A Potential Role of 15-Deoxy-Δ12,14-prostaglandin J2 for Induction of Human Articular Chondrocyte Apoptosis in Arthritis*

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The cyclopentenone prostaglandin (PG) J2 is formed within the cyclopentenone ring of the endogenous prostaglandin PG D2 by a nonenzymatic reaction. The PG J family is involved in mediating various biological effects including the regulation of cell cycle progression and inflammatory responses. Here we demonstrate the potential role of 15-deoxy-Δ12,14-prostaglandin J2 (15d-PG J2) in human articular chondrocyte apoptosis. 15d-PG J2 was released by human articular chondrocytes and found in joint synovial fluids taken from osteoarthritis or rheumatoid arthritis patients. Proinflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) up-regulated chondrocyte release of 15d-PG J2. PG D2 synthase mRNA expression was up-regulated by IL-1β, TNF-α, or nitric oxide. 15d-PG J2 induced apoptosis of chondrocytes from osteoarthritis or rheumatoid arthritis patients as well as control non-arthritis subjects in a time- and dose-dependent manner and in a peroxisome proliferator-activated receptor γ-dependent manner. Peroxisome proliferator-activated receptor γ expression was up-regulated by IL-1β and TNF-α. Inhibition of NF-κB, and the activation of p38 MAPK were also found to be involved in 15d-PG J2-induced chondrocyte apoptosis. Such signal pathways led to the activation of the downstream pro-apoptotic molecule p53 and caspase cascades. Together, these results suggest that 15d-PG J2 may play an important role in the pathogenesis of arthritic joint destruction via a regulation of chondrocyte apoptosis.

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‡The abbreviations used are: OA, osteoarthritis; PG, prostaglandin; 15d-PG J2, 15-deoxy-Δ12,14-prostaglandin J2; PPARγ, peroxisome proliferator-activated receptor γ; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor-α; SNP, sodium nitroprusside; PGDS, PG D2 synthase; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase; NF-κB, nuclear factor-κB; RA, rheumatoid arthritis; PI, propidium iodide; TGF, transforming growth factor; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription.
15d-PG J2 in the pathogenesis of arthritis was demonstrated (20, 21).

Here we investigated the possible role of 15d-PG J2 in chondrocyte viability and apoptosis. 15d-PG J2 was found to be secreted by human articular chondrocytes and induced potent chondrocyte apoptosis; this effect was PPAR-α-dependent. We also demonstrated that 15d-PG J2-induced chondrocyte apoptosis is dependent on the inhibition of nuclear factor-xB (NF-xB) and activation of mitogen-activated protein kinase (MAPK) pathway member p38 kinase.

### EXPERIMENTAL PROCEDURES

#### Materials

15-Deoxy-
\[\Delta^2\Delta^14\] prostaglandin J2, 15-PG J2, PG J2, PG E2, citicline, pigiglitzane, GW9662, and T0070907 were obtained from Cayman Chemical (Ann Arbor, MI), and sodium nitroprusside (SNP) was from Sigma. Interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and transforming growth factor-β1 (TGF-β1) were obtained from Wako (Osaka, Japan). benzoyloxycarbonyl-Val-Ala-Asp-(OCH3) fluoromethy ketone was purchased from R&D Systems (Minneapolis, MN). The minimum detectable concentration of 15d-PG J2 in this assay is typically less than 36.8 pg/ml. Cross-reactivity was only detectable for PG J2 (49.2%), Δ2-PG J2 (5.99%), and PG D2 (4.92%) but was less than 0.01% for other PGs according to the manufacturer's instructions.

Heparin (0.3 mg/ml) was added as an anticoagulant to the synovial fluid taken from 25 OA or 29 RA patients. The synovial fluids were then centrifuged at 10 min at 1000 °C, and washed by suspension and centrifugation in DMEM. Cells, debris, and apoptotic bodies were pelleted by centrifugation and then washed once with phosphate-buffered saline. The washed pellet was subsequently labeled with Guava ViaCount reagent (Guava, Hayward, CA). Viable, apoptotic, and dead cell populations were analyzed using a small desktop Guava personal cytometer with ViaCount and Express software (Guava). The system counts the stained nucleated events, then uses the forward scatter properties to distinguish free nuclei and cellular debris from cells to determine an accurate cell count (24). The collected chondrocytes were also labeled with 0.5 μg/ml annexin V-fluorescein isothiocyanate (Wako) and 2 μg/ml propidium iodide (Sigma) to document early stages of chondrocyte apoptosis. The fluorescein isothiocyanate signal of annexin V was detected at 518 nm by FL1 (fluorescein isothiocyanate detector), and propidium iodide fluorescence was detected at 620 nm by FL2 (phycoerythrin fluorescence detector).

#### Nuclear Morphology

The harvested primary passage chondrocytes were seeded into 8-well chamber slides (300 μl of cell suspension/well). When confluent, nuclear morphology was assessed by labeling stimulated or unstimulated adherent cells or 30 min at 37 °C with 1 μM Hoechst 33342 (Sigma). Cultures were then washed three times with phosphate-buffered saline and examined by fluorescence microscopy.

#### Cell Viability Assay

The chondrocytes were seeded into 6-well plates at a density of 1 × 10⁶ cells/well. After serum starvation for 24 h, they were treated with the indicated effectors. Untreated cells served as controls. After medium was transferred to a 15-ml conical tube, the adherent chondrocytes were removed by trypsin/EDTA treatment and transferred to the tube containing culture medium. Cells, debris, and apoptotic bodies were pelleted by centrifugation and then washed once with phosphate-buffered saline. The washed pellet was subsequently labeled with Guava ViaCount reagent (Guava, Hayward, CA). Viable, apoptotic, and dead cell populations were analyzed using a small desktop Guava personal cytometer with ViaCount and Express software (Guava). The system counts the stained nucleated events, then uses the forward scatter properties to distinguish free nuclei and cellular debris from cells to determine an accurate cell count (24). The collected chondrocytes were also labeled with 0.5 μg/ml annexin V-fluorescein isothiocyanate (Wako) and 2 μg/ml propidium iodide (Sigma) to document early stages of chondrocyte apoptosis. The fluorescein isothiocyanate signal of annexin V was detected at 518 nm by FL1 (fluorescein isothiocyanate detector), and propidium iodide fluorescence was detected at 620 nm by FL2 (phycoerythrin fluorescence detector).

#### Reverse Transcriptase (RT)-PCR and Real-time PCR Analysis

Total cellular RNA was extracted from confluent chondrocytes by a single-step guanidinium thiocyanate-phenol-chloroform method using Isogen (Nippon Gene, Toyama, Japan). RNA was recovered in diethyl cyanophosphonated water and quantified by spectrophotometry at 260 and 280 nm. For RT-PCR, RNA samples were reverse-transcribed to cDNA using reverse transcriptase (Invitrogen) and random hexamers (TaKaRa Biomedical, Osaka, Japan). The primer sequences as well as the number of cycles are shown in Table I. The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, 30–35 cycles of amplification (1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C) in an automated thermal cycler (TaKaRa Biomedical) followed by a final extension step of 5 min at 72 °C. The PCR products were separated on

### Table I

| Gene       | Primer          | Sequence               | Size (bp) | Cycle | Accession number |
|------------|-----------------|------------------------|-----------|-------|------------------|
| GAPDH      | Primer          | Sequence               | Size (bp) | Cycle | Accession number |
| PGDS-Hematopoietic | 5' | AGACACAGTGTGACGCG | 273       | 35    | NM_014485        |
| PGDS, brain | 3' | AGCCGCTATGACGCT    | 204       | 35    | BC005939         |
| PPARγ      | 5' | TCCCCTGCAACGAAAG   | 356       | 35    | BC006811         |
| p53        | 5' | AGCTCTTATCCGGATGG  | 300       | 35    | NM_000546        |
| GAPDH      | 5' | CCCCTCAGTATGAGAACC | 226       | 30    | NM_002046        |
Main-

Whitney U test.

Mann-Whitney U test. represent 50th percentiles; minescent (ECL) system (Amersham Biosciences). and rabbit anti-p38. The blots were developed using a horseradish phosphorylated kinase (ERK) 1/2, rabbit anti-ERK1/2, rabbit anti-phospho-p38, Cell Signaling Technology: goat anti-phospho- extracellular signal-regulated kinase (ERK) 1/2, rabbit anti-ERK1/2, rabbit anti-phospho-p38, immunoglobulin G as primary antibody served as a negative control.

1% agarose gels and photographed under ultraviolet excitation after ethidium bromide staining. PPARγ and p53 mRNA expression levels were also determined by quantitative real-time RT-PCR using fluorescence-labeled (Light Cycler-Fast Start DNA Master SYBR Green I, Roche Applied Science) primers and LightCycler software (Roche Applied Science). Normalized gene expression was calculated as the ratio between PPARγ or p53 and glyceraldehyde-3-phosphate dehydrogenase copy number.

**Immunocytochemistry**

Immunohistochemical staining was performed according to the manufacturer's protocol (goat ABC staining system; Santa Cruz Biotechnology, Santa Cruz, CA). Chondrocytes were cultured at a density of 5 × 10⁴ cells per well in 4-well chamber slides. Subconfluent chondrocytes were fixed with 10% methanol. The cells were incubated with primary goat polyclonal antibody to PG D2 synthase (PGDS) or PPARγ (Santa Cruz Biotechnology) at 4 °C overnight. The cell-bound antibody complexes were then visualized by development in a substrate solution containing 3,3'-diaminobenzidine to yield a red-brown reaction product. A dilution protocol (goat ABC staining system; Santa Cruz Biotechnology, Santa Cruz, CA). Chondrocytes were cultured at a density of 5 × 10⁴ cells per well in 4-well chamber slides. Subconfluent chondrocytes were fixed with 10% methanol. The cells were incubated with primary goat polyclonal antibody to PG D2 synthase (PGDS) or PPARγ (Santa Cruz Biotechnology) at 4 °C overnight. The cell-bound antibody complexes were then visualized by development in a substrate solution containing 3,3'-diaminobenzidine to yield a red-brown reaction product. A dilution of normal goat serum containing the same concentrations of nonspecific immunoglobulin G as primary antibody served as a negative control.

**Western Blotting Analysis**

Whole cell lysates were prepared from 1.5 × 10⁶ chondrocytes stimulated with the indicated effectors. The trypsinized adherent cells were pelleted by centrifugation and lysed with 0.1 ml of ice-cold lysis buffer containing 20 mM Tris-Cl, pH 7.4, 250 mM NaCl, 1% Nonidet P-40, and 0.1% SDS and supplemented with protease inhibitors and phosphatase inhibitors (10 mM NaF and 2 mM NaVO₄). The lysates were transferred to Eppendorf tubes, and the protein concentration was determined by Bradford assay. Similar amounts of protein were size-fractionated by 10% SDS-PAGE on polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk in phosphate-buffered saline, 0.1% Tween 20, the protein expression was determined using specific antibodies purchased from Cell Signaling Technology: goat anti-phospho- extracellular signal-regulated kinase (ERK) 1/2, rabbit anti-ERK1/2, rabbit anti-phospho-p38, and rabbit anti-p38. The blots were developed using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescent (ECL) system (Amersham Biosciences).

**Caspase-3 and NF-κB Activity Assay**

Caspase-3 activity was assayed on whole cell lysates by commercially available kit (Roche Applied Science) according to the manufacturer's protocols. Briefly, after treatment of cells (1–2×10⁶ cells in a 10-cm dish) with or without the indicated stimulation, the adherent chondrocytes were harvested by trypsinization and lysed for 1 min on ice in 200 μl of lysis buffer containing 10 mM dithiothreitol. The lysates were pooled and centrifuged, and the supernatant was used to determine caspase-3 activity. The fluorescence intensity was measured with an excitation filter of 420 nm and an emission filter of 535 nm. The whole cell lysates were also used for NF-κB p65 activity determination using a commercially available kit (Pierce). Briefly, the 96-well plate was coated with the bound NF-κB-biotinylated consensus sequence so that only the active form of NF-κB bound to the DNA sequence. Whole cell lysates were added to the plates and incubated with the specific primary antibody (NF-κB p65) followed by the secondary antibody, and the resulting signal was then captured by chemiluminescent detection.

**Statistical Analysis**

For all results shown at least three separate experiments with cells from different donors were performed. Within experiments, each individual measurement was performed in duplicate. Except where specifically mentioned, one-way analysis of variance was used to analyze the differences between different groups for the production of 15d-PGJ2 and the percentage of apoptosis. The data on 15d-PGJ2 concentrations in synovial fluids are nonparametric. Therefore, the Mann-Whitney U test was used to compare the difference between OA and RA patients. p < 0.05 was considered significant.

**RESULTS**

Production of 15d-PGJ2 by Chondrocytes and Concentrations in Synovial Fluid—We first investigated whether cultured human articular chondrocytes produce 15d-PGJ2 in vitro. As shown in Fig. 1, both OA and RA chondrocytes in the resting state released 15d-PGJ2, as assayed by ELISA (OA, 221 ± 14 pg/ml; RA, 297 ± 51 pg/ml). However, levels were lower than in normal chondrocyte cultures (579 ± 49 pg/ml). Stimulation with IL-1β (10 ng/ml) and SNP (2 mM) led to a significantly increased production of 15d-PGJ2 in OA, RA, and normal chondrocytes. We also found that 1 μM PG E2 slightly but significantly enhanced 15d-PGJ2 production levels. TNF-α (25 ng/ml) did not significantly augment 15d-PGJ2 production. On the other hand, 15d-PGJ2 levels were significantly decreased by TGF-β1 (10 ng/ml). In particular, 15d-PGJ2 levels in OA chondrocytes after TGF-β1 treatment were below the levels of detection (Fig. 1A).

15d-PGJ2 was detectable by ELISA in synovial fluids of 25 OA and 29 RA patients whereby one of the former had a much higher 15d-PGJ2 concentration than any of the others. On the whole, however, 15d-PGJ2 concentrations in synovial fluids were at pg/ml levels, and no obvious difference between OA and RA patients was observed (Fig. 1B).

PGD2 Synthase Expression and Its Regulation by Proinflammatory Cytokines—PGD2 is naturally and rapidly converted into PG J2 by nonenzymatic pathways, and PG J2 itself is rapidly metabolized to Δ14-PG J2 and 15d-PG J2 after elimination of one or two water molecules. Therefore, we assessed
Prostaglandin J2 Induces Chondrocyte Apoptosis

Prostaglandins of the J Series Induce Chondrocyte Apoptosis

Fig. 2. PGDS expression in osteoarthritic chondrocytes. A, RT-PCR results of brain PGDS expression in OA chondrocytes stimulated with IL-1β (10 ng/ml), TNF-α (25 ng/ml), or SNP (1 mM) for the indicated times. B, RT-PCR results of hematopoietic-PGDS expression in OA chondrocytes stimulated with IL-1β (10 ng/ml), TNF-α (25 ng/ml), or SNP (1 mM) for the indicated times. C–F, immunocytochemistry confirms the expression of PGDS in OA chondrocytes. Less immunoreactivity was found in untreated chondrocytes (C). Shown are the nuclear localization of PGDS in OA chondrocytes. Immunohistochemistry was performed using polyclonal anti-PGDS antibody. We found markedly enhanced expression of PGDS in OA chondrocytes stimulated with IL-1β (10 ng/ml, 48-hour stimulation) or TNF-α (25 ng/ml, 48-h stimulation). PGDS in OA chondrocytes was mainly localized to the nucleus and cytoplasmic region (Fig. 2, D and E). OA chondrocytes treated with SNP (1 mM, 48-h stimulation) also up-regulated PGDS, but the shape of the cells after treatment was irregular, and PGDS immunoreactivity appeared to be completely localized to the nucleus (Fig. 2F). These data provide evidence that human chondrocytes are potent producers of PG J2 series molecules upon activation of PGDS by inflammatory stimuli.

Prostaglandins of the J Series Induce Chondrocyte Apoptosis—Next we evaluated the potency of the PG J series to induce chondrocyte apoptosis. First, the characteristic nuclear morphological changes, such as nuclear condensation, cell shrinkage, and appearance of small membrane-bound bodies (apoptotic bodies) were observed by Hoechst staining (Fig. 3A). This suggests apoptotic nuclear changes of chondrocytes after stimulation with PG J series molecules. Cytometry analyses were performed to quantify apoptosis induced by 15d-PG J2, an end-product of the PG J series. OA chondrocyte apoptosis was first demonstrated by annexin V/propidium iodide (PI) staining. The inversion of phosphatidylserine from the inner to the outer plasma membrane occurs early in the apoptotic program and is often used as a specific marker of cells undergoing apoptosis. Flow cytometry analyses of fluorescein isothiocyanate-conjugated annexin V binding to OA chondrocytes are shown in Fig. 3B. Chondrocytes were counterstained with PI to distinguish between viable cells (annexin V−/PI−, lower left quadrant of the histograms), early apoptotic (annexin V+/PI−, lower right quadrant of the histograms), and late apoptotic or necrotic cells (annexin V+/PI+, upper right quadrant of the histograms). Early (annexin V+/PI+) and late apoptotic (annexin V+/PI+) chondrocytes constituted a much higher percentage of the total gated cells after stimulation with 10 μM 15d-PG J2 compared with unstimulated chondrocytes. The total apoptotic cell number counted by Guava staining was in accordance with annexin V/PI staining (Fig. 3, B and C).

As summarized in Fig. 4, 15d-PG J2 induced chondrocyte apoptosis in a dose-dependent manner at concentrations of 0–10 μM in RA, OA, and normal samples (Fig. 4A) and in a time-dependent manner in OA chondrocytes (Fig. 4B). The magnitude of apoptosis induction by 15d-PG J2 was similar in RA and OA chondrocytes. The arthritic chondrocyte apoptosis was further documented by significantly increased caspase-3 activity after stimulation with 15d-PG J2 (see Fig. 7E). Furthermore, the effects of PG E2, SNP, and several proinflammatory cytokines on cell viability were compared with the effect of 15d-PG J2. Added as an exogenous donor of nitric oxide (NO), 2 mM SNP exerted a potent apoptotic effect. 1 μM PG E2 also induced chondrocyte apoptosis. However, no significant apoptotic effect of IL-1β (10 ng/ml), TNF-α (25 ng/ml), or TGF-β1 (20 ng/ml) on chondrocytes was observed (Fig. 4C). We next
explored whether a low dose of 15d-PG J2 (0.1 µM) could enhance NO-induced chondrocyte apoptosis. Neither 0.1 µM 15d-PG J2 nor 0.2 mM SNP alone could induce chondrocyte apoptosis. Nevertheless, a synergistic effect was obtained in OA chondrocytes after combined 15d-PG J2 and SNP treatment (Fig. 4D).

**Induction of OA Chondrocyte Apoptosis by PPARγ Ligands**—The above data clearly demonstrated a proapoptotic effect of 15d-PG J2. Because 15d-PG J2 is a known ligand of PPARγ, we hypothesized that 15d-PG J2-induced apoptosis might depend on the PPARγ pathway. To investigate whether apoptosis is induced by PPARγ activation, we tested three different classes of PPARγ agonists, 15d-PG J2, ciglitazone, and pioglitazone, and the PPARγ antagonists GW9662 and T0070907 for their capacity to induce or alter chondrocyte apoptosis. The two synthetic compounds ciglitazone and pioglitazone did induce OA chondrocyte apoptosis, but were not as potent as 15d-PG J2 (Fig. 5A). Although apoptosis could still be observed when a much higher concentration of 15d-PG J2 (50 µM) was added to the cell cultures, a large amount of dead cells was present (unpublished data). This implies a cytotoxic effect at this high dose of 15d-PG J2. Here we show that pretreatment of OA chondrocytes with the PPARγ antagonist T0070907 (1 µM) decreased chondrocyte apoptosis by almost 50% (Fig. 5B). Moreover, 15d-PG J2-induced chondrocyte apoptosis was almost completely prevented by GW9662 (Fig. 5C), another PPARγ antagonist which is more potent than any of the other antagonists, according to the manufacturer. Taken together, these data suggested that activation of PPARγ is involved in chondrocyte apoptosis, and that PPARγ might mediate the apoptotic effect induced by 15d-PG J2.

**PPARγ Expression in Human Chondrocytes**—First, immunocytochemistry using the polyclonal anti-human PPARγ antibody was performed to evaluate the constitutive expression of
PPARγ in human chondrocytes. Fig. 6A shows the nuclear localization of PPARγ in OA chondrocytes, whereas the negative controls treated with nonimmune serum showed no positive reaction. Effects of the proinflammatory cytokines IL-1β and TNF-α on PPARγ mRNA expression were next investigated. Both IL-1β and TNF-α stimulation up-regulated the expression of PPARγ. Induction appeared after 1 h, peaked at 6 h, and thereafter decreased with time (Fig. 6, B and C). 15d-PG J2 stimulation also increased PPARγ expression level. The peak-fold increase occurred at 6 h and lasted until 12 h after stimulation (Fig. 6, B and C).

Evaluation of the Signaling Pathway Involved in 15d-PG J2-induced Apoptosis—NF-κB is a transcription factor interfering with the induction of apoptosis (25, 26). To determine the involvement of NF-κB in 15d-PG J2-induced chondrocyte apoptosis, three approaches were used in the present study. First, we assayed the active form of NF-κB p65 in OA chondrocytes stimulated with 15d-PG J2 for different periods of time. Significantly decreased NF-κB p65 activity was found after treatment with 10 μM 15d-PG J2. Inhibition became noticeable 1 h after treatment and lasted up to 6 h, after which p65 activity began to be restored, as compared with the control group (see Fig. 7A). Second, we measured the effect of NF-κB pathway inhibitors on 15d-PG J2-induced chondrocyte apoptosis. Bay11–7085, which specifically inhibits IkB-α phosphorylation and degradation from NF-κB complex, significantly enhanced the apoptotic effects induced by 10 μM 15d-PG J2 (see Fig. 9A). Third, Western blot analysis was used as a reliable readout of
NF-κB pathway proteins. As shown in Fig. 7B, 15d-PG J2 down-regulated the level of phosphorylated IκB-α protein, whereas it had no significant effect on the level of NF-κB in OA chondrocytes. These data show that inhibition of NF-κB activity is involved in chondrocyte apoptosis induced by 15d-PG J2.

We further studied the effects of 15d-PG J2 on MAPK activation pathways by focusing on ERK and p38 kinase, the two key kinases involved in cellular apoptosis (27). Changes in the activities of ERK1/2 and p38 kinase were assessed by Western blot analysis. Interestingly, 15d-PG J2 was found to differentially regulate the activities of the two subtypes of MAPK. P38 kinase activity was transiently increased, whereas ERK1/2 was inhibited after stimulation with 15d-PG J2, as determined by the phosphorylation status of the proteins. Levels of phosphorylated p38 kinase protein expression began to increase at 30 min, reached peak levels at 6 h, and were maintained up to 12 h after stimulation. Phosphorylated ERK1/2 protein expression began to decrease from 6 h after 15d-PG J2 stimulation and remained depressed for 24 h (Fig. 7B). Confluent OA chondrocytes were then challenged with MAPK inhibitors to evaluate which kinases are involved in the chondrocyte apoptosis induced by 15d-PG J2. OA chondrocytes were preincubated for 1 h with PD98059 (25 μM), a specific inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK). Thereafter, the cells were challenged with 10 μM 15d-PG J2 for 48 h. PD98059 was found to enhance apoptosis induced by 15d-PG J2, whereas 10 μM SB202190, a specific p38 MAPK inhibitor, significantly counteracted the apoptotic effect induced by 15d-PG J2 (see Fig. 9B).

To further elucidate the mechanism of 15d-PG J2-induced OA chondrocyte apoptosis, RT-PCR and quantitative real-time PCR analyses were performed to quantify the transcriptional expression of p53, a signaling molecule that is downstream of MAPK (27). p53 mRNA expression began to increase 30 min after 15d-PG J2 stimulation and was maintained up to 6 h after treatment (Fig. 7C). Western blotting analysis showed that p53 protein expression levels began to increase from 30 min after 15d-PG J2 stimulation and persisted up to 12 h (Fig. 7B). Significantly decreased p53 expression was found after the chemical inhibition of p38 MAPK, whereas no such effect was observed after ERK1/2 inhibition (Fig. 8), suggesting that only p38 MAPK contributed to the activation of the downstream molecule p53. We also examined caspase-3 activity, an important pro-apoptotic protease that was reported to be downstream of p53 (27). Compared with the control group, caspase-3 activity was significantly increased when 10 μM 15d-PG J2 was added to both RA and OA chondrocyte cultures (Fig. 7E). The observation that chondrocyte apoptosis was almost completely abrogated by the general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-(OCH3) fluoromethyl ketone further demonstrated that 15d-PG J2-induced chondrocyte apoptosis is caspase-dependent (Fig. 9C).

**DISCUSSION**

Our present study is the first to report the *in vitro* and *in vivo* production of 15d-PG J2 by human articular chondrocytes and that 15d-PG J2 potently induces apoptotic death in these cells, thus suggesting an important contribution of this eicosanoid to the pathogenesis and/or pathophysiology of arthritis in humans.

Prostaglandins are derived from fatty acids. The main sources of prostaglandins are arachidonates, which are released from membrane phospholipids by the action of phospholipases. Arachidonic acid is first converted to the unstable endoperoxide intermediate PG H2 by cyclooxygenases and subsequently converted to related products, including PG D2, PG E2, PG F2, PG I2, and thromboxane A2, by the action of specific enzymes.
PG synthases. PG D2 is short-lived and is rapidly metabolized in vivo through different pathways including conversion to PG J series molecules. The apparent half-life of PG D2 in the blood has been reported to be 1.5 min (28). 15d-PG J2 is abundantly produced by mast cells, platelets, and macrophages and has been proposed as a key immunoregulatory lipid mediator (29). Recently Shibata et al. (30) reported the endogenous production of 15d-PG J2 in human atherosclerotic lesions and hypothesized that this compound is involved in atherosclerotic inflammation. In the present study we clarified the ability of human articular chondrocytes to produce 15d-PG J2, a regulatory lipid involved in inflammatory responses. We found that both types of PGDS, an enzyme that regulates PG J2 synthesis, are expressed in OA chondrocytes (Fig. 2). Increased levels of mRNA after stimulation with IL-1β/H9252 or TNF-α/H9251 were observed only for hematopoietic-PGDS, which was originally recognized as a splenic enzyme expressed by antigen-presenting dendritic cells as well as in mast cells (31). On the other hand, mRNA for brain PGDS, which is reported to be involved in the regulation of sleep and pain responses (32), was not altered after cytokine stimulation. These data suggest that hematopoietic PGDS is enhanced in the inflammatory milieu of joint diseases, whereas the brain-type PGDS is not induced. The expression of PGDS was also documented by immunocytochemistry, which demonstrated the localization of PGDS in cartilage. Because PGDS is abundant in articular chondrocytes, especially when challenged with inflammatory cytokines, it is likely that the cyclopentenone PG D2 metabolites are locally produced, may reach functionally significant levels during inflammation, and may play a role in cartilage destruction. The finding that significant amounts of 15d-PG J2 accumulated in the culture medium of human articular chondrocytes when challenged with an inflammatory stimulus (Fig. 1A) suggests a potential autocrine action of 15d-PG J2, which might be involved in chondrocyte destruction. In this regard, recent data suggest that 15d-PG J2 is a key regulator of negative feedback in the arachidonate cascade of the cyclooxygenase pathway (33). Our study also showed that another prostaglandin analogue, PG E2, enhanced the release of 15d-PG J2 by chondrocytes (Fig. 1A), suggesting interactions between different members of the PG family. These findings may provide new insights into the feedback mechanism of the arachidonate cascade and the regulatory role of 15d-PG J2 in inflammatory responses.

Thus far, controversy still remains regarding the role of 15d-PG J2 in inflammatory responses (21, 34). Moreover, the in vivo significance of endogenous 15d-PG J2 has even been questioned (18) because of the technical difficulties in determining its in vivo levels. However, our present study demonstrated the presence of 15d-PG J2 in arthritic synovial fluids, although at picomolar levels, using a recently developed ELISA assay (Fig. 1B). Hence, technical improvements to the experimental system, especially regarding sensitivity, will help our better understanding of the role of the cyclopentenone prostaglandins in vivo. In addition, it should be noted that arachidonate metabolism is greatly increased under several pathogenic conditions, including hyperthermia and inflammation, and local PG con-
centrations in the micromolar range have been detected at the sites of the acute inflammation (30). Systemic or local administration of nonsteroidal anti-inflammatory drugs may prevent PG secretion in patients. In particular, it is very important that articular chondrocytes secrete PG J2 in an autocrine fashion, because these cells are embedded in the extracellular matrix, which prevents them from having contact with pro-apoptotic stimuli in synovial fluids or synovial tissues. Thus, autocrine release of PG J2 might play a pivotal role in inducing chondrocyte apoptosis. It has been proposed that 15d-PG J2 might act as a “dual agent” regulating cyclooxygenase-2 expression in human chondrocytes (35). It is therefore possible that 15d-PG J2 might have diverse effects on chondrocyte metabolism, depending on the different cellular circumstances. Thus, the role of 15d-PG J2 in the regulation of cartilage metabolism is complex and remains under intense investigation. Our present study demonstrated the pro-apoptotic effect of 15d-PG J2 in articular chondrocytes, suggesting a catabolic role of 15d-PG J2 in the cartilage. The potency of apoptosis induction by 15d-PG J2 is consistent with reports on various cell lines, such as human hepatic myofibroblasts (36), vascular endothelial cells, (16) and synoviocytes (21). However, it has been claimed that the in vitro dose used in these experiments is far higher than the levels of 15d-PG J2 detectable in vivo. In this context we demonstrated that a low dose of 15d-PG J2 cooperated with low dose SNP treatment to enhance the apoptotic effect (Fig. 4D). Specifically, in a comparative study, neither IL-1β nor TNF-α alone could induce chondrocyte apoptosis. Instead, these cytokines induced the release of 15d-PG J2 by the chondrocytes.

**Fig. 7.** Signaling pathways involved in chondrocyte apoptosis induced by 15d-PG J2. Serum-starved OA chondrocytes were incubated with 15d-PG J2 (10 μM) for the indicated time periods. Chondrocytes treated with vehicle served as controls. A, DNA binding activity of NF-κB p65 was determined by chemiluminescent assay as described under “Experimental Procedures.” B, NF-κB, phosphorylated IκB-α (pIκB-α), ERK1/2, phosphorylated ERK1/2 (p-ERK1/2), p38, phosphorylated p38 (p-p38) and p53 expression were detected by Western blotting analysis. C, RT-PCR analysis of p53 expression. D, real-time RT-PCR analysis of p53 expression. E, caspase-3 activity of OA and RA chondrocytes was increased after stimulation with 15d-PG J2. Values are expressed as mean ± S.D. of three separate experiments. **, p < 0.01 versus control (Con). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
However, the amount of 15d-PG J2 secretion was unlikely to have been high enough to induce chondrocyte apoptosis by itself. Similarly, levels of 15d-PG J2 in synovial fluid were insufficient for apoptosis induction. Nevertheless, 15d-PG J2 was shown to cooperate with other proinflammatory factors such as NO, which together would be able to mediate enhanced apoptotic effects. In addition, we confirmed that not only 15d-PG J2 but also PG J2 and \( \Delta^{12} \)-PG J2 show pro-apoptotic effects (data not shown), implying that the concomitant presence of these factors might mutually enhance their catabolic effects. These results collectively emphasize that there could be a hitherto unrecognized role of 15d-PG J2 and other cyclopentenones in cartilage degradation in arthropathies such as OA and RA despite their very low levels thus far detected.

Of the naturally occurring PPAR\( \gamma \) agonists, 15d-PG J2 is among the most potent for both transactivating PPAR\( \gamma \) (13, 37) and inducing apoptosis (38). We, therefore, sought to determine whether 15d-PG J2 exerted its effects via activation of PPAR\( \gamma \). Constitutive expression of PPAR\( \gamma \) in human articular chondrocytes was documented here (Fig. 6A) and has also been reported by others (21). Furthermore, we showed that the PPAR\( \gamma \) mRNA expression level was up-regulated by the proinflammatory cytokines IL-1\( \beta \) or TNF-\( \alpha \) (Fig. 6, B and C). Our present study provided evidence that the transcriptional activation of PPAR\( \gamma \) is a critical event in 15d-PG J2-induced chondrocyte apoptosis (Fig. 5). Specifically, the two synthetic PPAR\( \gamma \) agonists ciglitazone and pioglitazone both induced chondrocyte apoptosis, although the effects of these two compounds were not as potent as 15d-PG J2. On the other hand, 15d-PG J2-induced chondrocyte apoptosis was abrogated by the PPAR\( \gamma \) antagonist GW9662 or T0070907. These data provided pharmacological evidence that PPAR\( \gamma \) is critical for chondrocyte apoptosis. In this regard, however, controversy still exists as to the molecular mechanisms of 15d-PG J2 activity. It can also exert effects that are independent of PPAR\( \gamma \), for example, induction of formation of reactive oxygen species that lead to cell apoptosis.

![Fig. 8. p38 MAPK contributed to the activation of p53. OA chondrocytes were incubated with PD98059 (25 \( \mu \)M) or SB202190 (10 \( \mu \)M) for 1 h, then with 15d-PG J2 (10 \( \mu \)M) for a further 6 h. A, real-time RT-PCR analysis of p53 expression. B, Western blotting analysis of p53 protein expression. *, \( p < 0.05 \) versus 15d-PG J2 treatment only. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.](image1)

![Fig. 9. Effect of pharmacological antagonism of NF-\( \kappa \)B, MAPK, and caspase-3 on chondrocyte apoptosis induced by 15d-PG J2. A, effect of specific I\( \kappa \)B-\( \alpha \) inhibitor Bay11-7085 (20 \( \mu \)M) on OA chondrocyte apoptosis. B, divergent effects of ERK inhibitor PD98059 (25 \( \mu \)M) and p38 kinase inhibitor SB202190 (10 \( \mu \)M) on OA chondrocyte apoptosis. C, caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe) fluoromethyl ketone (ZVAD-fmk) completely blocked the apoptotic effects of 15d-PG J2 on OA chondrocytes. Serum-starved OA chondrocytes pretreated with antagonists for 1 h followed by coincubation with 10 \( \mu \)M 15d-PG J2 for 48 h are shown. Apoptotic cell number was quantified by the Guava ViaCount assay. \( p < 0.05 \) (*) and \( p < 0.01 \) (**) versus control; \( p < 0.05 \) (#) and \( p < 0.01 \) (##) versus 15d-PG J2 treatment alone. The data represent the results of three separate experiments conducted with three different donors.](image2)
Reduced chondrocyte apoptosis. The findings of decreased NF-κB activity (Fig. 7, A) and inhibition on p53 expression confirmed the interrelationship of MAPK and p53. In our study, p38 kinase contributed to activation of the downstream p53, whereas ERK1/2 seemed not to have such an effect (Fig. 8). Kim et al. (27) previously reported that increased p53 expression was accompanied by ERK1/2 inhibition in rabbit chondrocytes after stimulation with SNP; therefore, use of different cell types or stimuli might account for the different regulatory effects observed. The above results collectively suggest that 15d-PGJ2-induced chondrocyte apoptosis is accomplished via a p53 and caspase-3-dependent pathway, as depicted in Fig. 9B.

In conclusion, our present study demonstrated the ability of human articular chondrocytes to produce PGJ2 in an autocrine fashion and a potential role of 15d-PGJ2 in the induction of chondrocyte apoptosis. Because this implies an involvement of chondrocyte apoptosis in the pathogenesis of arthritis, pathways affected by prostaglandin analogues may be an important focus of investigation to establish novel therapeutic strategies for preventing cartilage degradation in joint diseases, in the pathogenesis of which the cyclooxygenase/PG system is closely involved.

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