytes were hardly observed in WT ND mice (Fig. 3a, b, c). Simultaneously, hypertrophy of the synovium accompanied by infiltration of inflammatory cells (Fig. 3c arrows) was also enhanced in the Sirt6± ND (Fig. 3c, d, e). The osteophyte formation was further enhanced by HFD in Sirt6± mice (Fig. 3a, b) in association with further hypertrophy of the synovial tissues (Fig. 3c, d, e). These findings suggested inflammation triggered by global haploinsufficiency of Sirt6 was further enhanced by HFD.

3.5. Chondrocyte apoptosis and senescence

Chondrocyte apoptosis is increased in OA cartilage and is anatomically linked to proteoglycan depletion [10]. The number of TUNEL positive cells was elevated by HFD in WT and Sirt6± mice (Fig. 4a, b). Furthermore, Sirt6± mice exhibited spontaneous increased apoptotic cells even in the ND group (Fig. 4a, b). On the other hand, in Prx-1Cre;Sirt6f/f mice, TUNEL positive cells were not increased in the articular cartilage (Fig. 4a, b). To address the role of Sirt6 in articular chondrocyte senescence, the expression of PAI-1 protein was evaluated by immunohistochemistry. PAI-1 positive chondrocytes were rarely observed in 2-month-old mice (Fig. 4d). Both Sirt6± mice at 9 months of age and Prx-1Cre++;Sirt6f/f demonstrated higher levels of PAI-1 expression (Fig. 4c, d). The HFD caused a more notable increase in PAI-1 expression in Sirt6± mice. PAI-1 expression in the chondrocytes was significantly increased in WT aged mice (Fig. 4c, d), suggesting that PAI-1 expression in chondrocytes increases with age. These results indicate enhanced PAI-1 expression in Sirt6± mice and Prx-1Cre++;Sirt6f/f mice may mimic the ageing process of the joint cartilage.

4. Discussion

Here we revealed the roles of Sirt6 in the etiology of metabolic syndrome, including osteoarthritis. First, Sirt6 haploinsufficiency promoted the expression of inflammatory cytokines in the IPPF in association with impaired glucose tolerance. With the background of 129sv, we performed a glucose tolerance test (GTT) on wild-type (WT) and Sirt6± mice. The 129 strain is reported to develop features of metabolic syndrome, such as obesity, hyperinsulinemia, and glucose intolerance, after being fed a high-fat diet (HFD) for over 18 weeks [11]. From the result of our GTT, we confirmed Sirt6± mice were more susceptible to metabolic syndrome after being fed HFD.

Second, enhanced inflammation of the IPPF was associated with accelerated OA progression in Sirt6± mice. In Sirt6± mice, advanced degeneration of the articular cartilage (Fig. 2) and increased apoptosis (Fig. 4a, b) were observed, in many cases, irrespective of administration of HFD. In Sirt6± mice, the expression level of inflammatory cytokines increased in the IPPF (Fig. 1c). Whereas TNF-α and IL-6 induce apoptosis in chondrocytes, we previously reported Sirt6 had no effect on chondrocyte apoptosis [8]. This phenotype is consistent with that of Prx-1Cre++;Sirt6f/f mice, in which TUNEL positive cells were not increased in the articular chondrocytes (Fig. 4a). The enhancement of the inflammatory cytokines in the IPPF was observed only in Sirt6± mice and not in Prx-1Cre++;Sirt6f/f mice, as Sirt6 expression was comparative in the IPPF of Prx-1Cre++;Sirt6f/f mice (Fig. 1d). The expression levels of CoIX and MMP13 were also spontaneously enhanced in Sirt6± mice, in which the expression of TNF-α was enhanced in the IPPF. TNF-α induces IL-6 mRNA in OA chondrocytes [12]. Treatment of OA chondrocytes with IL-6 results in enhanced production of MMP-13 protein [12]. In contrast, in Prx1Cre;Sirt6f/f mice, the expression levels of CoIX and MMP13 were decreased or unaffected. In our previous study, we revealed Sirt6 deletion inhibits the expression of CoIX through the inhibition of Indian hedgehog (Ihh) by suppressing the affinity of AT14 to the Ihh promoter [8]. This result is consistent with the aforementioned inhibition of ColX expression in Prx1Cre;Sirt6f/f mice. Given that positive regulation of MMP13 expression by Ihh was previously reported [13], we believe that Ihh is also likely to contribute to the unchanged MMP13 expression in Prx1Cre;Sirt6f/f mice. Considering these facts, the increased CoIX, MMP-13 expression and apoptotic activity in Sirt6± chondrocytes may be caused by enhanced IPPF inflammation and not as the result of chondrocyte cell autonomous regulation.

When Sirt± mice were fed HFD, an increase was observed in osteophyte and synovium formations (Fig. 3a), in addition to a further increase in the expression level of the cartilage degeneration marker MMP13 (Fig. 2f, g). These findings imply that the expression levels of inflammatory cytokines such as TNF-α and IL-6 in the IPPF are higher in HFD-fed Sirt6± mice than in ND-fed Sirt6± mice. In other words, the co-existence of Sirt6 haploinsufficiency and obesity worsens the inflammation in the IPPF.

The IPPF contains adipocytes and immune cells such as lymphocytes, monocytes, and granulocytes [14]. The IPPFs from OA patients contain increased amounts of inflammatory cytokines [15]. Thus, the IPPF has been shown to play an important role in the initiation and progression of knee-OA. Overexpression of SIRT6 in
Fig. 2. Histological analysis of Sirt6 mutants and WT mice with normal diet and high fat diet. (a); Sections of articular cartilage from indicated genotypes, diets stained with Safranin-O. (b) OA severity of medial tibial plateau based on OARSI scoring for mice. (c) mRNA expression of aggrecan in the epiphyseal primary chondrocyte of Sirt6 knocked down and the control. (d, f); Immunostaining for ColX and MMP-13 in the knee joint of indicated genotypes and diet. (e) The number of hypertrophic chondrocytes at the deep layer of tibial cartilage of indicate genotypes. (g) The number of MMP-13 positive chondrocytes at the superficial layer of the joint cartilage in the indicated genotypes and diets. Scale bar: 100 μm. Values are the mean ± 1 SD of five mice per group. * = P < 0.05 the Turkey–Kramer test or the Man–Whitney U test.
mice renders protection from various metabolic disorders caused by HFD-induced obesity [16]. Many inflammatory cytokines, including TNF-α, are implicated in HFD-induced inflammation of the fat tissues. TNF-α is produced by macrophages, lymphocytes, and also by adipose tissue, although its expression level is relatively low in humans [17]. Thus, locally present macrophages may play important roles in the elevated expression levels of these cytokines. Macrophages derived from the bone marrow of Sirt6-deficient mice

![Fig. 3. High magnification of the anterior edge of the medial tibial plateau in the HFD and the control diet mice at twelve weeks after the diet initiation. (a, c) Representative Safranin-Ostained sections (a) and HE-stained sections (c). (b, d, e) Quantification of osteophytes (b), synovial cells (d) and synovial tissue area (e) at anterior edge of tibial plateau of indicated genotypes and diets. (a, c) Arrowheads represent osteophytes developed at the anterior edges of the medial tibial plateau. Arrows represent synovial tissues. Scale bar: 100 μm. Values are the mean ± 1 SD of five mice per group. * = P < 0.05 with the Turkey-Kramer test or the Mann-Whitney test.](image-url)
Fig. 4. (a, b): TUNEL staining for the femorotibial joints (a) and the number of TUNEL positive cells in the superficial layer of articular cartilage (b) of indicated genotypes and diets. (c, d): Immunostaining for PAI-1 in the tibial plateau (c) and the rates of PAI-1-positive cells per all the chondrocytes in the superficial layer of the articular cartilage (d) of indicated genotypes and diets. Scale bar: 100 μm. Values are the mean ± 1 SD of five mice per group. * = P < 0.05 vs 2 mWT; ND, ** = p < 0.05 vs 18mo-WT; ND with the Turkey–Kramer test or the Mann–Whitney U test.
exhibit increased MCP-1, IL-6, and TNFα expression levels and are hypersensitive to LPS stimulation through activation of c-JUN signaling [18]. Furthermore, Sirt6 was reported to directly promote the secretion of TNF-α by removing the fatty acyl modification of TNF-α [19]. Combined with our data, Sirt6 may function as an anti-inflammatory factor in the course of HFD-induced OA development through the action on the macrophages in the IPFP.

Third, we demonstrated PAI-1 protein expression was dominantly observed at the superficial layer of the articular chondrocytes of Sirt6−/− mice. Consistently, PAI-1 expression is increased in the human OA cartilage [20]. We previously reported the expression of senescence marker genes are increased in epiphyseal chondrocytes from young Sirt6−/− mice [8]. Seneescent cells secrete inflammatory cytokines as a result of a behavior termed the senescence-associated secretory phenotype (SASP) [21]. We previously revealed the expression of these cytokines was enhanced in Sirt6−/− chondrocytes [8]. Considering the fact that the SASP contributes to the onset of osteoarthritis [22], we believe that, in aged mice, increased expression of PAI-1 in chondrocytes contributes to the progression of knee osteoarthritis. Thus, in the normal aged mice, increased expression of PAI-1 in chondrocytes contributed to the progression of cartilage degeneration in Sirt6−/− mice.

In conclusion, our results provide clues that Sirt6 prevents articular cartilage degradation and IPFP inflammation caused by metabolic syndrome. We believe these findings imply that, in humans, the risk of knee osteoarthritis increases as the level of Sirt6 activity decreases with age and that the combination of senility and obesity makes the elderly more susceptible to the worsening of knee osteoarthritis as compared to the young. These results support a potential therapeutic application of a SIRT6 agonist in the IPFP in human OA.

Author contributions

Conceived and designed the experiments: YA. Performed the experiments: MA, ZA, MI, JP, YA. Analyzed the data: MA, YA, JP. Contributed reagents/materials/analysis tools: YH, DK, AO, SM. Wrote the paper: YA, MA.

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