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β-Catenin regulates expression of cyclooxygenase-2 in articular chondrocytes☆

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Abstract

Pro-inflammatory cytokine such as interleukin (IL)-1β causes inflammation of articular cartilage via induction of cyclooxygenase (COX)-2 expression. We investigated in this study the role of β-catenin in the IL-1β regulation of COX-2 expression in articular chondrocytes. IL-1β increased expression of COX-2 and induced accumulation and nuclear translocation of transcriptionally competent β-catenin. Inhibition of β-catenin degradation by the treatment of cells with LiCl or proteasome inhibitor stimulated expression of COX-2, indicating that transcriptionally active β-catenin is sufficient to induce COX-2 expression. This was demonstrated further by the observation that ectopic expression of transcriptionally competent β-catenin stimulated expression of COX-2. Levels of β-catenin and COX-2 protein were increased in osteoarthritic and rheumatoid arthritic cartilage, suggesting that β-catenin may play a role in the inflammatory responses of arthritic cartilage. Taken together, our data suggest that accumulation of transcriptionally active β-catenin contributes to the expression of COX-2 in articular chondrocytes.

Keywords: Chondrocytes; β-Catenin; Cyclooxygenase-2; Interleukin-1β; Cartilage; Arthritis

Arthritic joints produce large amounts of prostaglandins (PGs) that are involved in cartilage inflammation [1]. The rate-limiting step of PG production is the initial conversion of arachidonic acid to PGH2 by cyclooxygenases (COX). COX-1 and COX-2 are encoded by distinct genes and have different physiological functions [2]. The constitutively expressed COX-1 plays a homeostatic function, whereas COX-2 is rapidly induced by various extracellular stimuli. Regulation of COX-2 expression has been shown to occur at both transcriptional and post-transcriptional levels, although the molecular mechanisms of this up-regulation remain unclear. IL-1β acts as a major pro-inflammatory cytokine that plays a key role in cartilage inflammatory responses [3–5] and is known to induce COX-2 expression in articular chondrocytes [6–10] contributing to the pathophysiology of arthritis [11–15]. Administration of COX-2 inhibitor has been shown to repress joint inflammation in animal models of arthritis [13–15].

β-Catenin, initially identified as a cytoskeletal component that participates in cadherin-mediated cell adhesion, is also engaged in the regulation of gene expression by acting as a transcriptional co-activator of the transcription factor T cell-factor (TCF)/lymphoid-enhancer-factor (LEF) [16,17]. Depending on the cell type, β-catenin as a transcriptional co-activator regulates the expression of many genes including cyclin D1 [18], connexin-43 [19], and fibronectin [20]. Cytosolic β-catenin not associated with cadherin in cell–cell junctions is incorporated into a large complex that includes the APC tumor suppressor protein, the glycogen synthase kinase (GSK)-3β, and the axin/conductin protein, which results in subsequent proteolytic degradation of

☆ *Abbreviations:* COX, cyclooxygenase; GSK-3β, glycogen synthase kinase-3β; IL, interleukin; TCF/LEF, T cell-factor/lymphoid-enhancer-factor.

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β-catenin by the ubiquitin–proteasome system. However, in the presence of Wnt signals β-catenin escapes from ubiquitin-dependent proteolytic degradation by the inhibition of GSK-3β and the accumulated β-catenin translocates to the nucleus in association with members of the TCF/LEF family of transcription factors to stimulate transcription of target genes [16,17].

The possibility that COX-2 is either a direct or indirect target gene of β-catenin has been suggested, based on the observation that mutation of APC gene or activation of Wnt signaling, conditions that cause accumulation of transcriptionally active β-catenin, stimulates COX-2 expression in epithelial cells [21–23]. However, there is as yet no direct evidence that indicates COX-2 is regulated by β-catenin. We therefore investigated a role of β-catenin in the regulation of COX-2 expression in articular chondrocytes treated with IL-1β. To reveal the functional significance of β-catenin accumulation in the pathophysiology of arthritis, we also examined β-catenin accumulation and COX-2 expression in arthritic cartilage and cartilage explants treated with IL-1β. We report herein that IL-1β-induced accumulation of transcriptionally active β-catenin is sufficient to cause COX-2 expression in articular chondrocytes, suggesting a positive regulatory role for β-catenin in COX-2 expression in articular chondrocytes.

Materials and methods

Culture of rabbit articular chondrocytes and cartilage explants. Rabbit articular chondrocytes were released from cartilage slices by enzymatic digestion as described previously [24]. The cells were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine calf serum and then plated on culture dishes at a density of 5×10^4 cells/cm^2. The medium was changed every 1.5 days and cells reached confluence in 4.5 days. Cartilage explants (~125 mm^3) were organ cultured in complete medium for 48 h in the absence or presence of 5 ng/ml IL-1β.

Arthritic cartilage. Human osteoarthritic joint cartilage was obtained from patients undergoing total knee arthroplasty. Experimental rheumatoid arthritis was induced in 12-week-old male DBA/1 mice obtained from Jackson Laboratory (Bar Harbor, ME). Six mice were injected s.c. at the base of the tail with 200 μg (in 150 μl) bovine type II collagen dissolved in 0.05 M acetic acid at 4 °C and emulsified in an equal volume of complete Freund's adjuvant. Three weeks after primary immunization, mice were boosted i.p. with 200 μg type II collagen. Mice were observed daily for the onset of arthritis and compared to control mice. Immunized mice developed rheumatoid arthritis 2 weeks after the first immunization. Mice were sacrificed 7 weeks after primary immunization and their hind legs were fixed in 10% NBF and decalcified, after which they were used for immunohistochemical study.

Immunohistochemistry and immunofluorescence microscopy. Rabbit joint cartilage explants or arthritic cartilage were fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C, washed with PBS, dehydrated with graded ethanol, embedded in paraffin, and sectioned at 4 μm thickness. The sections were stained by standard procedures using antibodies against β-catenin or COX-2 (BD Transduction Laboratories, Lexington, KY) and visualized by developing with a kit purchased from DAKO (Carpinteria, CA), following the procedure recommended by the manufacturer. Expression and distribution of β-catenin and COX-2 in rabbit articular chondrocytes were determined by indirect immunofluorescence microscopy, as described previously [25]. Briefly, chondrocytes were fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature. The cells were permeabilized and blocked with 0.1% Triton X-100 and 5% fetal calf serum in PBS for 30 min. The fixed cells were washed and incubated for 1 h with antibody (10 μg/ml) against β-catenin or COX-2. The cells were washed, incubated with rhodamine- or fluorescin-conjugated secondary antibodies for 30 min, and observed under a fluorescence microscope.

Western blot assay. Whole cell lysates were prepared by extracting proteins using a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS, supplemented with protease and phosphatase inhibitors. The proteins (30 μg/lane) were size-fractionated by SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Expression of proteins was detected using antibodies purchased from the following sources: rabbit phospho-GSK-3β polyclonal antibody from New England Biolabs and β-catenin and COX-2 monoclonal antibodies from BD Transduction Laboratories.

Transfection and reporter assays. Retroviral vector (5 μg) containing cDNA for S37A β-catenin [26] was transfected to articular chondrocytes using LipofectaminePLUS (Gibco-BRL, Gaithersburg, MD), following the procedure recommended by the manufacturer. The transfected cells, which were cultured in complete medium for 24 or 48 h, were used for further assay as indicated for each experiment. To investigate β-catenin-TCF/LEF signaling, cells were transiently transfected with 1 μg TCF/LEF reporters, TOPFlash (optimal LEF-binding site) or FOPFlash (mutated LEF-binding site) [27] (Upstate Biotech., Lake Placid, NY), and 1 μg pCMV-β-galactosidase. Following incubation with IL-1β for 48 h, luciferase activity was measured and normalized for transfection efficiency using β-galactosidase activity.

Results

Accumulation and transcriptional activation of β-catenin in articular chondrocytes treated with IL-1β

Treatment of articular chondrocytes with IL-1β induced COX-2 expression in a dose- and time-dependent manner (Fig. 1). To determine the role of β-catenin in IL-1β-induced COX-2 expression, we first examined expression and/or accumulation of β-catenin protein. Treatment of chondrocytes with IL-1β resulted in significantly increased levels of β-catenin in a dose-dependent manner (Fig. 2A, upper panel). The increase in β-catenin was apparent ~4 h after IL-1β treatment (Fig. 2A, lower panel). Northern blot analysis of β-catenin indicated that IL-1β did not alter β-catenin transcript levels (Fig. 2A, upper panel), indicating post-transcriptional regulation of β-catenin accumulation. β-Catenin in untreated cells is localized predominately in cell–cell contacts, whereas IL-1β treatment induced a dramatic localization and accumulation of β-catenin to the nucleus (Fig. 2B). Consistent with the nuclear translocation of β-catenin, transcriptional activity of β-catenin was increased by 5-fold as determined by TOPFlash assay, an artificial β-catenin/TCF-responsive promoter reporter gene (Fig. 2C). Taken together, these data

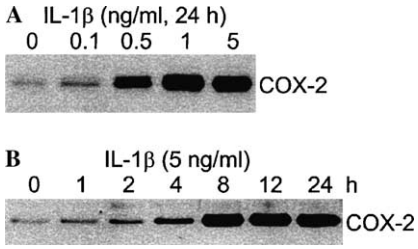

Fig. 1. IL-1β induces COX-2 expression in articular chondrocytes. Rabbit joint articular chondrocytes were treated with the indicated concentrations of IL-1β for 24 h (A) or with 5 ng/ml IL-1β for the indicated time period (B). Expression of COX-2 was determined by Western blot analysis. Results of a typical experiment are presented from at least four independent experiments.

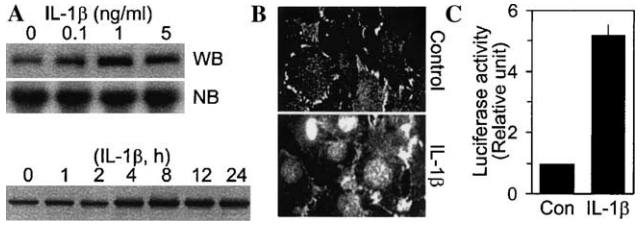

Fig. 2. IL-1β induces the accumulation of transcriptionally active β-catenin in chondrocytes. (A) Rabbit joint articular chondrocytes were treated with the indicated concentrations of IL-1β for 24 h (upper panel) or with 5 ng/ml IL-1β for the indicated time periods (lower panel). Expression of β-catenin was determined by Western blot (WB) or Northern blot (NB) analysis. (B) Chondrocytes were treated with vehicle alone or 5 ng/ml IL-1β for 24 h and localization of β-catenin was determined by indirect immunofluorescence microscopy. (C) To measure transcriptionally active β-catenin, chondrocytes were transfected with TCF/LEF reporter gene (TOPFlash) and treated with vehicle alone as a control (Con) or 5 ng/ml IL-1β for 24 h. TCF/LEF reporter gene activity was monitored by a luminometer. The data shown in (A) and (B) represent results of a typical experiment conducted at least four times with similar results. Data shown in (C) represent average values with standard deviations ($n = 3$).

indicate that IL-1β can induce the nuclear translocation and transcriptional activation of β-catenin in articular chondrocytes.

β-Catenin accumulation causes COX-2 expression

We next determined mechanisms of β-catenin accumulation and its effects on COX-2 expression. Phosphorylation of GSK-3β was significantly increased in cells treated with IL-1β, which was detectable ~10 min after IL-1β treatment (Fig. 3A). Because GSK-3β activity is primarily responsible for the degradation of β-catenin via a ubiquitin–proteasome pathway, the inhibition of GSK-3β (i.e., increased phosphorylation) suggests that post-translational accumulation of β-catenin contributes to the increased levels of β-catenin in IL-1β-treated cells. To characterize further post-translational accumulation of β-catenin, the activity of GSK-3β was blocked by LiCl treatment. LiCl treatment of chondrocytes inhibited GSK-3β activity, as demonstrated by its increased phosphorylation, and resulted in the accumulation of β-catenin and increased expression of COX-2 (Fig. 3B). Accumulation of β-catenin and increase in COX-2 expression were more dramatic when degradation of β-catenin was blocked by the inhibition of 26S proteasome with MG132 (Fig. 3C). Therefore, accumulation of β-catenin appears to contribute to the increased COX-2 expression in articular chondrocytes.

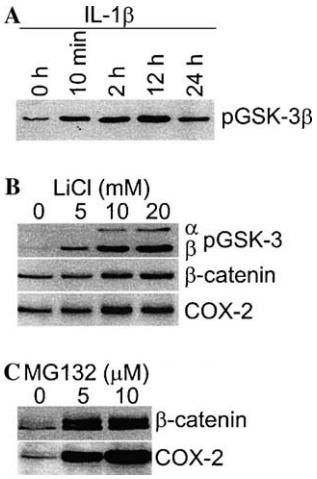

Fig. 3. Inhibition of β-catenin degradation induces COX-2 expression. (A) Rabbit joint articular chondrocytes were treated with 5 ng/ml IL-1β for the indicated time periods and phosphorylation of GSK-3β was determined by Western blot analysis. (B) and (C) Chondrocytes were treated with the indicated concentrations of LiCl (B) or MG132 (C) for 24 h to inhibit GSK-3β and 26S proteasome, respectively. Phosphorylation of GSK-3β and expression of β-catenin and COX-2 were determined by Western blot analysis. The data represent the results of a typical experiment conducted at least three times with similar results.

To directly determine whether the nuclear accumulation of transcriptionally active β-catenin is involved in the regulation of COX-2 expression, chondrocytes were transfected with S37A β-catenin, a non-ubiquitinatable stable form of β-catenin. Ectopic expression of β-catenin was transcriptionally competent as determined by the reporter gene assay (Fig. 4A) and caused an increase in expression of COX-2 but not connexin-43 (Fig. 4B). Immunofluorescence double staining of β-catenin and COX-2 in chondrocytes transfected with S37A β-catenin indicated that cells highly expressing β-catenin are also strongly positive for COX-2 staining, whereas cells that do not express β-catenin (indicated by arrow head) are negative for COX-2 staining (Fig. 4C). Therefore, ectopic expression of transcriptionally active β-catenin appears to be sufficient to induce COX-2 expression in articular chondrocytes.

Increased levels of β-catenin and COX-2 in cartilage explants culture and arthritic cartilage

To examine the in vivo significance of β-catenin-mediated COX-2 expression in cartilage, explants of rabbit

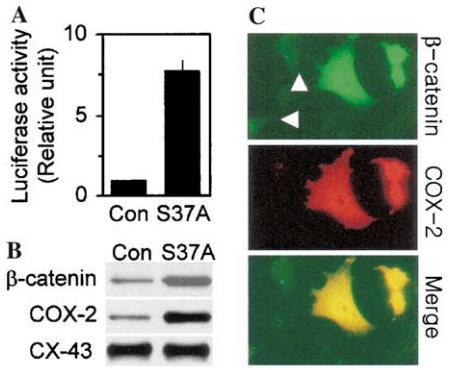

Fig. 4. Ectopic expression of β-catenin or β-catenin degradation induces COX-2 expression. (A) and (B) Chondrocytes were transfected with empty vector as a control (Con) or S37A β-catenin (S37A). Following 48 h incubation in complete medium, TCF/LEF activity (A) and expression levels of β-catenin, COX-2, and connexin-43 (CX-43) (B) were determined by TOPFlash assay and Western blot analysis, respectively. (C) Chondrocytes were transfected with S37A β-catenin, cultured for 48 h, double stained for β-catenin and COX-2, and analyzed by immunofluorescence microscopy. The data represent average values with standard deviation (A) or the results of a typical experiment ((B) and (C)) conducted at least three times with similar results.

joint cartilage were cultured in the absence or presence of IL-1β, the primary inflammatory cytokine involved in cartilage destruction. IL-1β treatment of cartilage explants induced increased β-catenin protein levels with a concomitant increase in COX-2 expression (Fig. 5A). We also examined the expression levels of β-catenin in human osteoarthritic cartilage obtained from patients undergoing total knee arthroplasty. Relatively normal parts of the joint (i.e., undamaged parts of the osteoarthritic joint) showed undetectable expression of β-catenin and COX-2 (Fig. 5B). However, expression of β-catenin and COX-2 was significantly increased in osteoarthritis-affected part of cartilage. The increase in β-catenin protein and COX-2 levels was also evident in experimental rheumatoid arthritic cartilage, caused by type II collagen injection in DBA/1 mice (Fig. 5C). Taken together, these results suggest that the accumulation of β-catenin may contribute to the cartilage destruction during arthritic disease.

Discussion

In this study, we demonstrated that IL-1β causes accumulation and transcriptional activation of β-catenin that is sufficient to cause COX-2 expression in articular chondrocytes. We also showed that expression levels of β-catenin and COX-2 were increased in arthritic cartilage or in cartilage explants treated with IL-1β. These results suggest that accumulation of β-catenin may contribute to the inflammatory responses of cartilage by inducing COX-2 expression in chondrocytes of arthritis-affected cartilage.

β-Catenin appears to be accumulated in IL-1β-treated cells by post-translational modification because inhibition of GSK-3β or 26S proteasome increased β-catenin protein levels. The increase in β-catenin levels is more evident in cells treated with 26S proteasome inhibitor compared with that in cells treated with LiCl. The effects of LiCl on GSK-3β phosphorylation are obvious, yet there is less significant increase in β-catenin levels. This suggests that other mechanisms rather than GSK-3β may regulate β-catenin accumulation. Nevertheless, the levels of β-catenin accumulation are well co-related to the increase in COX-2 expression, indicating a positive role of β-catenin in COX-2 expression. A role of β-catenin in COX-2 expression has been suggested, based on the observation that accumulation of β-catenin accompanies COX-2 expression. For instance, activation of Wnt signals such as Wnt-1 and -3 [21–23] or mutation in the APC gene [28] results in the accumulation of both β-catenin and COX-2 expression in epithelial cells. However, there is no direct evidence to date that indicates direct regulation of COX-2 expression by β-catenin accumulation. It has been shown that ectopic expression of β-catenin causes only weak activation of the COX-2 promoter [22] or no activation [23] in epithelial cells, raising a question regarding the role of

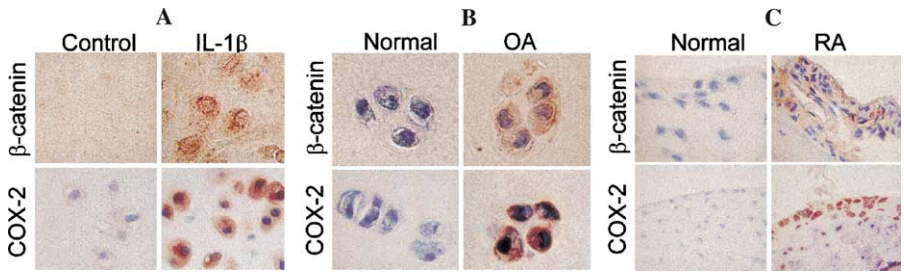

Fig. 5. Increased levels of β-catenin and COX-2 in arthritic cartilage and cartilage explants treated with IL-1β. (A) Expression of β-catenin and COX-2 proteins was detected from control or 5 ng/ml IL-1β-treated explants of rabbit joint cartilage for 72 h. (B) and (C) Expression of β-catenin and COX-2 proteins was detected from undamaged part of osteoarthritic cartilage (normal) and arthritis-affected human joint cartilage (OA) (B) and normal and experimental rheumatoid arthritic cartilage (RA) (C). Tissue sections were counter stained with hematoxylin, except a sample used for the staining of β-catenin in cartilage explant culture. Results of a typical experiment are presented.

β-catenin in COX-2 expression. However, our current observation that increased accumulation of β-catenin (caused by the ectopic expression of transcriptionally competent β-catenin, the inhibition of GSK-3β, or the inhibition of 26S proteasome) leads to increased expression of COX-2 clearly indicates a positive role of β-catenin in COX-2 expression in articular chondrocytes. To the best of knowledge, this is the first direct indication that suggests regulation of COX-2 expression by β-catenin.

The mechanism by which β-catenin activates expression of COX-2 in articular chondrocytes is currently unknown. Although the sequence of the rabbit COX-2 promoter is not known, the sequence of the human COX-2 promoter contains two potential TCF/LEF binding sites with partial homology with the canonical TCF/LEF motif: ACTTTGATC and TCTTGTAG compared with CCTTTGA/TA/TC [27]. If the rabbit promoter is similar, there is the possibility of direct regulation of the COX-2 promoter by β-catenin in rabbit articular chondrocytes. However, it may be equally possible that β-catenin regulates COX-2 expression indirectly, i.e., β-catenin-LEF/TCF modulates target genes that in turn induce COX-2 expression.

Several inflammatory mediators, including nitric oxide and PG, have been implicated in the disease process of osteo- and rheumatoid arthritis. PGs are produced by cytokine-stimulated chondrocytes in arthritis-affected cartilage via induction of COX-2 expression [11–15]. Our current observation of increased expression of COX-2 in arthritis-affected chondrocytes is consistent with the reports by Amin et al. [11] and Jacques et al. [29], showing increased expression and activity of COX-2 in osteoarthritic cartilage. Based on the observation that accumulation of β-catenin induces COX-2 expression during in vitro culture, it is likely that high levels of β-catenin in arthritic cartilage might be associated with increased COX-2 expression.

Acknowledgments

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