Expression and regulation of peroxiredoxin 5 in human osteoarthritis

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Abstract **Reactive oxygen species (ROS) are implicated in the pathogenesis of osteoarthritis (OA). However, little is known about the antioxidant defence system in articular cartilage. We investigated the expression and regulation of peroxiredoxin 5 (PRDX5), a newly discovered thioredoxin peroxidase, in human normal and osteoarthritic cartilage. Our results show that human cartilage constitutively expresses PRDX5. Moreover, the expression is up-regulated in OA. Inflammatory cytokines tumour necrosis factor α and interleukin 1 β contribute to this up-regulation by increasing intracellular ROS production. The present study suggests that PRDX5 may play a protective role against oxidative stress in human cartilage.
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Key words: Osteoarthritis; Peroxiredoxin 5; Oxidative stress; Reactive oxygen species; Articular cartilage; Antioxidant

1. Introduction

Osteoarthritis (OA) is the most common form of arthritis and is characterised by chronic pain and significant disability. Although the aetiology of OA is multifactorial, the key pathological feature of OA is articular cartilage degradation [1]. The pathological processes of cartilage degradation, including the molecular processes that impair cartilage homeostasis, are still largely unknown.

Several studies have demonstrated that chondrocytes are able to generate reactive oxygen species (ROS) under certain conditions [2–4]. The over-production of ROS molecules including superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and nitric oxide ($NO^{\bullet}$) may contribute to the degradation of cartilage matrix. ROS can inhibit proteoglycan and hyaluronic acid syntheses [5], fragment hyaluronan [6] and enhance matrix protein degradation [7]. ROS also damage cell membranes [8] and induce apoptosis of synoviocytes [9]. Although there is information regarding the pathological role of ROS in cartilage degeneration, little is known about the antioxidant defence system and particularly about any ROS scavengers in articular cartilage. The redox status of human chondrocytes in OA is also largely undetermined.

To prevent toxicity by ROS, mammalian cells possess a well-coordinated antioxidant enzyme system. Examples of antioxidant enzymes that have been well characterised are the superoxide dismutases (SOD) which catalyse superoxide to H_2O_2 [10]. SOD play a critical but limited role in detoxifying ROS, as H_2O_2 can cause oxidative stress if the levels of the H_2O_2 detoxification enzymes are insufficient or depleted. Catalase and glutathione peroxidases are widely investigated H_2O_2-scavenging enzymes. Recently, a novel family of peroxidases, the peroxiredoxins (PRDX), was identified in many living organisms. Six isoforms of PRDX have been identified in mammals [11–13], all of which participate directly in eliminating H_2O_2 and neutralising other oxidising chemicals [14]. A study on the crystal structure of human peroxiredoxin 5 (PRDX5), also known as PrxV/AOEB166/PMP20/ARC1 [12,15–17], suggested that PRDX5 may have a broader activity against ROS compared with other isoforms of PRDX and other antioxidant enzymes [18]. We have previously shown that the expression of PRDX5 is up-regulated in degenerative human tendon [19]. The expression of this enzyme in human cartilage, particularly in osteoarthritic cartilage, has not been investigated.

To understand better the mechanisms by which human chondrocytes are protected against oxidative stress, we investigated the expression of PRDX5 in normal and osteoarthritic human cartilage, and investigated the regulation of PRDX5 expression in human chondrocytes. We were particularly interested in determining if inflammatory cytokines play any role in the regulation of PRDX5 expression.

2. Materials and methods

2.1. Tissue collection and culture

Human tissue collection in this study was approved by the South Eastern Sydney Area Health Service Ethics Committee, Australia. Human osteoarthritic cartilage ($n = 4$) was removed from knee joints of patients with OA undergoing total knee-replacement surgery. Significant OA was identified by preoperative radiography. Normal human cartilage ($n = 4$) was removed immediately adjacent to the insertion site of supraspinatus tendon as part of the surgical procedure to reattach the tendon to bone. Significant OA was excluded by plain anterior–posterior and lateral radiographs of the shoulder, and by arthroscopic examination of the glenohumeral joint prior to open rotator culf repair.

For RNA and protein extraction, cartilage tissues were snap-frozen in liquid nitrogen upon collection and stored at $-80°C$ until RNA extraction and tissue homogenisation were performed. For tissue culture, cartilage explant discs were made using a 4-mm-diameter disposable biopsy punch (SMS Inc., Columbia, MD, USA) with an average thickness of 2 mm. Three discs were placed in each well of 24-well

plates (1 ml final volume). Chondrocytes were isolated from cartilage by collagenase digestion [20] and plated in 25-cm² flasks (5×10^5) containing Dulbecco's modified essential medium (DMEM) supplemented with 10% foetal bovine serum. Primary chondrocyte monolayer cultures and cartilage explants were incubated at 37°C in a 5% CO_2 humidified atmosphere. Culture medium was replaced with serum-free DMEM for all experiments.

To mimic in vivo pathological conditions, tumour necrosis factor α (TNFα) 100 ng/ml, and interleukin 1 β (IL-1β) 10 ng/ml (R&D System, Minneapolis, MN, USA) were added to explant and chondrocyte cultures. Catalase (500 U/ml) (Sigma, St. Louis, MO, USA) was used as an H_2O_2 scavenger and added to chondrocyte culture 2 h before TNFα stimulation. Tissue and cells were harvested at 3, 6, 12, 24 and 48 h after the addition of TNFα or IL-1β for total RNA extraction, protein preparation and intracellular H_2O_2 determination.

2.2. Northern blotting

Total RNA was isolated from human chondrocytes using Trizol reagent (Life Technologies, Melbourne, Australia) following the manufacturer's instructions. Denatured RNA samples (20 μg) were fractionated by electrophoresis in a denaturing 1% (w/v) agarose gel, transferred to a GeneScreen Plus nylon membrane (NEN Life Science Products, Boston, MA, USA), cross-linked using an ultraviolet cross-linker (Ultra-Lum, Carson, CA, USA), and hybridised with ^{32}P-labelled human PRDX5 cDNA probe. The blots were subsequently stripped and reprobed with ^{32}P-labelled human β-actin cDNA. All probes were radiolabelled by random priming (Promega, Sydney, Australia).

2.3. Western blotting

Human cartilage tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle, and homogenised in five volumes of Tris–HCl buffer (pH 7.4) containing a protease inhibitor cocktail (2 μg/ml leupeptin, 5 μg/ml pepstatin A, 2 mg/ml of ethylenediamine tetraacetic acid (EDTA)–Na_2, 500 μg/ml AEBSF and 1 μg/ml E-64) (Sigma). Chondrocyte lysate was prepared by sonication in the same homogenisation buffer. Proteins in the tissue homogenates or cell lysate were denatured by boiling for 5 min in 2% (w/v) sodium dodecyl sulfate (SDS) sample buffer and fractionated by electrophoresis on 12.5% (w/v) SDS–polyacrylamide gel. The fractionated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane, and the blots were blocked with 5% (w/v) non-fat dry milk TTBS solution containing 25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% (w/v) Tween 20. After washing in TTBS, the blots were incubated for 1 h with polyclonal anti-PRDX5 antibody [19] at 1:2000 dilution, followed by horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Chemicon, Melbourne, Australia). Immunoreactive bands were detected by enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). The membranes were stripped with Restore Western Blot Stripping Buffer (Pierce) and reprobed using a rabbit anti-human actin antibody (Sigma) as a house-keeping control. The net intensity of the PRDX5 band was analysed by Bio-Rad Quantity One image quantification system (Bio-Rad, Hercules, CA, USA). PRDX5 protein level was expressed by the ratio of the net intensity of the PRDX5 band to actin band of the same sample.

2.4. Intracellular H_2O_2 assay

Intracellular H_2O_2 generation was assessed in chondrocytes by a flow cytometric technique based on the methods of Sattler et al. [21], in which 2′,7′-dichloro-fluorescin-diacetate (DCF-DA) (Sigma) was used. DCF-DA is a cell-permeable dye commonly used to monitor intracellular changes in ROS (more specifically for H_2O_2). DCF-DA readily diffuses into cells where it is hydrolyzed to the non-fluorescent derivative 2′,7′-dichloro-fluorescin (DCF) and is trapped within the cells. DCF becomes highly fluorescent when oxidised by either H_2O_2 or superoxide. Cellular fluorescence intensity is directly proportional to the level of intracellular H_2O_2 produced by the cells and can be monitored by flow cytometry. Chondrocytes were freed from culture flasks by trypsin–EDTA. Cells (10^6) were incubated with 5 μM of DCF-DA for 5 min at 37°C and subsequently washed twice in cold phosphate-buffered saline before analysis by a flow cytometer (FAC-Sort, Becton Dickinson System, San Jose, CA, USA) with Cell Quest Software (Becton Dickinson). The fluorescence of oxidised DCF was measured with an excitation wavelength of 488 nm and emission wavelength of 525 nm. Mean fluorescence intensity (MFI) for 10 000 cells was recorded for each sample. Fluorescence relative variance

(FRV) was calculated for each sample (S) compared with control (C; loaded cells without TNFα treatment): $FRV = [(MFI_{(S)} - MFI_{(C)})/MFI_{(C)}$.

2.5. Statistical analysis

Comparison of PRDX5 expression in normal and osteoarthritic cartilage was made by Mann–Whitney Rank Sum Test using SigmaStat (Jandel Scientific, San Rafael, CA, USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. PRDX5 protein is constitutively expressed in human cartilage and up-regulated in osteoarthritic cartilage

A rabbit antibody was raised against recombinant human PRDX5 [19]. With this antibody, PRDX5 protein expression was readily detected by Western blot of cartilage tissue homogenates (Fig. 1). To semi-quantify the PRDX5 protein expression in both normal and osteoarthritic cartilage, all blots were reprobed with an anti-actin antibody and the results were expressed as a density ratio of PRDX5:actin. It was observed that osteoarthritic cartilage had more fibrin tissue and less cellularity than normal cartilage. Even if the same amount of total protein were loaded for Western blotting, one would expect less cellular protein in osteoarthritic cartilage than in normal cartilage. Therefore, the normalisation of PRDX5 protein with actin is important for the semi-quantification. Fig. 1 shows a significantly higher PRDX5 protein level in osteoarthritic cartilage than in normal cartilage, suggesting an up-regulation of PRDX5 in OA.

3.2. PRDX5 expression is regulated by inflammatory cytokines

To explore the mechanisms by which the PRDX5 expression in human chondrocytes is regulated, we introduce inflammatory cytokines to the cartilage explant culture and primary chondrocyte culture, as cytokines have been shown to be implicated in OA. As shown in Fig. 2, TNFα and IL-1β up-regulated PRDX5 protein expression in the cartilage explant
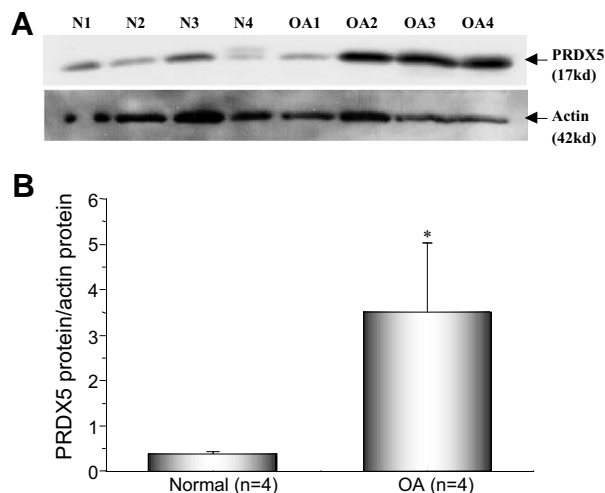

Fig. 1. Expression of PRDX5 protein in human articular cartilage. A: PRDX5 protein bands (upper panel) and actin bands (lower panel) detected by Western blotting in normal (N) and osteoarthritic cartilage (OA). B: Comparison of PRDX5 protein levels in normal and osteoarthritic cartilage ($n = 4$) by densitometry analysis. PRDX5 protein level was expressed by the ratio of the net intensity of the PRDX5 band to the actin band of the same sample. Bars represent mean ± S.E.M. *$P < 0.05$ OA vs normal.
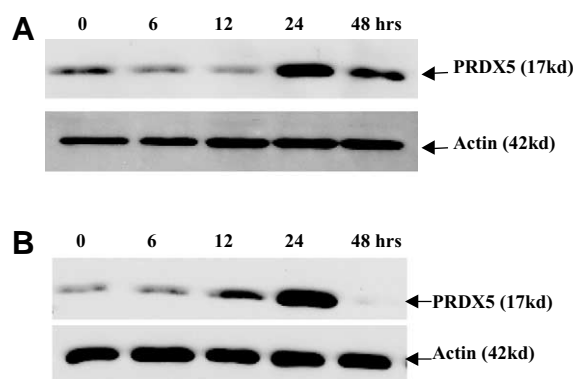
Fig. 2. Effects of TNFα and IL-1β on PRDX5 protein expression in human articular cartilage explant culture. Cartilage explants from OA patients were precultured with TNFα (100 ng/ml; panel A) or IL-1β (10 ng/ml; panel B) for the indicated times, and subject to Western blot analysis using anti-PRDX5 and anti-actin antibodies as described in Section 2.3.

culture 24 h after the cytokine challenge. At 48 h, the PRDX5 protein levels decreased to near baseline in TNFα-treated cartilage and below baseline in IL-1β-treated cartilage. A similar phenomenon was observed in primary chondrocyte culture (Fig. 3). Both PRDX5 mRNA and protein expression started to increase 3 h after TNFα stimulation, and reached their peak levels at 12 and 24 h. The protein level returned to baseline at 48 h, while the mRNA level remained high.

3.3. H_2O_2 might be an important mediator for the PRDX5 up-regulation in human chondrocytes

Cytokines can induce intracellular H_2O_2 production in chondrocytes. We hypothesised that the increased PRDX5 expression in stimulated chondrocytes was a cellular response to intracellular flux of H_2O_2. To test this hypothesis, intracellular H_2O_2 induction was measured using a flow cytometric technique. Catalase was introduced to the chondrocyte culture system to test if scavenging H_2O_2 would affect the PRDX5 expression level. As shown in Fig. 4A, the intracellular H_2O_2 level in cultured human chondrocytes started to rise 3 h after a TNFα challenge, reached its peak at 6 h, and started to decline at 12 h when the PRDX5 protein expression reached its peak (Fig. 4B). The addition of catalase to the chondrocyte
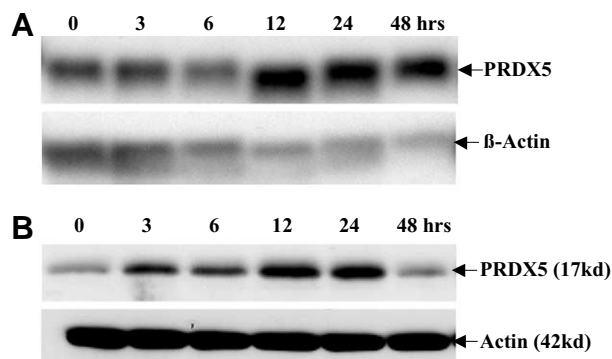

Fig. 3. Effects of TNFα on PRDX5 mRNA and protein expression in cultured human articular cartilage chondrocytes. Chondrocytes isolated from OA cartilage were precultured with TNFα (100 ng/ml) for the indicated times. A: PRDX5 mRNA was detected by Northern blot analysis. B: PRDX5 protein expression was analysed by Western blot.
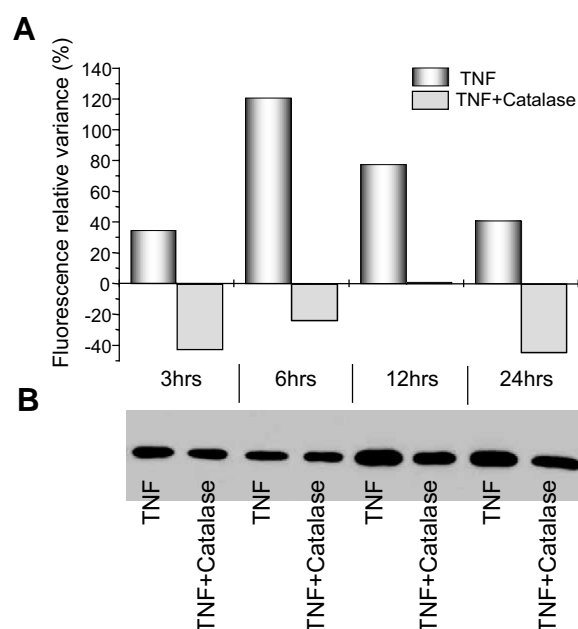

Fig. 4. Effect of catalase and/or TNFα on chondrocyte H_2O_2 production and PRDX5 protein expression. A: Flow cytometric analysis of intracellular H_2O_2 production by chondrocytes treated with TNFα (100 ng/ml) or TNFα+catalase (500 U/ml) for the indicated times. Catalase was added to culture 2 h before the addition of TNFα. B: Western blot analysis of PRDX5 protein from chondrocytes treated as above. Results were representatives of three separate experiments using chondrocytes isolated from three OA cartilages.

culture prior to exposure to TNFα significantly inhibited TNFα-induced intracellular H_2O_2 production (Fig. 4A), as well as the enhanced PRDX5 protein expression (Fig. 4B, at 12 and 24 h), suggesting that H_2O_2 might be an important mediator for the cytokine-induced PRDX5 up-regulation in human chondrocytes.

4. Discussion

Articular chondrocytes, embedded in an avascular matrix, are exposed to a low partial oxygen pressure and exhibit a predominantly anaerobic metabolism [22]. Accordingly, the ROS defence mechanisms in articular chondrocytes are generally weak and chondrocytes are susceptible to attacks by ROS. However, chondrocytes have the potential to increase their antioxidant status. Previous studies on chondrocytes antioxidant mechanisms have shown that human chondrocytes constitutively express SOD and catalase [23]. Our study provides the first evidence that human chondrocytes also express PRDX5 and the expression is significantly higher in osteoarthritic cartilage compared with normal cartilage.

To explore the regulatory mechanisms of the PRDX5 expression, we introduced the inflammatory cytokines TNFα and IL-1β to cartilage explant cultures and primary chondrocyte cultures, as these cytokines have been shown to stimulate chondrocyte ROS production [3,24] and to be implicated in OA [25]. Our results demonstrate that IL-1β and TNFα are important modulators of PRDX5 up-regulation. During the time course observation of PRDX5 expression, we noted a discrepancy between the PRDX5 mRNA and the protein levels in TNFα challenged chondrocytes. Indeed, the PRDX5 protein level returned to baseline 48 h after TNFα stimula-

tion, while the mRNA level remained high (Fig. 3). This may be due to a translational block for the synthesis of PRDX5 protein, which may have happened 48 h after initial PRDX5 up-regulation as a feed-back protection mechanism, as physiological levels of ROS are essential for many biochemical processes, including signal transduction [26], cell differentiation and immunity [27]. Translational regulation has been reported in many mammalian cells and is believed to be responsible for the discrepancy between mRNA and protein levels [28,29]. More experiments are necessary to define the translational regulation PRDX5 protein in human chondrocytes.

The pathways by which PRDX5 is up-regulated in OA are not yet known. It is possible that the up-regulation of PRDX5 is a response to increased levels of ROS in OA, as there is increasing evidence showing that over-production of ROS is implicated in the pathogenesis of OA [4,7,30,31]. Our study has shown that a TNFα/IL-1β challenge significantly increases intracellular H_2O_2 production, followed by enhanced PRDX5 expression. When the H_2O_2 scavenger catalase was introduced into the primary chondrocyte culture, intracellular H_2O_2 was inhibited and this was accompanied by the inhibition of PRDX5 protein expression, suggesting that H_2O_2 might be an important mediator for the cytokine-induced PRDX5 up-regulation.

The significantly increased expression of PRDX5 in OA may act as a protection to cartilage against ROS-induced oxidative damage. Increased SOD activity has been reported in OA [32]. Dismutation of the anion superoxide to H_2O_2 by SOD is a well-described defence mechanism against oxidative stress at the cellular level. However, reduction of H_2O_2 by the Fenton reaction to the highly toxic hydroxyl radical can be, in some circumstances, even more harmful to the cell. H_2O_2 detoxifying enzymes, including PRDX5, can metabolise and eliminate H_2O_2, partially avoiding the conversion of H_2O_2 to hydroxyl radical. Among the H_2O_2 detoxifying enzymes, catalase is known to be present only in peroxisomes [33]; glutathione peroxidase is restricted to the mitochondrial intermembrane space in very limited amounts [34]; PRDX5, however, is present in a broader range of intracellular locations, including mitochondria, peroxisomes and cytosol [12]. The functional significance of PRDX5 localisation in organelles needs further investigation.

In conclusion, our study has demonstrated that the antioxidant enzyme PRDX5 is constitutively expressed in human chondrocytes. The expression is up-regulated in OA. Inflammatory cytokines TNFα and IL-1β may be responsible for this up-regulation via the stimulation of intracellular H_2O_2 production. 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.

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