ABSTRACT

Aging-related osteoarthritis (OA) is the most common type of arthritis. Chondrocyte senescence has been linked with the pathogenesis of OA. Here, we examined the expression of GPR39 in chondrocytes and its modulatory effect on IL-1β-induced cellular senescence. We show that GPR39 is moderately expressed in human chondrocytes and its expression is repressed by the pro-inflammatory cytokine IL-1β. The GPR39 agonist TC-G 1008 mitigates IL-1β-induced chondrocyte senescence. Mechanistically, we show that TC-G 1008 mitigates IL-1β-induced cell cycle arrest at G1 phase by suppressing the expression of p53, p21, PAI-1, and K382 acetylation of p53. Moreover, we show that TC-G 1008 treatment restores IL-1β-induced inhibition of SIRT1 and the silencing of SIRT1 abolishes the function of TC-G 1008 on p53 acetylation and senescence, suggesting that the function of GPR39 signaling is mediated by SIRT1 in chondrocytes. Altogether, our findings implicate that the activation of GPR39 signaling ameliorates IL-1β-induced chondrocyte senescence and the GPR39 agonist TC-G 1008 could have the potential to modulate aging-associated OA.

Introduction

Osteoarthritis (OA) is the most common form of arthritis as well as one of the most common aging-related diseases. OA affects a large amount of the population over 60 [1]. Aging is the most important contributor to the development of OA. In aged joint tissue, matrix degradation often affects the mechanical properties of the joint and causes bone or cartilage damage, which ultimately leads to OA [2]. Cellular senescence of chondrocytes has been closely linked with the pathogenesis of OA. Inflammatory tissue-derived mediators such as cytokines can increase the production of matrix metalloproteinases by chondrocytes, resulting in the degradation of cartilage. Most importantly, inflammatory mediator-associated chondrocyte senescence has a significant contribution to OA [3]. Senescence of chondrocytes possesses a variety of biomarkers, including senescence-associated beta-galactosidase (SA-β-gal), expressions of p21, PAI-1 and p53 [4]. SIRT1, a member of the NAD+-deacetylase family, is important for the aging process [5]. SIRT1 is able to suppress cellular senescence by interacting physically with p53 and inducing deacetylation of p53 [6]. SIRT1 has been found to be reduced in OA chondrocytes. This reduction is linked with increased chondrocyte death in OA cartilage [7,8]. Thus, a better understanding of chondrocyte biology and how to prevent chondrocyte senescence plays a key role in OA treatment.

GPR39 is a conserved orphan GPCR and is distinct from other GPCRs, which makes it a unique drug target. It has been reported that GPR39 can be found in various tissues and involved in various physiological processes, mostly known for its involvement in metabolic regulation [9]. The development of the GPR39 agonist TC-G 1008 provides a new method to research GPR39 [10]. GPR39 has been linked with the pathogenesis of depression and plays a key role in the antidepressant mechanism of action. TC-G 1008 has displayed an antidepressant-like effect by increasing the expression of BDNF [11]. Administration of TC-G 1008 has been reported to improve the survival rate in lipopolysaccharide (LPS)-induced sepsis rodent model by increasing the expression of interleukin (IL)-10 [12]. Recently, GPR39 was reported to be expressed in osteoblasts and play an important role in bone matrix composition and structural integrity [13]. Both chondrocytes and osteoblasts originate from a common osteochondroprogenitor. While chondrocytes secrete cartilage matrix, osteoblasts secrete bone matrix, but they can also transdifferentiate each other [14]. Based on their similarity, we hypothesize that GPR39 would play a role in chondrocytes. In this study, we utilized the availability of TC-G 1008 and investigated the function of this receptor in chondrocyte senescence.

Materials and methods

Reagents

We obtained human-derived IL-1β from R&D Systems and TC-G 1008 from Sigma-Aldrich, St. Louis, MO. IL-1β was dissolved in...
0.1% BSA-PBS solution. 5, 10, and 20 ng/ml IL-1β, and 5 and 10 μM TC-G 1008 were used to treat cells. The human chondrosarcoma cell line SW1353 and colon adenocarcinoma cell line Caco-2 were from ATCC, Manassas, VA, USA. The cell transfection Lipofectamine RNAi Max reagents were from Thermo Fisher Scientific, Waltham, MA, USA.

**Cell culture and treatment experiments**

The primary cells, SW1353 cells, and Caco-2 cells were cultured in DMEM/HAM F12 (Sigma-Aldrich) containing 10% FBS. For IL-1β treatment experiments, chondrocytes were stimulated with IL-1β (5, 10, 20 ng/ml) for 24 h. For TC-G 1008 co-treatment experiments, 5 and 10 μM TC-G 1008 were used to treat cells for 24 h.

**SA-β-gal determination**

After treatment, 0.25% glutaraldehyde was used to fix chondrocytes. Cells were then stained with the SA-β-gal staining buffer at 4°C overnight [15]. After three washes, signals were visualized using a microscope.

**Real-time PCR analysis**

RNA was isolated from cultured chondrocytes using a kit (No.74004, Qiagen, Gaithersburg, MD). Isolated RNA (1 μg) was used to produce cDNA using a kit (#1708840, Bio-Rad, Hercules, CA). For semi-quantitative PCR, GPR39 primers were used to amplify a 110 bp fragment from its total cDNA mix. For real-time PCR analysis, synthesized cDNA and an SYBR green mix kit (Thermo Fisher Scientific, Waltham, MA) were used to assess the total mRNA expression of human GPR39 and GAPDH on the ABI7500 platform. The relative expression of GPR39 was normalized to GAPDH and presented as fold change using the $2^{-ΔΔCT}$ method [16].

**Western blot analysis**

Chondrocytes were lysed with RIPA buffer, 20 μg samples were run on 10% SDS-PAGE [17] and transferred to PVDF membranes, which were then blocked with 5% non-fat milk for 2 h at RT. The membrane was then probed with primary antibodies and HRP-linked secondary antibody. Blots were visualized with an ECL kit (Thermo Fisher Scientific, Waltham, MA).

**Immunoprecipitation**

After stimulation, cell lysates were prepared and centrifuged at 18,000×g. The supernatant was collected. A total of 10 μg p53 antibody or negative vehicle IgG was mixed with 100 μL cell lysates at 4°C overnight. Then, 100 μL Protein A/G PLUS-Agarose was used and incubated for 2 h at 4°C. The beads were washed and dissolved. Samples were used for Western blot analysis.

**Flow cytometry**

After stimulation, the cell cycle of chondrocytes was analyzed using flow cytometry. Briefly, after fixation with ethanol, cells were permeabilized with 0.1% Triton X-100 and stained with propidium iodide [18]. The patterns of cell cycles were counted via flow cytometry.

**SIRT1 knockdown**

To silence SIRT1 in human chondrocytes, we used a SIRT1 siRNA-based approach to target the SIRT1-specific DNA sequence. We delivered SIRT1 siRNA (siSIRT1: 5'-CCACCUG AGUUGGAUGAUA-3') into chondrocytes by treating the cells with Lipofectamine RNAi Max reagents. The transfected cells were allowed to grow for 48–72 to silence SIRT1 expression. The efficiency of knockdown was verified by blotting SIRT1 protein with its specific antibody.

**Statistical analysis**

Experimental data are expressed as means ± SD. Statistical analysis was performed by ANOVA and $p < .05$ was determined to be statistically significant.

**Results**

**GPR39 is expressed in chondrocytes**

To investigate the role of GPR39 in chondrocyte senescence, we examined its expression in different chondrocytes. In our experiment, Caco-2 intestinal cancer cell line was used as a reference, as it is known to express GPR39. Our data show that GPR39 was moderately expressed in both primary chondrocytes and SW1353 cells, and its transcript (Figure 1(A)) and protein (Figure 1(B)) levels in chondrocytes were comparable to those of intestinal cells for mRNA.

**IL-1β represses GPR39 expression in chondrocytes**

Next, we challenged chondrocytes with the cytokine IL-1β and assessed the response of GPR39 to IL-1β. Our data demonstrate that both GPR39 mRNA and proteins were...
repressed when the chondrocytes were treated with the different dosages of IL-1β. At the mRNA level, IL-1β suppressed GPR39 transcription (Figure 2(A)). The same doses of IL-1β had a similar suppressive effect on GPR39 protein expression (Figure 2(B)).

**GPR39 agonist TC-G 1008 mitigates IL-1β-induced chondrocyte senescence**

Age-associated chondrocyte senescence is a major risk factor for OA. We investigated whether the change in GPR39 expression is involved in chondrocyte senescence. To correct the reduction in GPR39 expression in the IL-1β experiment, we co-treated the chondrocytes with the specific GPR39 agonist TC-G 1008 and compared the senescence population using SA-β-gal marker staining. We took the representative images and calculated the percentage of SA-β-gal-positive cells in different experiments. Compared to non-treated cells, 24 h IL-1β treatment induced roughly more than 2.5-fold the number of SA-β-gal positive cells. Meanwhile, co-treatment with the two doses of TC-G 1008 (5, 10 μM) for the same length of time significantly suppressed this induction (Figure 3).

**TC-G 1008 attenuates IL-1β-induced G1 phase cell cycle arrest**

One of the hallmarks of senescent chondrocytes is the loss of replicative potential. We assessed the profile of cell cycles under the different treatment conditions using IL-1β and TC-G 1008. As shown in Figure 4, G1 phase cells accounted for about 50% of the total population of non-treated chondrocytes, while IL-1β induced about 70% G1 phase cell arrest. Co-treatment with the two doses of TC-G 1008 significantly ameliorated G1 phase arrest induced by IL-1β. Correspondingly, TC-G 1008 treatment ameliorated IL-1β-induced shortening of the S and G2/M phases.

**TC-G 1008 suppresses IL-1β-caused induction of p53, p21, and PAI-1**

Next, we examined the effect of GPR39 activation by TC-G 1008 on cell cycle proteins. As shown in Figure 5(A), IL-1β exposure induced about 4–6 fold expression of p53, p21, and PAI-1 at the mRNA levels, which was inhibited by TC-G 1008. Consistently, IL-1β treatment induced about 3–4 fold expression of p53, p21, and PAI-1 proteins, but co-treatment with the two doses of TC-G 1008 significantly inhibited this
increase (Figure 5(B)). These data indicate that the amelioration of IL-1β-induced G1 phase arrest by TC-G 1008 could be mediated via inhibition of key regulatory proteins.

**TC-G 1008 inhibits IL-1β-induced K382 acetylation of p53**

We further explored the molecular mechanism of TC-G 1008 on p53 protein expression. As previously reported, the post-transcriptional modification of p53 including acetylation is vital to its capacity to guide the cell cycle. We assessed the acetylation level of p53 K382 in this scenario. As shown in Figure 6 and referenced to non-treated chondrocytes, IL-1β induced roughly more than 3-fold K382 acetylation of p53. However, the co-treatment experiment revealed that TC-G 1008 almost abolished this increase by IL-1β, with the higher dose being more effective.

**TC-G 1008 mitigates the inhibition of IL-1β on SIRT1**

Finally, we explored the co-treatment effect of the GPR39 agonist and IL-1β on the SirT1 family protein SIRT1, which is responsible for the deacetylation of p53. As a well-established aging-related protein, SIRT1 is important for the senescence of chondrocytes. Compared to non-treated chondrocytes, IL-1β inhibited about 70% of the total SIRT1 protein. However, co-treatment with the two doses of TC-G 1008 mitigated this inhibition (Figure 7(A)). We then investigated the influence of SIRT1 silencing in this experiment. Our knockdown experiment showed that roughly 60% of SIRT1 was silenced (Figure 7(B)). We assessed the p53 acetylation level and SA-β-gal-positive populations in SIRT1-silenced chondrocytes. When compared to non-treated cells, IL-1β still led to about 3-fold induction of p53 K382 acetylation and co-treatment with TC-G 1008 reversed this increase in SIRT1-silenced chondrocytes. However, co-treatment with 10 μM TC-G 1008 did not show any influence on the senescent population in SIRT1-silenced chondrocytes (Figure 7(D)).

**Discussion**

Our understanding of the pathophysiological mechanism of OA remains limited. Common treatment options are unable to cure the disease completely. In our effort to study the molecular mechanism of chondrocytes and their involvement in OA, we found that GPR39 activation by its agonist TC-G 1008 could mitigate IL-1β-induced chondrocyte senescence. GPR39 activity has been reported in epithelial and neuronal cells and zinc ion has been identified as the endogenous agonist of GPR39 [19,20]. TC-G 1008 has a chemical name of 2-pyridylpyrimidines and was originally developed to enhance GLP-1 levels in type 2 diabetic conditions. In macrophages, GPR39 exhibits anti-inflammatory capacity through increasing IL-10 production. TC-G 1008 administration in mice reduces IL-10 induction by stimulated peritoneal macrophages [12]. In epithelial cells, treatment with either TC-G
1008 or zinc ions increases tight junction assembly [21]. Recent studies show that TC-G 1008 has an anti-depressive-like effect [11,22]. This evidence indicates that GPR39 activity has pleiotropic effects in different cell types and TC-G 1008 appears to be a very efficient agonist of GPR39 signaling.

Our study reveals new roles of GPR39 agonist in chondrocytes. These findings implicate that TC-G 1008 is a beneficial factor that can suppress IL-1β-induced chondrocyte senescence. Firstly, we show that the presence of TC-G 1008 suppresses SA-β-gal activity in IL-1β-induced chondrocyte senescence. SA-β-gal activity is an essential hallmark of cellular senescence [23] and the inhibitory effect of TC-G 1008 in chondrocyte senescence shows that inflammation-associated chondrocyte senescence could be mitigated by GPR39 activation, implying potential new roles for this compound in modulating cellular senescence. Secondly, the action of TC-G 1008 on IL-1β-induced chondrocyte G1 phase arrest and key cell cycle proteins are connected. TC-G 1008 makes senescent chondrocytes to regain their replicative capability but also recovers decreased p53, p21, and PAI-1 expression to normal levels. Upregulation of these proteins is a critical step for the induction of senescence [24]. It is possible that the amelioration of the altered cell cycle in senescent chondrocytes by TC-G 1008 could be mediated by its recovery of p53, p21, and PAI-1 proteins. Thirdly, our study demonstrates that the modulation of TC-G 1008 on SIRT1 and p53 acetylation is connected. It has been shown that SIRT1 is the critical regulator of p53 deacetylation and the downregulation of SIRT1 could cause lower acetylation levels of p53 [25]. Thus, the mitigation of TC-G 1008 on IL-1β-induced p53 acetylation could be mediated by its restoration of IL-1β-mediated SIRT1 reduction. Moreover, we verify that SIRT1 expression is required for the anti-senescence effect of TC-G 1008 in chondrocytes, as our data show that deficiency of SIRT1 abolishes the effect of TC-G 1008 on p53 acetylation and SA-β-gal activity. SIRT1 is a well-known regulator of senescence, aging, and longevity.

Figure 7. The specific GPR39 agonist TC-G 1008 restores IL-1β-induced inhibition of SIRT1. (A) Cells were treated with IL-1β (10 ng/ml) or TC-G 1008 (5, 10 μM) for 24 h. Expression of SIRT1 was determined by Western blot analysis; (B) Cells were transfected with SIRT1 siRNA. Successful inhibition of SIRT1 was revealed; (C) Blockage of SIRT1 abolished the inhibitory effects of TC-G 1008 in p53 K382 acetylation; (D) Silencing of SIRT1 abolished the inhibitory effects of TC-G 1008 on SA-β-Gal staining (*, #, $, p < .01 vs. previous control group).
In chondrocytes, SIRT1 is required for the differentiation of chondrocytes from progenitor cells [27]. The modulation of the SIRT1-p53 acetylation axis by TC-G 1008 reveals that SIRT1 is required in GPR39 signaling-mediated suppression of chondrocyte senescence and other survival mechanisms. We propose that GPR39 can sense extracellular senescence signals and deliver messages to call on the SIRT1-p53 signaling pathway to protect chondrocytes from senescent stimuli.

In summary, the activation of GPR39 signaling by its agonist TC-G 1008 shows promising protection in chondrocytes. As a recent study showed that GPR39 actually is expressed in osteoblasts and is required for their differentiation and that GPR39 knockout mice have weaker bones as a result of altered bone matrix composition and structure [13]. Therefore, we conclude that GPR39 signaling plays an indispensable role in the maintenance of joint tissue. Our study implies that the GPR39 activation-mediated protection by its agonist TC-G 1008 compound has beneficial roles in countering chondrocyte senescence and the activation of the GPR39 pathway provides a unique therapeutic target in the treatment of OA and bone-related diseases. Further, mechanistic and animal model research would shed more light on the therapeutic potential of TC-G 1008 in joint tissue diseases.

Disclosure statement
No potential conflict of interest was reported by the authors.

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