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Original Contribution

Knockdown of peroxiredoxin 5 inhibits the growth of osteoarthritic chondrocytes via upregulating Wnt/β-catenin signaling

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ABSTRACT

Peroxiredoxin 5 is a member of the peroxiredoxin family, which has been shown to act as an antioxidant whose main function is to reduce reactive oxygen species in cells. Peroxiredoxin 5 has been found to be abnormally elevated in human osteoarthritic chondrocytes. However, the detailed mechanism by which peroxiredoxin 5 modulates human osteoarthritic chondrocytes' survival has not been elucidated. In the current study, we demonstrated that peroxiredoxin 5 knockdown activated osteoarthritic chondrocytes apoptosis, and decreased scavenging of endogenous reactive oxygen species. Furthermore, silencing of peroxiredoxin 5 resulted in an altered expression of proteins associated with Wnt signaling. Collectively, these results demonstrated that the regulatory effects of peroxiredoxin 5 can be partially attributed to Wnt/β-catenin signaling.

Introduction

Osteoarthritis (OA) is the most prevalent articular disorder and it is increasingly becoming a major cause of disability in aged people. Multiple factors are believed to cause OA, such as trauma, abnormal mechanical loading, failure of nutrient supply, and genetic predisposition [1]. Current available drugs to treat OA are predominantly directed toward the symptomatic relief of pain and inflammation but they do little to reduce joint destruction [2]. Therefore, the discovery of molecules essential to the initiation and progression of OA as well as new therapeutic strategies to treat OA are important for improving the prognosis and therapy of OA patients.

The pathological processes responsible for OA initiation and progression are very complex and poorly understood, despite the extensive research efforts into this complex disease. Several studies have revealed that reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals, and nitric oxide can contribute to the onset and progression of OA by inducing chondrocyte death and matrix degradation [3–5]. To prevent toxicity by ROS, chondrocytes possess a well coordinated antioxidant enzyme system formed by superoxide dismutase (SOD), catalase, and glutathione peroxidase. Recently, a novel family of peroxidases, the peroxiredoxins (Prdxs), was identified in many living organisms. Six isoforms of Prdxs have been identified in mammals, all of which participate directly in eliminating H_2O_2 and neutralizing other oxidizing chemicals. Peroxiredoxin 5 (Prdx5) is a thioredoxin peroxidase which is highly expressed in many tissues [6]. Like other 2-Cys peroxiredoxins, Prdx5 requires a thioredoxin [7] as a reducing partner. Prdx5 is upregulated in degenerative human tendon and its overexpression protects human tendon cells against apoptosis [8,9]. Normal human cartilage also constitutively expresses Prdx5 [10,11]. Moreover, Prdx5 is found to be elevated in OA cartilage and its expression is upregulated by IL-1 and TNF-α. This IL-1/TNF-α stimulating effect is fully inhibited by catalase, indicating that H_2O_2 might be an important mediator for the cytokine-induced Prdx5 upregulation. It has been suggested that Prdx5 may play a protective role against oxidative stress involved in the pathogenesis of OA, and may have therapeutic value in the prevention and treatment of OA [12]. Despite these insights, the biological functions of Prdx5 in osteoarthritic chondrocytes have not been fully characterized, and the underlying mechanisms remain poorly understood.

Wnt signaling constitutes one of the most critical biological processes during cell fate assignment and homeostasis [13]. It is essential for bone homeostasis and its effects in chondrogenic differentiation and cartilage formation are complex [14]. Recent studies further suggest the relevance of Wnt signaling in human

Abbreviations: Prdx5, peroxiredoxin 5; ROS, reactive oxygen species; OA, osteoarthritis; H_2O_2, hydrogen peroxide; SOD, superoxide dismutase; Prdxs, peroxiredoxins; DMEM, Dulbecco's modified Eagle's medium; NAC, *N*-acetyl-L-cysteine; 7-AAD, 7-amino-actinomycin D; MTT, 3-(4, 5-dimethylthiozol-2yl)-2, 5-diphenyltrazolium bromide

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OA [15–17]. Because Wnt pathways are involved in both cartilage and bone formation that include a role in regulation of chondrocyte hypertrophy in the growth plate, dysregulation of Wnt pathways in adult tissues could contribute to the chondrocyte hypertrophy seen in OA and pathologic changes in cartilage and bone [18]. Wnt/β-catenin signaling is described as part of the canonical Wnt pathway [19]. β-Catenin is a key element in driving Wnt/β-catenin signaling [20]. In humans, 10 frizzled receptors and at least 19 Wnt proteins are known. For instance, Frizzled-2 is expressed in synovial tissue of arthritic cartilage [21]; it also influences cell proliferation and the response to activating stimuli. Elevated expression of Frizzled-2 has been linked to tissue regeneration and hyperplasia in an animal model of atherosclerosis [22]. Wnt-4 is expressed within developing joints [23,24] and it also has been found to be differentially expressed during specific stages of mesenchymal condensation and/or chondrocyte differentiation [24–27]. Furthermore, hyperexpression of Wnt-4 blocks mesenchymal condensation [28]. The Wnt protein binds to Frizzled family receptors and low density lipoprotein receptor-related protein (LRP) 5/6 can activate the Wnt signaling pathway, which results in the activation of dishevelled (Dvl) family proteins. The activation of Dvl leads to the inhibition of glycogen synthase kinase (GSK)-3β (the enzyme responsible for proteosomal β-catenin degradation). Without Wnt signaling, GSK-3β is thought to phosphorylate and consequently induce the degradation of β-catenin, thus keeping intracellular β-catenin levels low [29]. When the kinase activity of GSK-3β is suppressed, nonphosphorylated β-catenin can accumulate in the cytoplasm, since it is no longer continuously phosphorylated and degraded by the proteosome. The accumulating β-catenin migrates to the nucleus, where it binds to transcription factors, such as lymphoid enhancing factor/T-cell factor, to generate a transcriptionally active complex that targets genes such as Myc, cyclin D1, matrix metalloproteinase MMP-3, and CD44 [30]. Increased levels of β-catenin have been observed in degenerative cartilage, suggesting that a diminished capacity to limit Wnt signaling might contribute to cartilage loss [31–33].

In recent studies, ROS have been implicated in the regulation of Wnt signaling [34,35]. Several research works have shown that treatment of cells with H_2O_2 to induce ROS-dependent signaling inhibits β-catenin/TCF transcriptional activity [36,37]. In addition, a recent study indicated that Wnt/β-catenin signaling is involved in increased apoptosis and decreased proliferation in colorectal cancer cells by Prdx2 knockdown [38]. Based on the above research results, we speculated that the regulatory role of Prdx5, in the survival of osteoarthritic chondrocytes, may be associated with the Wnt/β-catenin signaling. In this study, we investigated the expression of Prdx5 in human normal and osteoarthritic chondrocytes, and examined if Prdx5 plays a role in the survival of osteoarthritic chondrocytes and whether these effects are mediated by the Wnt/β-catenin signaling.

Materials and methods

Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, NY). Collagenase, dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiozol-2yl)-2, 5-diphenyltrazolium bromide (MTT), and XAV-939 were from Sigma–Aldrich (St. Louis, MO, USA). pTOPflash reporter plasmid was obtained from Upstate Biotechnology (NY, USA). Fetal bovine serum (FBS) was obtained from HyClone (Shanghai, China). Annexin V-PE and a 7-AAD (7-amino-actinomycin D) double-staining apoptosis detection kit and Total protein extraction kit were purchased from KeyGEN (Nanjing, China). Reactive oxygen species assay kit and *N*-acetyl-L-cysteine (NAC) were purchased from Beyotime (Jiangsu, China). PrimeScript RT Reagent kit, Trizol, and SYBR Premix Ex Taq II were purchased from TaKaRa (Dalian, China). Pierce BCA protein assay kit was from Pierce Biotechnology (USA). Lentiviral constructs expressing Prdx5 shRNA (Prdx5-shRNA-LV) were purchased from Shanghai Genechem Co., Ltd., China. The Prdx5 and Frizzled-2 polyclonal rabbit antibody were purchased from Abcam (UK). The Wnt-4, β-catenin, GSK-3β, p-GSK-3β^{ser9}, MMP-13, cyclin D1, Lamin B1, and β-actin antibodies were purchased from Epitomics (CA, USA). Alexa Fluor 488-conjugated goat anti-rabbit IgG was from ZSGB-BIO (Beijing, China).

Tissue specimens and histologic evaluation

OA cartilage samples ($n=8$) were obtained from joints of patients with OA undergoing knee replacement surgery at the Department of Orthopedics, The First Affiliated Hospital of Chongqing Medical University. Human normal articular cartilage samples were obtained from 8 patients with knee joint fractures undergoing orthopedic operations at the Department of Orthopedics, The First Affiliated Hospital of Chongqing Medical University, and excluded patients with a history of joint disease. The study was approved by the Medical Ethics Review Committee of The First Affiliated Hospital of Chongqing Medical University. Informed consent was given by all patients involved. Research has been performed in accordance with the Declaration of Helsinki involving human material.

For histological analysis, samples were fixed in 4% paraformaldehyde, decalcified, and then processed through a series of increasing ethanol concentrations for dehydration. These samples were subsequently paraffin-embedded, and 4 μm sections were stained with hemotoxylin and eosin (HE). Histological scoring of the cartilage was performed by two blinded investigators and determined according to the Mankin scoring system [39].

Cell culture

For explant cultures and chondrocytes, the articular cartilage was minced and digested in 0.15% (w/v) collagenase in DMEM supplemented with 10% FBS, 2% penicillin/streptomycin (Beyotime, Jiangsu, China) for 16 h at 37 °C as described previously [40]. The digest was centrifuged and the cells were resuspended in FBS-enriched DMEM and cultured in flasks at 37 °C in 5% CO_2. Subcultures were performed with 0.25% trypsin ethylenediaminetetraacetic acid (Gibco) and first passage cells were used.

Construction of Prdx5-shRNA lentiviral vector and transduction of chondrocytes with shRNA

Three pairs of specific shRNA sequences which target the three sites of Prdx5 coding sequences and one pair of negative control oligonucleotide sequence were cloned into the lentiviral expression vector pMU6-MCS-Ubi-EGFP plasmid (restricted by HpaI and XhoI). The sequence of three siRNAs candidates were as follows: si-001, sequence GGA ATC GAC GTC TCA AGA GGT; si-002, sequence CCA CTC TTG AGA CGT CGA CAA; si-003, sequence GCC TTG AGA CGT CAT CGA TGA. Bacterial clones with insertions of siRNA oligonucleotides were identified using PCR and sequenced with vector primers GCC CCG GTT AAT TTG CAT AT (forward) and CAC CCA AGA TCT GGC CTC (reverse). After identification by restriction and sequencing, the pMU6-MCS-Ubi-EGFP vector plasmid and two helper plasmids pHelper1.0 and pHelper2.0 were cotransfected into the 293T cells, and the lentiviral supernatant particles were packaged. Osteoarthritic chondrocytes and normal chondrocytes were seeded in 6-well plates at a concentration of