Original Article

Effect of puerarin on TIMP3, MMP-9 expression and methylation in chondrocytes of rat osteoarthritis

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Abstract: Puerarin has potential protective effects on osteoarthritis, but leaving the detailed mechanism unknown. Methylation level at the promoter region of genes has been found to be related with osteoarthritis. Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-3 (TIMP-3) have been found to be related with chondrocyte injury. This study thus investigated if puerarin may affect the methylation level at promoter region of these genes to decrease matrix degradation and protect chondrocytes. Rat osteoarthritis model was generated by intraarticular cavity injection of iodacetic acid, and treated with Puerarinat the second day. Two weeks later, chondrocyte tissues were collected from keen joint and quantified for protein and mRNA levels of TIMP-3 and MMP-9 using Western blotting and real-time quantitative PCR, respectively. Genomic DNA was processed by sodium bisulfite, and was tested for promoter region methylation of TIMP-3 and MMP-9 genes. Puerarin can up-regulate TIMP-3 and down-regulate MMP-9 levels in injured chondrocytes. Methylation specific PCR showed the correlation between such modulation and the methylation of promoter regions at TIMP-3 and MMP-9 genes. Puerarin can alter the methylation level of TIMP-3 and MMP-9 genes, thus decreasing degradation of cellular matrix and exerting protective role.

Keywords: Puerarin, osteoarthritis, methylation, TIMP-3, MMP-9

Introduction

Osteoarthritis is one degenerative osteoarticular disease in aged people. It can be caused by multiple reasons but with unclear pathogenesis so far. It is most commonly manifested as damage of articular chondrocytes, thickening and sclerosis of subchondral bone and osteophyte formation at articular peripheral regions. Current studies have revealed the higher activity of matrix metalloproteinases (MMPs) which are caused by inflammation as important factors during progression of osteoarthritis. MMP-9 is one enzyme that was closely correlated with osteoarthritis, and can promote chondrocyte damage via collagen degradation, and facilitate angiogenesis in osteoarthritis tissues inducing pannus [1]. Tissue inhibitor of metalloproteinase-3 (TIMP-3) is one insoluble protein that can bind with extracellular matrix, and a specific inhibitor for MMP-9 to suppress its expression, and thus having protective effects on chondrocyte injury.

Puerarin is one effective compound of isoflavone facility extracted from Pueraria lobate (Willd.). Current studies revealed its potency for improving immune function [2], enhancing cardiac function, protecting cardiomyocytes, anti-tumors [3, 4], suppressing pressure and anti-coagulation of platelets [5]. In addition, puerarin also had satisfactory anti-inflammation activity in both in vivo and in vitro studies [6, 7]. The down-regulation of MMP-9 has obtained significant effects in decreasing bone absorption and facilitating bone formation [8, 9].

Recent studies have revealed the critical roles of MMP-9 and TIMP-3 gene expression and epigenetic modification in the pathogenesis of osteoarthritis. The roles of puerarin in methylation status of TIMP-3 and MMP-9 genes, and its correlation with expression regulation of TIMP-3 and MMP-9 in chondrocytes of osteoarthritis are still unclear. This study thus generated an osteoarthritis rat model by intraarticular cavity of iodacetic acid, and investigated the effect of
Puerarin in osteoarthritis

All animals were divided into four groups: normal control, puerarin, model and model + puerarin. All animals were fasted 12 hours before surgery. Animals were anesthetized by 3% sodium pentobarbital (30 mg/kg). Rats were then fixed in a supine position, and received 0.1 mL iodacetic acid (1 mg) while equal volume of saline water used. Puerarin was given by intra-gastric cannulation starting from the second days after surgery. Equal volumes of saline were given to control animals.

Sample collection

Two weeks after treatment, all rats were sacrificed and knee arthritis chondrocytes were removed, which were frozen at -80°C for further usage.

Western blotting

100 mg chondrocyte samples were homogenized in liquid nitrogen and mixed with 1 mL RAPI lysis buffer for 15-min incubation. After centrifugation at 12,000 g for 10 min, supernatants were saved to quantify protein concentration using BCA method. Protein samples were mixed with 2× loading buffer and denatured in boiling water for 5 min. Proteins were separated in SDS-PAGE and then transferred to PVDF membrane. The membrane was blocked in 5% defatted milk powder for 2 hours at room temperature. Primary antibody against TIMP-3 or MMP-9 (1:1,000) was added for overnight incubation at 4°C. On the next day, the membrane was washed in TTBS for 3 times (10 min each). HRP-labeled goat anti-rabbit secondary antibody was added for 2-hour incubation at room temperature. After TTBS washing (3 times ×10 min), ECL method was used to develop the membrane. The florescent intensity was recorded for analysis.

Expression level of mRNA

Chondrocytes were homogenized in liquid nitrogen, and mixed with lysis buffer for 5-min iced incubation. Lysates were mixed with 0.2 mL chloroform for 15-sec vortex followed by 4°C centrifugation for 15 min. The upper phase was saved and mixed with 0.5 mL isopropanol. Under room temperature, the mixture was incubated for 10 min and centrifuged for 10 min.

Materials and methods

Reagents and drugs

Puerarin was purchased from Aladdin (Shanghai, China) with its structure shown in Figure 1. Iodacetic acid was purchased from Sigma (LA, US). Real-time quantitative PCR kit was produced from TransGen Biotech (Beijing, China). Genomic DNA Isolation Kit was purchased from Biovision (LA, US). Methylation Universal kit was produced by Qiwu Bioscience (Shanghai, China). Primers for PCR, methylation specific PCR, and real-time quantitative PCR kit were provided by TaKaRa (Dalian, China).

Animals

A total of 40 male SD rats (2 months old, body weight 200 to 250 g) were provided by Laboratory Animal Research Center, Peking University.

puerarin on methylation level of TIMP-3 and MMP-9 gene promoter, in addition to the protective effect against osteoarthritis-induced chondrocyte injury.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>TIMP-3</td>
<td>Forward: 5’-GCCTTCTGCAACTCGACATC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-CGTGTGACCTTGCCATCATA-3’</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Forward: 5’-CAACATCACTATGGATCC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-TGGGTAGAGTCTCTCGCT-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5’-CGCGAGAAGATGACCCAGAT-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’-GCACCTGTGGGCGTACAGG-3’</td>
</tr>
</tbody>
</table>

Figure 1. Chemical structure of puerarin.
The supernatants were discarded, with washing by 1 mL ethanol for three times. The RNA pellet was re-suspended in 20 μL DEPC water.

Primers for TIMP3, MMP-9 and β-actin as previously recorded [10, 11] were synthesized by Sigma (US) as shown in Table 1. PCR reaction was performed in a 50 μL system following manual instruction of test kit. PCR parameters were: 55°C for 30 min, followed by 95°C denature for 5 min, and 40 cycles each containing 95°C denature for 30 sec, 55°C annealing for 30 sec and 72°C elongation for 50 sec. The reaction ended with 72°C elongation for 5 min. Real-time PCR amplification curve was plotted. The gene expression level was determined by 2-ΔΔCt method, in which Ct values of target gene and internal reference gene were compared. The expression level in control group was set as 1.0, for comparing the expressional profile of model and drug treatment groups.

**Methylation specific PCR**

Genomic DNA was extracted using test kits. After modification by methy-sulfite, methylation specific PCR primers as reported [12] were synthesized by Toyobo. Sequences were: Methyl-TIMP-3-F, 5’-CGTTT CGTA TT TTTT TCGGT TC-3’; Methyl-TIMP-3-R, 5’-CCGAAA AACCC CGCCT CG-3’; Non-Methyl-TIMP-3-F, 5’-TTTTG TT TTTT TTATT TTTTT GGTGT T-3; Non-Methyl-TIMP-3-R, 5’-CCCCC AAAAA CCCCA CCTCA-3’; Methyl-MMP-9-F, 5’-GAAGT TCGAA ATTATG TTTGG TTAAC-3’; Methyl-MMP-9-R, 5’-TCCCG AAATAA CTATATTATTAA ACC GTT TAAAG TTATTT TATAT -3’; Non-Methyl-MMP-9-F, 5’-AGTTT GAAAT TAGTT TGGTT AATGT-3’; Non-Methyl-MMP-9-R, 5’-CC-TCC CAAAT AACTA ATAT TATATCAATA-3’. Amplification was performed on those chondrocytes with or without methylate enzyme under the following conditions: 95°C pre-denature for 5 min, followed by 40 cycles each containing 95°C denature for 30 sec, 60°C annealing for 30 sec and 72°C elongation for 50 sec. All experiments were performed in triplicates.

**Statistical methods**

SPSS 19.0 software was used to analyze all collected data. Each experiment was conducted in at least triplicates. Measurement data were presented as mean ± standard deviation (SD). Between-group-comparison was performed in student t-test. One-way analysis of variance (ANOVA) was used for multiple group comparison, followed by post-hoc Dunnt t test. A statistical significance was defined when P<0.05.

**Results**

Puerarin regulates TIMP-3 and MMP-9 gene expression

Model rats had down-regulated TIMP-3 expression and elevated MMP-9 expression. After puerarin treatment, TIMP-3 level was elevated while MMP-9 level was decreased (P<0.05, Figure 2).
Puerarin in osteoarthritis

Effect of puerarin on mRNA levels of TIMP-3 and MMP-9

Puerarin up-regulated TIMP-3 mRNA level, which was suppressed in osteoarthritis model rats, but down-regulated MMP-9 mRNA that was enhanced in model animals (P<0.05, Figure 3).

TIMP-3 and MMP-9 methylation status

Using methylation specific PCR, we measured the methylation level of promoter regions of TIMP-3 and MMP-9 genes. Compared to normal group, model rats had significantly elevated TIMP-3 expression level, while MMP-9 methylation level was significantly depressed (P<0.05, Figure 4). The intervention of puerarin partially reversed such trends.

Discussion

Puerarin is natural active compound extracted in Pueraialabata (wild) ohwi of Leguminosae family. There are more than 20 different plants containing puerarin, and are mainly distributed in sub-tropical Asia. About one dozen of such plants have been found in China. The pharmaceutical role of puerarin has been studied well, as its can inhibit MMP-1 expression in hylth-induced rabbit osteoarthritis model, and can inhibit the degradation of collagen in extracellular matrix, in addition to the anti-inflammatory role by NF-κB signaling pathway. In clinics, the combined treatment using puerarin and intraarticular activity injection of steroid drugs can improve the long-term efficacy of osteoarthritis.

Epigenetics modification of DNA can be regulated by external environment regarding mutated sites and levels, thus making it as one acquired inheritance. Study has proved the correlation between promoter methylation in various genes and the occurrence of osteoarthritis [13, 14]. In this study, we investigated the role of puerarin on protein levels of TIMP-3 and
MMP-9 in impaired chondrocyte tissues, and illustrated the possible involvement of puerarin in modulating methylation level of promoter regions in TIMP-3 and MMP-9 genes for regulating gene expression.

TIMPs consist of TIMP-1, 2, 3 and 4. TIMP-3 is located on the membrane and can bind with extracellular matrix for adhesion onto basal membrane, thus reducing matrix degradation caused by MMP-9 up-regulation [15]. TIMP-3 is expressed in chondrocytes from both normal people and osteoarthritis patients. In addition to inhibiting MMP-9 induced cellular matrix degradation, it can also facilitate the basal matrix synthesis and mitosis, as well as regulate cell differentiation and suppress apoptosis [16]. Other studies have also found the enhancement of osteoarthritis susceptibility by TIMP-3 gene knockout [17].

MMPs are members of zinc-dependent endopeptidase, and can degrade different components of extracellular matrix including core proteins of proteoglycan, fibronectin, gelatin, elastin and interstitial collagen, thus breaking the network structure of collagen and making chondrocytes attacked by inflammatory cytokines for inducing apoptosis and eventually chondrocyte breakage [18]. The regulatory mechanisms of MMPs include transcriptional modification, potential activation of MMPs and inhibition of TIMPs. Recent studies found the important role of epigenetic control in mediating MMPs level and the occurrence of osteoarthritis [19]. Previous study has reported the demethylation of 104-CpG island in MMP-13 gene promoter region in osteoarthritis patients and consequent elevation of MMP-13 expression and chondrocyte degradation [20].

Osteoarthritis is caused by various biological factors, inflammatory cytokines and free radicals. The major adverse effect is the disruption of joint homeostasis and dysfunction. MMPs elevation and degradation of collagen are critical steps in the disease progress and major targets for drugs. This study tested both protein and mRNA levels of TIMP-3 and MMP-9 in damaged articular cartilage tissues, in addition to the promoter methylation level. Our results demonstrated the modulation of puerarin on methylation level of promoter regions of TIMP-3 and MMP-9 genes for regulating gene expression level, thus providing protection on damaged chondrocytes in osteoarthritis. This study provided further evidences for clinical treatment of osteoarthritis using puerarin.

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Disclosure of conflict of interest

None.

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