Original Article
Effects of gamma-glutamyl carboxylase gene overexpression on the differentiation of chondrocytes from osteoarthritis rabbits

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Abstract: Background: Gamma-glutamyl carboxylase (GGCX) was apparently decreased in cartilage tissue from OA patients. However, whether GGCX overexpression has functions in regulating chondrocytes from osteoarthritis (OA) model is still unknown. Objective: This study aimed to explore the effects of GGCX gene overexpression on cell differentiation of chondrocytes from osteoarthritis (OA) rabbits, and to assess the mechanisms. Methods: Chondrocytes were isolated from OA rabbit and identified by Alizarin red staining. The isolated chondrocytes were divided into three groups: normal control, GGCX overexpression and vehicle. Lenti-viral encoding GGCX vector was applied to overexpress GGCX expression. Apoptosis of the cells after viral transfection was detected by flow cytometry. GGCX, matrix metalloproteinase 13 (MMP13), type X collagen, type II collagen, tumor necrosis factor (TNF) and interleukin-1β (IL-1β) were detected by real-time PCR and western blot. Results: Compared with vehicle, lenti-viral encoding GGCX significantly elevated GGCX expression in OA chondrocytes in both mRNA and protein levels (P<0.05). Subsequently, GGCX overexpression remarkably increased type II collagen, while decreased MMP13, type X collagen, TNF and IL-1β expression in both mRNA and protein levels (P<0.05). Especially, GGCX overexpression prohibited the apoptosis of OA chondrocytes compared with vehicle (P<0.05). Conclusion: GGCX overexpression could regulate the balance of extracellular matrix, promote proteoglycan synthesis in chondrocytes, which might be related to the decreased apoptosis. This study provides new therapeutic concept for osteoarthritis.

Keywords: Chondrocytes, GGCX overexpression, type II collagen, type X collagen

Introduction

Primary osteoarthritis (OA) is manifested by limited joint mobility, joint pain and joint bone hyperplasia. Various forms of treatments, such as non-steroidal anti-inflammatory drug therapy and glucosamine capsules were applied in the treatment of OA [1, 2]. However, these methods are featured by temporary relief of symptoms [3]. Therefore, the treatment of osteoarthritis of knee joint is now becoming an urgent problem.

Cartilage is a type of highly-differentiated tissue, including chondrocytes and extracellular matrix. Cartilage matrix was mainly composed of collagen fibers, proteoglycans, water and other [4]. In the embryonic stage, mesenchymal cells begin to coalesce with each other, further differentiate into chondrocytes and secrete cartilage matrix, finally promoting the formation of cartilage tissue [5]. The volume of chondrocytes trended to increase, and entered the stage of hypertrophic differentiation after maturation, accompanied by increased type X collagen, and decreased type II collagen. Hypertrophic chondrocytes are characterized by increased secretion of type X collagen and matrix metalloproteinases (MMPs) [6]. In the hypertrophic cartilage, 45% of the total collagen was type X collagen, which therefore is regarded as an important marker in endochondral ossification [7, 8].

Gamma-glutamyl carboxylase (GGCX) is a key enzyme in the process of carboxylation of MGP and physiologically activated by vitamin K to perform its function [9]. Uncarboxylated MGP
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Table 1. The primers for the genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>GGCX</td>
<td>TATGTCATGCTGGCCAGCA</td>
<td>TGAAGAGGCGCTCCACGCT</td>
</tr>
<tr>
<td>MMP13</td>
<td>GGAAGACTTCCAGTGGGAGCAG</td>
<td>CGGATCGCTGGTTGTCTTCA</td>
</tr>
<tr>
<td>Type X collagen</td>
<td>AAGCTTACCCAGCGTGGAGG</td>
<td>CAAACCAGATCCAGGCAGCA</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>CGGTCCACTTCCATTCCCCAGA</td>
<td>CAACACAGGTGGTAAGTAGCATGAT</td>
</tr>
<tr>
<td>TNF</td>
<td>CTGAGATATGATCCCGGGA</td>
<td>GCCACAGGGTTGACTAGAT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>ATGACCTGTATCTTGGAGGCC</td>
<td>TGTGGGCAGGAACGTGCTT</td>
</tr>
<tr>
<td>β-actin</td>
<td>AGTGCCAGCTGAGCATCCTC</td>
<td>TGCGCTCAACAGTCGGCCCTAG</td>
</tr>
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</table>

 ucMGP) is elevated in OA patients [10] and GGCX performs critical function in carboxylating MGP. Moreover, GGCX was apparently decreased in cartilage tissue from OA patients [11, 12]. In this study, we transferred lentivirus encoding GGCX to chondrocytes and aimed to investigate the protective effects of GGCX overexpression on the differentiation of OA chondrocytes. This study would provide a theoretical basis for the application of GGCX overexpression in the treatment of osteoarthritis.

Materials and methods

Cell isolation and identification

Chondrocytes were isolated from OA rabbits as previously demonstrated [13]. After culturing, the cells were identified by Alizarin red staining. The animal use and experimental protocol were under the approval of Ethics Committee of Nanchang University.

Groups and treatments

OA chondrocytes were divided into normal control, vehicle control and GGCX overexpression groups. The transfection of lentivirus was conducted as previously described [14]. Briefly, OA chondrocytes were cultured in six-well plates in a density of 10^5/well. When the cell confluence reached 80%, lentivirus with GGCX vector or vehicle vector were added into the wells. Six h later, the medium was refreshed into normal DMEM (10% serum). After 48 h culturing in CO_2 incubator, the cells were collected and western blotting and real-time PCR were applied to detect the expression of GGCX, MMP13, type X collagen, type II collagen, tumor necrosis factor (TNF) and interleukin-1β (IL-1β). Apoptosis of the cells after viral transfection was detected by flow cytometry.

Flow cytometry

Cells underwent trypsinization and collection. After centrifuge in 1000 r/min for 3 min, the cells were washed by PBS and fixed in 75% iced ethanol. PI and Annexin V-FITC were added into 200 μl cell suspension. After incubation in room temperature for 45 min, the cells were detected by flow cytometry (BD, Franklin Lakes, New Jersey, USA) and data were analyzed by Cell Quest Software (BD, USA).

Real-time PCR

Total RNA was extracted from the cells using Trizol reagent. RNA concentrations were determined by spectrophotometrical method, and 1 μg total RNA was reversely transcribed by an avian myeloblastosis virus reverse-trascrip-tase kit (Promega, Madison, WI, USA). The expressions of target genes were normalized to β-actin. The primers were listed in Table 1.

PCR was carried out using a 7500 real-time PCR system (Applied Biosystems), with initial hold step (95°C for 10 min) and 40 cycles of a two-step PCR (95°C for 15 s and 60°C for 1 min). The amount of target gene expression was normalized to GAPDH and calculated following a calibrator (2^-ΔΔCt) as previously described [15].

Western blot

Protein was abstracted from the cells for western blotting as previously described [16]. The antibodies, including anti-GGCX, MMP13, type X collagen, Type II collagen, TNF and IL-1β (1:3000) (Abcam, USA) were incubated overnight at 4°C. After washing, the membranes were incubated with the second antibody conjugated with horseradish peroxidase. ECL reagent kit was applied to assist the staining. The blots were scanned by ChemiDocTM XRS (Bio-Rad, USA) and grey density was analyzed by Image J 7.0 software.

Statistical analysis

Data were presented as means ± standard deviations. One-way analysis of variance with
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Results

Structural changes of the chondrocytes after GGCX overexpression

As shown in Figure 1, the cells in control group adhered to the wells in a fibroblast-like pattern, and in spindle, triangular and squamelliform shapes. Among them, spindle and triangular shapes were typical. Nuclei were oval and located in central with clear boundary. One or two nucleoli were visible and no calcium salt deposition was observable. However, the transfected cells displayed classical cell nodules stained in orange red with blurry cell boundaries.

GGCX overexpression prohibits apoptosis of chondrocytes

As shown in Figure 2, viral encoding GGCX could promote GGCX expression in chondrocytes after 48 h incubation in both mRNA and protein levels. As shown in Figure 3, apoptotic rate in control group was about 26%. GGCX overexpression but not vehicle could apparently decrease the apoptosis (GGCX overexpression: 18%; Vehicle: 27%, P<0.05).

GGCX overexpression decreases the expression of MMP13, type X collagen, TNF and IL-1β, but promotes the expression of type II collagen

GGCX overexpression significantly decreased the expression of MMP13, type X collagen, TNF and IL-1β in mRNA level (P<0.05) (Figure 4), while promoted the expression of type II collagen in mRNA level (P<0.05) (Figure 4). As shown in Figure 5, GGCX overexpression significantly decreased the expression of MMP13, type X collagen, TNF and IL-1β in protein level (P<0.05), while promoted the expression of type II collagen in protein level (P<0.05) (Figure 5).

Discussion

Chondrocytes could secret extensive type II collagen to support their survival or differentiation [17]. However, abnormal chondrocytes in osteoarthritis patients were in hypertrophic phenotype and the ability to secret type II collagen was reduced. By contrast, the ability to secret type X collagen was facilitated in that type of cells [18]. As reported, type X collagen is rarely observed in normal cartilage tissue, but extensively detected in OA tissue [19]. In this study, we isolated chondrocytes from OA cartilage tissues. Genetic method was applied to overexpress GGCX expression.
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Lenti-virus encoding GGCX could apparently facilitate GGCX expression. After transfection with GGCX vector, morphologic of chondrocytes remarkably recovered to normal condition.

Phenotype of chondrocytes was also distinguished by staining type II collagen and type X collagen. Our data displayed that GGCX overexpression significantly increased type II collagen.

Figure 3. GGCX overexpression decreases apoptosis of chondrocytes. *P<0.05 compared with vehicle.

Figure 4. GGCX overexpression promotes type X collagen, decreases MMP-13, type II collagen, TNF and IL-1β expression in mRNA level.
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Figure 5. GGCX overexpression promotes type X collagen, decreases MMP-13, type II collagen, TNF and IL-1β expression in protein level.

In addition, studies have shown that the synthesis and secretion of type X collagen may lead to chondrocyte apoptosis in articular cartilage [20]. Chondrocytes encountering pathological stimuli in osteoarthritis would become hypertrophic phenotype. Cellular redox state was altered and signaling pathway for apoptosis was activated, finally contributing to apoptosis of chondrocyte [21]. Amling et al demonstrated that reduction of type X collagen could prohibit the apoptosis of chondrocytes [22]. In our study, apoptosis was detected after GGCX overexpression in OA chondrocytes. After transfection with GGCX, apoptotic rate was significantly reduced. As type X collagen is also decreased after GGCX overexpression, the apoptosis-inhibition is likely through inhibiting the synthesis of type X collagen [23].

The stability of extracellular matrix in cartilage is important for chondrocyte survival, and matrix metalloproteinases (MMPs) are important elements to stabilize cell matrix. Destruction of cartilage is also due to the abnormality of MMPs. MMPs in osteoarthritis were significantly promoted as reported [24]. The degradation of collagen type II in the matrix leads to the destruction of MMPs, while MMP13 is one of the typical MMPs. Therefore, MMP13 has become one of the most commonly detected indicators in the pathogenesis of osteoarthritis [25]. Our study also examined MMP13 expression using real-time PCR and Western blot. We found that overexpression of GGCX significantly decreased MMP13 expression, revealing that GGCX overexpression likely improves chondrocyte survival by down-regulating MMP13 expression.

Studies have shown that IL-1β can affect the synthesis of extracellular matrix components and structural proteins, such as proteoglycans and type II collagen [26, 27]. In addition to the effect on structural protein synthesis, IL-1β can also affect MMPs, especially MMP13, resulting in the destruction of chondrocytes [28, 29]. In addition to the induction by MMPs family enzymes, MMP5 could be regulated by IL-1β and TNF, to promote the expression of nitric oxide and production of nitric oxide synthase, reduce proteoglycan synthesis, thereby inducing apoptosis of chondrocytes [30, 31]. The results of this study also demonstrated that the expressions of IL-1β and TNF were down-regulated after GGCX overexpression.

In normal cartilage tissue, extracellular matrix are decomposed and synthesized in a dynamic balance. By contrast, type II collagen was reduced, while type X collagen was facilitated in the abnormal chondrocytes. Meanwhile, the amount of MMPs was also increased, as well as IL-1β and TNF. Kamekura et al [32, 33] found that expressions of MMP13 and type X collagen were significantly increased in the osteoarthritis model [34]. Overexpression of IL-1β and TNF would decrease proteoglycan synthesis, thereby inducing cell apoptosis and destruction of cartilage, and contributing to osteoarthritis.

In conclusion, lenti-viral encoding GGCX could improve the balance of extracellular matrix, promote proteoglycan synthesis in chondrocytes, which might be related to the decreased apoptosis. This study implicates that GGCX could serve a therapeutic target for osteoarthritis.

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Disclosure of conflict of interest

None.
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