Published in final edited form as:

Pathol Res Pract. 2011 April 15; 207(4): 225–231. doi:10.1016/j.prp.2011.01.007.

Senescence of chondrocytes in aging articular cartilage: GADD45 β mediates p21 expression in association with C/EBP β in senescence-accelerated mice

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Summary

Growth arrest and DNA damage-inducible protein 45 β (GADD45 β) is expressed in normal and early osteoarthritic articular cartilage. We recently reported that GADD45 β enhances CCAAT/enhancer binding protein β (C/EBP β) activation *in vitro*. This study was undertaken in order to determine whether GADD45 β is expressed with C/EBP β in aging articular cartilage. We also investigated whether the synergistic expression of GADD45 β and C/EBP β may be involved in the mechanism of chondrocyte senescence. Senescence-accelerated mice (SAMP1) were used as a model of aging. GADD45 β , C/EBP β , and p21 were analyzed by immunohistochemistry. A luciferase reporter assay using ATDC5 cells was performed in order to examine p21 as a target gene of the GADD45 β /C/EBP β cascade. GADD45 β exhibited increased expression in the aging articular cartilage of SAMP1 mice compared to that in control mice. The co-localization of GADD45 β and C/EBP β was confirmed by double immunostaining. The synergistic mechanisms of GADD45 β and C/EBP β on the gene regulation of p21, a molecule related to cellular senescence, were verified by a p21-luciferase reporter assay. Co-expression of C/EBP β and p21 was confirmed. These observations suggest that the synergism between GADD45 β and C/EBP β may play an important role in cellular senescence in the aging articular cartilage.

Keywords

SAMP1; immunohistochemistry; osteoarthritis

Introduction

The aging of articular cartilage is a process distinct from osteoarthritis (OA). In the early stages of OA, a transient increase in chondrocyte proliferation and an increase in metabolic activity are associated with a local loss of proteoglycans. This occurs initially at the cartilage surface, along with a cleavage of type II collagen, resulting in chronic cartilage degeneration [1]. During these processes, both catabolic and anabolic responses occur in chondrocytes, which are the sole cellular phenotypes in articular cartilage. Since the OA process usually progresses in elderly patients, it is essential to understand the cellular senescence in articular

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The authors declare no conflicts of interest.

cartilage. However, few reports have described the cellular senescence of articular chondrocytes because of the difficulty in distinguishing aging cartilage and the early stage of OA [2, 3, 4]. In the present study, we investigated the molecular features of senescent chondrocytes in aging articular cartilage, but not in OA cartilage.

In order to study the senescent cartilage separately from OA cartilage, we used the senescence-accelerated mouse (SAM) model, which is a multifactorial model of accelerated aging that is widely used in aging research [5]. The SAM prone (SAMP) strain has an accelerated senescence process, loss of behavioral activity, impaired physiologic functions, higher oxidative stress, impaired mitochondrial function, and a short lifespan [6, 7, 8]. Among SAMP strains, the SAM prone 1 (SAMP1) exhibits the most pronounced acceleration of senescence and short-life [6]. SAMP1 shows a high incidence of age-associated microscopic amyloid deposition in synovial joints and intervertebral discs, and the lesion is reported to be similar histologically to that in humans [9, 10, 11].

Using this animal model, we focused on growth arrest and DNA damage inducible (GADD) 45 β , which is a member of the GADD45 family of small (18 kD) proteins that are associated with cell growth control, cell death, and cellular response to genotoxic stress [12, 13]. Previously, we have reported that GADD45 β is expressed in human samples from normal and early-stage OA articular cartilage [14]. We also reported that GADD45 β enhances the CCAAT/enhancer binding protein β (C/EBP β) activation in terminally differentiating chondrocytes [15]. C/EBP β belongs to the larger family of basic leucine zipper transcription factors, which are involved in the regulation of differentiation, inflammation, hematopoiesis, and endoplasmic reticulum stress [16, 17]. Recently, p21, one of the molecular markers of cellular senescence [18], was identified as a down-stream target of C/EBP β signaling by microarray analysis using mouse mesenchymal C3H10T1/2 cells [19]. Therefore, the current study was undertaken in order to examine whether GADD45 β /C/EBP β signaling may be involved in the mechanism of chondrocyte senescence in aging articular cartilage, especially in the regulation of p21 gene expression, using aging articular cartilage from the SAM model.

Materials and Methods

Animals and experimental procedures

Male SAMP1 and Institute of Cancer Research mice (ICRM) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) at 6 and 12 weeks of age, respectively. We previously reported that age-related muscle morphological changes in SAMP1 occur from 50 weeks of age, and voluntary motor behavior declines with age [7]. Therefore, we examined the articular cartilage of SAMP1 at 58 weeks of age ($n = 5$) as a model of senescent articular cartilage, and at 25 weeks of age ($n = 5$) as a model of pre-senescent articular cartilage. Age-matched ICRM were examined as normal senescence models ($n = 5$ at 58 weeks of age, $n = 5$ at 25 weeks of age). The following experimental protocol was approved by the ethics board of the Institute of Laboratory Animal Sciences of Kagoshima University.

Sample Preparation

The knee joints, including the patella and the joint capsule in both the legs, were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH, 7.4) at 4 °C overnight. The fixed specimens were decalcified using Kalkitox™ solution containing ethylenediaminetetraacetic acid (EDTA) (Wako Pure Chemical Industries, Ltd., Japan) for 2-3 days at 4 °C. After decalcification, the specimens were neutralized in 5% sodium sulfate solution at 4 °C overnight, washed in water, dehydrated, and embedded in paraffin. The embedded tissues were aligned so that the frontal plane sections were cut in the proximal-to-

distal direction at a thickness of 5 μm . Standardized serial sections were collected from the medial tibio-femoral joint.

Histological and Immunohistochemical Analysis

The serial sections were stained with hematoxylin and eosin (H.E.) and toluidine blue in order to observe histological changes in the articular cartilage. Immunohistochemistry was performed according to the indirect immunoperoxidase method. In brief, after deparaffinization, hydration, and blockage of endogenous peroxidase, the specimens were incubated for 20 min with 10% skim milk in phosphate buffered saline (PBS) in order to block specific sites and then individually incubated at 4 °C overnight with the following primary antibodies: goat polyclonal anti- β -galactosidase (β -gal) antibody (C-20; 1:100; Santa Cruz Biotechnology, Inc.), goat polyclonal anti-human GADD45 β antibody (C-18; 1:1000; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-rat C/EBP β antibody (C-19; 1:400 Santa Cruz Biotechnology, Inc.), and a p21 mouse monoclonal antibody raised against amino acids 1-159 representing the full length of p21 of mouse origin (F-5; 1:150; Santa Cruz Biotechnology, Inc.). After rinsing, the sections were reacted with biotinylated anti-goat IgG, anti-rabbit IgG, or anti-mouse IgG (all at a dilution of 1:200), which was followed by the avidin biotin complex (ABC) kit (Vector Laboratories, Inc.), according to the manufacturer's instructions. The immunoreactivity was visualized with diaminobenzidine and hydrogen peroxide. A light counterstaining was performed with H.E. The specificity of the immunohistochemical procedures was confirmed by incubation of sections with non-immune serum instead of the primary antibody. Hypertrophic chondrocytes in the growth plate of 1-day-old C57BL/6 mice were used as positive controls for GADD45 β , C/EBP β , and p21 immunostaining (data not shown) [4, 20, 21]. Co-localization of GADD45 β and C/EBP β immunoreactivities was observed using Confocal Laser Scanning Microscopy (CLSM; Olympus, FV500 Japan). After reaction with the 2 primary antibodies followed by washing, the sections were incubated with Alexa Fluor 546-conjugated anti-goat IgG antibody (1:200 in PBS) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (1:200 in PBS) for 60 min. For co-localization of C/EBP β and p21, after reaction with the 2 primary antibodies and washing, the sections were incubated with FITC-conjugated anti-rabbit IgG antibody (1:200 in PBS) and Alexa Fluor 546-conjugated anti-mouse IgG antibody (1:200 in PBS) for 60 min. All sections were counterstained with 4', 6-diamino-2-phenylindole (DAPI; Invitrogen, Japan, Tokyo).

Quantitative Analysis

Histological studies were performed at 2 sites of the medial tibio-femoral joint: the tibial peripheral site (TP) and the tibial apposed site (TA). The TP was defined as the site 400 μm from the attachment of the medial collateral ligament and as a dynamic weight-bearing site. In contrast, TA was defined as the one-third central part of the medial tibio-femoral joint and as a static weight-bearing site (Fig. 1). The number of GADD45 β -positive chondrocytes was counted separately in the superficial and deep cartilage zones. Four serial rectangles that were 60- μm deep and 100- μm long (total, 400- μm long) were superimposed over the immunostained sections. The superficial cartilage was defined as the area from the cartilage surface to a depth of 60 μm . The deep cartilage was defined as the area more than 60 μm from the cartilage surface to a depth of 120 μm . The immunostained sections were digitized through a 20 $\times$ microscope objective using a microscope and camera. The number of GADD45 β -positive chondrocytes in each rectangle was counted manually using an optical microscope by 3 individuals, who were blinded to the groups from which the specimens were obtained.

Luciferase Reporter Assays

In order to define the effects of C/EBP β and GADD45 β on target gene expression, luciferase reporter plasmids containing the p21 gene promoter were transfected into ATDC5 cells in combination with expression plasmids encoding GADD45 β and C/EBP β , alone or together. The ATDC5 cells were purchased from RIKEN BioResource center (Tsukuba, Japan) and cultured as described previously [16]. The luciferase reporter construct that was driven by a 2.5-kb p21 promoter sequence was a gift from De Xu Cao (University of Alabama, USA). The GADD45 β and C/EBP β expression plasmids have been described previously [16]. After incubation for 24 h, cell extracts were analyzed for luciferase activity using the dual-luciferase reporter assay system (Promega Corporation). All data were normalized to the value of pRL-TK Renilla luciferase activity [mean + SD, n = 3].

Statistical Analysis

Statistical comparisons were performed with one-way analysis of variance (ANOVA) using StatView version 5.0. If significance was achieved, a post-hoc Fisher's protected least significant differences (PLSD) test was performed in order to determine where significant differences existed. Significance was set at $p < 0.05$.

Results

Osteoarthritic changes, such as fibrillation, fissure, cluster formation, and osteophyte formation, were not observed in the articular cartilage of either SAMP1 or ICRM at 25 weeks or 58 weeks of age. However, metachromasy of the matrix was observed in SAMP1 at 25 weeks and 58 weeks of age, indicating the degeneration of aggrecan in the articular cartilage. Furthermore, immunohistochemistry using the anti- β -gal antibody revealed that the expression of senescence-associated β -gal in SAMP1 was higher than that in ICRM (Fig. 2). These results suggest that the articular cartilage of SAMP1 is a preferable model of the aging of articular cartilage, but not for OA cartilage.

At 58 weeks of age, GADD45 β -positive cells were observed in the articular cartilage of both SAMP1 and ICRM (Fig. 3A-D). More chondrocytes were labeled in the SAMP1 than in the ICRM in both the TP and TA. The expression patterns in SAMP1 and ICRM were identical (TP > TA, superficial zone > deep zone) (Fig. 2E).

C/EBP β immunoreactivity was also strongly present in GADD45 β -positive chondrocytes in the TA and TP of SAMP1. In SAMP1, co-localization of GADD45 β and C/EBP β was confirmed by confocal laser scanning microscopy (CLSM) in clusters of chondrocytes (Figure 4A-D). Interestingly, the expression of C/EBP β was detected in the nuclei of chondrocytes, whereas GADD45 β expression was observed in not only the nuclei of chondrocytes but also the cytoplasm around the nuclei (Fig. 4E-J).

In order to examine whether GADD45 β could participate in transactivating the p21 promoter in synergy with C/EBP β , we used a luciferase reporter construct driven by 2.5 kb of the p21 promoter sequence. GADD45 β and C/EBP β overexpression alone had a slight effect, 1.6- and 1.4-fold, respectively, whereas co-transfection of GADD45 β with C/EBP β synergistically enhanced p21 promoter activity by 4.1-fold (Fig. 5A). Importantly, co-localization of C/EBP β and p21 in the SAMP1 articular chondrocytes was confirmed immunohistochemically (Figure 5B-D).

Discussion

The physiological features of senescent chondrocytes are poorly understood because articular chondrocytes maintain a low turnover rate in the replacement of cartilage matrix

protein, even in young cartilage. Previous investigators have indicated that replicative senescence of chondrocytes occurs *in vivo* [2, 3]. Studies of articular chondrocytes from human donors revealed that senescence-associated β -gal activity increased with aging, whereas telomere length decreased with aging. Since these changes have also been reported in OA, the exact mechanism of chondrocyte senescence and its implications with regard to OA pathogenesis remain unclear [2, 3, 4]. Therefore, the present study was undertaken in order to investigate the physiological features of senescent chondrocytes in aging cartilage, which are distinct from OA.

In the present study, both GADD45 β and C/EBP β were expressed in senescent chondrocytes. Co-expression of C/EBP β and p21 was confirmed by immunohistochemistry. Synergistic mechanisms of GADD45 β and C/EBP β on the gene regulation of p21, a molecule related to cellular senescence, were verified by a p21-luciferase reporter assay. These observations suggest that synergism between GADD45 β and C/EBP β may play an important role in cellular senescence, or at least in the gene regulation of p21, in the aging articular cartilage.

We previously reported that the expression of GADD45 β was found in all chondrocytes throughout normal articular cartilage, in addition to its localization in early OA cartilage at sites peripheral to the lesion and in chondrocyte clusters and osteophytes in human samples [15]. The expression patterns of GADD45 β and C/EBP β in the present study were similar to those of early stage of OA (Fig. 3: TP > TA, superficial zone > deep zone) [15]. These results suggest that the lower expression levels of GADD45 β in the TA of SAMP and in the TP/TA of ICRM exhibited endogenous expression levels, and, on the other hand, the higher expression levels in the TP of SAMP1 exhibited a pathological status due to chondrocyte senescence. The endogenous levels of GADD45 β protect against TNF- α -induced cell death and play a role as a survival factor and as a feedback regulatory molecule during acute cell stress. On the other hand, elevated expression of GADD45 β , as in the TP of SAMP1, induces important signals that serve as the molecular link common to pathways of cartilage remodeling, such as in endochondral ossification, early OA, or abnormal mechanical stress [20, 22].

Cellular senescence is defined by the irreversible loss of the division potential of somatic cells and a variety of associated phenotypic changes. A DNA damage response is the most prominent initiator of senescence, and this response is characterized by induction of cell cycle arrest through the activation of checkpoint proteins, notably p53 and p21 [23]. The induction of p21 is mediated by p53, which is a tumor suppressor that regulates the induction of growth arrest associated with cellular senescence, and which was shown recently in an irradiation apoptosis model [12, 24, 25]. Interestingly, Passes et al. described a close relationship between p21 and GADD45 in the process of cellular senescence. They described the feedback loop mechanism of mitochondrial dysfunction and the production of reactive oxygen species (ROS) through the serial signaling of GADD45/p38/GRB2/TGFBR2/TGF β and p21, resulting in the establishment of the senescent phenotype [23]. In the present study, we hypothesized that p21 was a down-stream target of the GADD45 β /C/EBP β pathway. Recently, p21 was identified as a down-stream target of C/EBP β signaling by microarray analysis using mouse mesenchymal C3H10T1/2 cells [19]. Taken together with these facts, the expression of p21 in chondrocytes of SAMP1 that co-express GADD45 β and C/EBP β suggest their involvement in the molecular mechanism of chondrocyte senescence in aging cartilage.

The retinoblastoma protein (Rb) is an important downstream mediator of growth arrest for p21 [26]. p21 mediates p53-dependent G₁ arrest by inhibiting the activity of cyclin-dependent kinases, which phosphorylate the *Rb* gene products; unphosphorylated Rb does

not release E2F and does not induce G₁ entry into the cell cycle [27]. Dai et al. investigated the mechanism of cellular senescence of articular cartilage and concluded that catabolic stresses, IL-1 β , and H₂O₂ induce the up-regulation of p53 and p21 and the down-regulation of phosphorylated Rb in human and rat osteoarthritic chondrocytes [28]. In the present study, the expression of Rb was not confirmed in immunohistochemistry (data not shown); however, gene expression profiling or mechanical stress models that induce OA by unilateral ligament and anterior cruciate ligament (ACL) transection should be studied in order to elucidate the mediation of Rb in chondrocyte senescence of SAMP1 in the future.

Pathologically, the major feature of OA is chronic cartilage degeneration [1]. In the early stages of OA, a transient increase in chondrocyte proliferation and increased metabolic activity are associated with a local loss of proteoglycans, which occurs initially at the cartilage surface and which is followed by cleavage of type II collagen. The characteristic features of OA cartilage destruction, such as fibrillation, have not been observed in SAMP1. Although metachromasy of matrix was observed in SAMP1, which showed the degeneration of aggrecan in articular cartilage, chronological changes were not detected by immunohistochemistry for β -gal, GADD45 β , and C/EBP β between 25 weeks and 58 weeks of age in SAMP1. It is probably because cellular senescence develops considerably slowly from an initiation stage toward fully irreversible, phenotypically complete senescence [26].

In conclusion, the synergistic function of GADD45 β and C/EBP β in the regulation of p21 transcription may play an important role in the cellular senescence in the aging articular cartilage.

Acknowledgments

This research was supported by KAKENHI-20591787(KI) and National Institutes of Health grant R01-AG022021 (MBG).

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Figure 1.

Cartilage assessment sites in tibial articular cartilage. TA: central site of medial plateau, which is a weight-bearing site that contacts the femoral condyle; TP: peripheral site, which is the medial edge of the tibial articular cartilage.

Figure 2. Histology of articular cartilage of the tibia, showing senescent features of SAMP1. Toluidine blue staining and β -galactosidase immunostaining at 25 weeks and 58 weeks of age of ICRM and SAMP1. Bar = 60 μ m (all panels).

Figure 3.

Immunohistochemical detection of GADD45 β at TA (A) and TP (B) in controls (ICRM), and at TA (C) and TP (D) in SAMP1 articular cartilage. GADD45 β was detected in the chondrocytes of superficial and deep cartilage and was more strongly expressed in the TP in SAMP1 than in the control samples. Bar = 30 μ m (all panels). Quantitative analysis of GADD45 β -positive chondrocytes in articular cartilage (E). The number of GADD45 β -positive chondrocytes in SAMP1 was significantly increased in comparison with that of control mice. GADD45 β -positive chondrocytes in superficial cartilage were significantly increased in comparison with those of deep cartilage in TP. TC: tibia central site. TP: tibia peripheral site. Values are means (standard deviation). * $P < 0.05$, which is significant when compared with control values. † $P < 0.05$, which is significantly different compared with the TP (superficial) of SAMP1 values.

Figure 4.

Hematoxylin and eosin staining (A) of TP site of SAMP1. Confocal microscopic observation of C/EBPβ immunostaining (B and F, green), GADD45β (C and G, red) and co-localization of GADD45β and C/EBPβ in chondrocytes (D and J, yellow) in the same area. High magnification of chondrocytes (E-J). Nuclei (E, blue), double staining with DAPI and anti-C/EBPβ (H) or anti-GADD45β (I). The anti-C/EBPβ immunostaining was detected in the nuclei of chondrocytes (H); however, GADD45β expression was observed in not only the nuclei but also the cytoplasm of chondrocytes (J). Bar = 50 μm (A, B, C, D) and 20 μm (E, F, G, H, I, J).

Figure 5.

Up-regulation of p21 promoter activity by GADD45 β in synergy with C/EBP β (A). The luciferase reporter construct driven by a 2.5-kb p21 promoter sequence was transfected in ATDC5 cells with expression plasmids encoding GADD45 β and C/EBP β , alone or together. All data were normalized to value of pRL-TK Renilla luciferase activity [mean + SD, n = 3]. Colocalization of GADD45 β , C/EBP β , and p21 in the TP of SAMP1 articular cartilage. Confocal microscopic observation of C/EBP β (B, green), p21 (C, red), and co-localization of C/EBP β and p21 in chondrocytes (D, yellow) in serial sections of the TP of SAMP1. Bar = 100 μ m.