Activation of Interleukin-1 Signaling Cascades in Normal and Osteoarthritic Articular Cartilage

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Interleukin (IL)-1 is one of the most important catabolic cytokines in rheumatoid arthritis. In this study, we were interested in whether we could identify IL-1 expression and activity within normal and osteoarthritic cartilage. mRNA expression of IL-1 β and of one of its major target genes, IL-6, was observed at very low levels in normal cartilage, whereas only a minor up-regulation of these cytokines was noted in osteoarthritic cartilage, suggesting that IL-1 signaling is not a major event in osteoarthritis. However, immunolocalization of central mediators involved in IL-1 signaling pathways [38-kd protein kinases, phospho (P)-38-kd protein kinases, extracellular signal-regulated kinase 1/2, P-extracellular signal-regulated kinase 1/2, c-Jun NH₂-terminal kinase 1/2, P-c-Jun NH₂-terminal kinase 1/2, and nuclear factor κ B] showed that the four IL-1 signaling cascades are functional in normal and osteoarthritic articular chondrocytes. *In vivo*, we found that IL-1 expression and signaling mechanisms were detectable in the upper zones of normal cartilage, whereas these observations were more pronounced in the upper portions of osteoarthritic cartilage. Given these expression and distribution patterns, our data support two roles for IL-1 in the pathophysiology of articular cartilage. First, chondrocytes in the upper zone of osteoarthritic articular cartilage seem to activate catabolic signaling pathways that may be in response to diffusion of external IL-1 from the synovial fluid. Second, IL-1 seems to be involved in normal cartilage tissue homeostasis as shown by identification of baseline expression patterns and signaling cascade activation. (*Am J Pathol* 2007, 171:938–946; DOI: 10.2353/ajpath.2007.061083)

toid arthritis, but it is also synthesized during osteoarthritic joint disease.^{1,2} Increased levels of this cytokine have been detected in synovial fluids from patients with rheumatoid arthritis and osteoarthritis,^{3,4} and its up-regulation in osteoarthritic cartilage tissue has also been reported.⁵ Osteoarthritic chondrocytes also seem to express higher levels of IL-1 receptor type 1⁶ compared with normal chondrocytes. Inhibitors of interleukin-1 converting enzyme, a protease essential for IL-1 β processing, are able to reduce collagen-induced arthritis.⁷ However, cellular responses to IL-1 β are clearly not dominating the overall gene expression pattern of osteoarthritic chondrocytes throughout the tissue⁸ (unpublished data). In addition, contradicting data exist on a potential up- and down-regulation of IL-1 β in osteoarthritic cartilage.^{5,9,10} Subsequently, the question of the importance of IL-1 in regulating osteoarthritic cartilage degeneration is largely unresolved.

IL-1 is known to produce a plethora of effects in chondrocytes including i) a significant reduction in the expression of anabolic genes such as *aggrecan* and *collagen type II*^{11–13}; ii) up-regulation of various catabolic genes such as matrix degrading proteases (matrix metalloproteinase-1, -3, -13, and ADAMTS-4)^{13–15}; and iii) strong induction of intercellular mediators such as leukemia inhibitory factor and IL-6.^{16–18} IL-1 activity within cells is mainly mediated by four classic cellular signaling pathways,¹⁹ three of which belong to the mitogen-activated protein kinase (MAPK) pathways. These are channeled by three key enzymes among others: c-Jun NH₂-terminal kinase (JNK) 1/2 (jun kinases), 38-kd protein kinases (p38), and extracellular signal-regulated kinase (ERK) 1/2. A fourth signaling pathway that mediates IL-1 signaling involves nuclear factor κ B (NF- κ B), which is also the typical mediator of tumor necrosis factor- α signaling within cells. Whereas the MAPKs are activated by phosphorylation, active NF- κ B is produced by its release from the inhibitory protein I κ B. Here, I κ B is phosphorylated and

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Interleukin (IL)-1 is thought to be one of the most important catabolic cytokines produced by chondrocytes in rheuma-

degraded, thereby permitting NF- κ B to translocate into the nucleus and function as a transcription factor. In contrast, the MAPKs phosphorylate a large variety of transcription factors such as c-jun and c-fos in a relatively specific manner.¹⁹ Previous reports have shown the presence of these mediators *in vitro* in normal¹⁶ and osteoarthritic chondrocytes.²⁰ Regarding the expression and activation *in vivo*, only preliminary studies of two samples exist, suggesting that activated P-JNK is present in osteoarthritic cartilage but not in normal cartilage.²¹ In this study, we were interested in identifying the major IL-1 signaling pathways that are active in chondrocytes of normal and osteoarthritic human adult articular cartilage.

Materials and Methods

Tissue Specimens

For the mRNA expression analysis, cartilage from human femoral condyles of the knee joints was used. Normal articular cartilage ($n = 13$; 39 to 76 years; mean age, 58.6 years) and early degenerated cartilage ($n = 14$; 49 to 91 years; mean age, 69.1 years) were obtained from donors at autopsy within 48 hours of death. Osteoarthritic cartilage samples from late stage osteoarthritic joint disease were obtained from patients undergoing total knee replacement surgery ($n = 11$; 61 to 76 years; mean age, 70.7 years). Cartilage was considered normal if it showed no significant softening or surface fibrillation. Early degenerated cartilage was defined as cartilage that showed moderate fibrillation and softening but no advanced erosion of the articular cartilage. Only this cartilage was taken for the study and not the peripheral areas showing no obvious signs of degeneration. Late-stage osteoarthritic cartilage was derived from patients undergoing knee arthroplasty due to complete destruction of the articular cartilage in major portions of the joints. Cases of rheumatoid arthritis were excluded from the study. Only primary degenerated and not regenerative cartilage (osteophytic tissue) was used.

Histomorphology and Histochemistry

Hematoxylin and eosin and toluidine blue staining were performed on all tissue sections to evaluate matrix abundance, cellularity, and the content of glycosaminoglycans. Histopathological grading was performed according to the Mankin grade.²²

Immunohistochemistry

Conventional immunohistochemical studies were performed on paraformaldehyde-fixed and paraffin-embedded specimens of normal ($n = 8$) and late-stage osteoarthritic ($n = 12$) articular cartilage using a streptavidin-biotin complex technique (Biogenex, San Ramon, CA) with alkaline phosphatase as the detection enzyme (Biogenex), as described previously.²³

To obtain optimal staining results for the antibodies, we used various enzymatic pretreatments including hyaluronidase (2 mg/ml in phosphate-buffered saline, pH 5, for 60 minutes at 37°C; Boehringer, Mannheim, Germany); pronase (2 mg/ml in phosphate-buffered saline, pH 7.3, for 60 minutes at 37°C; Sigma, Munich, Germany); chondroitinase avidin-biotin complex (0.25 U/ml in 0.1 mol/L Tris-HCl, pH 8, for 60 minutes at 37°C; Sigma); or bacterial protease XXIV (0.02 mg/ml; phosphate-buffered saline, pH 7.3, for 60 minutes at 37°C; Sigma). The optimal assay conditions as well as the source of the antibodies are shown in Table 1.

All immunohistochemical slides were evaluated by two different observers, independently. Staining intensity was evaluated for four different regions: uppermost superficial zone (which is missing in osteoarthritic cartilage), intermediate zone (upper third of the cartilage), and deep zone. The deep zone was further divided into upper and lower deep zones (middle and lower third of the cartilage). The staining intensity was estimated using a qualitative scale (0, no staining; 1, very weak (maybe focally); 2, weak staining in the majority of cells; 3, intermediate staining intensity in the majority of cells; 4, strong stain in most cells; and 5, very strong staining in the majority of cells).

Confocal Laser Scanning Microscopy

Immunofluorescence analysis and confocal scanning microscopy were performed using a Leica TCS SP1 microscope (Leica Microsystems GmbH, Heidelberg, Germany) as described previously.²⁴ Fluorochrome-labeled secondary antibodies (fluorescein isothiocyanate, Texas Red; and Cy-5, Dianova, Hamburg, Germany) were used for visualization of the antigens. Nuclear DNA was counterstained with propidium iodide.

Cell Isolation—Stimulation with IL-1 β

For *in vitro* studies, macroscopically normal articular cartilage ($n = 3$) was taken from the weight-bearing areas of

Table 1. Primary Antibodies and Enzymatic Pretreatments Used for Immunohistochemical Analysis

Antigen	Type	Dilution	Digestion	Source
Phospho-ERK1/2	r	1:100		Promega, Mannheim, Germany
Phospho-p38	r	1:100		Promega
Phospho-JNK1/2	r	1:100	Pt	Promega
ERK1/2	r	1:5000	Pt	Santa Cruz, Heidelberg, Germany
p38	r	1:100		Abcam, Cambridge, UK
JNK1/2	r	1:500	Pt	Cell Signaling Technology, Danvers, MA
NF- κ B-p65	r	1:500	Pt	Delta Biolabs, Gilroy, CA

r, rabbit polyclonal; Pt, protease XXIV [0.02 mg/ml, phosphate-buffered saline, pH 7.3, 60 minutes at room temperature (Sigma)].

Table 2. Sequences of Primers and Probes for Quantitative Online PCR Experiments

	Accession no.	Primers	nmol/L	Probe	Mg mmol/L
Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	Forward: 5'-GAAGGTGA AGGTCGGAGTC-3'	50	5'-CAAGCTTCCCGTTCT CAGCC-3'	5.5
		Reverse: 5'-GAAGATG GTGATGGGATTTC-3'	900		
IL-1 β	NM_000576	Forward: 5'-CTTTGAAGC TGATGCCCTAAA-3'	300	5'-TCCAGGACCTGGA CCTCTGCCCTCT-3'	5.0
		Reverse: 5'-AGTGGTGT CGGAGATTTCGTA-3'	900		
IL-6	NM_000600	Forward: 5'-TGACAAACAAA TTCGGTACATCCT-3'	50	5'-CAGCCCTGAGAAA GGAGACATGTAACAA-3'	5.5
		Reverse: 5'- TCTGCCAG TGCCTCTTTGCT-3'	900		

the femoral condyle and tibial plateau under aseptic conditions at autopsy and within 48 hours of death (mean age, 61.8 years). Cartilage pieces were finely diced, and chondrocytes were enzymatically isolated from associated matrix as previously described.¹⁴ After 48 hours of recovery time, the cells were treated with 0.01 to 10 ng/ml IL-1 β (Biomol, Hamburg, Germany) for 96 hours in 10% fetal calf serum. Cultures for isolation of RNA were washed once in phosphate-buffered saline, and cells were lysed in 100 μ l/10⁶ cells Qiagen RLT lysis buffer (Qiagen, Hilden, Germany), containing 1% β -mercaptoethanol. Lysates were stored at -20°C.

Tissue Cultures—Stimulation with IL-1 β

Cartilage from three normal donors (mean age, 68.7 years, within 48 hours of death) were finely diced into 2 $\times$ 2 $\times$ 2-mm³ pieces and washed with Dulbecco's modified Eagle's medium/Ham's F-12 media (Gibco, Eggenstein, Germany). Before stimulation with IL-1 β , cartilage explants were cultured overnight in Dulbecco's modified Eagle's medium/Ham's F-12 media supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany). Cartilage plugs were then stimulated with 10 ng/ml IL-1 β for 0, 5, 30, and 60 minutes in Dulbecco's modified Eagle's medium/Ham's F-12 media supplemented with 10% fetal calf serum. After stimulation, cartilage plugs were fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) and processed as described above.

RNA Isolation

RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) with an on-column DNase digestion step according to the manufacturer's instructions. Total RNA from cartilage tissue was isolated as described previously.²⁵ RNA was analyzed by ethidium bromide staining of RNA separated in 1.2% agarose gels and stored at -20°C.

cDNA Synthesis

First-strand cDNA was synthesized using 2 μ g of total RNA; 400 U of Moloney murine leukemia virus reverse

transcriptase; RNase H Minus (Promega, Mannheim, Germany); 2 mmol/L deoxynucleoside-5'-triphosphate (Roth, Karlsruhe, Germany); and 200 ng of random primers (Promega) in a total volume of 40 μ l.

Quantitative Polymerase Chain Reaction (PCR) Using TaqMan Technology

TaqMan PCR was used to detect human IL-1 β , IL-6, and glyceraldehyde-3-phosphate dehydrogenase in human articular cartilage RNA samples as described previously.^{14,26} The primers (MWG Biotech, Ebersberg, Germany) and TaqMan probes (Eurogentec, Seraing, Belgium) were designed using Primer Express software (Applied Biosystems, Darmstadt, Germany). To be able to obtain quantifiable results for all genes, specific standard curves using sequence-specific control probes were performed in parallel to the analyses. Thus, for each gene, a gene-specific cDNA fragment was amplified by the specific primers (Table 2) and cloned into a pGEM T Easy (Promega) or a pCRII TOPO (Invitrogen, Karlsruhe, Germany) vector. The cloned amplification product was sequenced for confirmation of correct cloning. Cloned standard probes were purified using the QIAfilter Midi Plasmid Kit (Qiagen) and linearized by restriction digest. Linearized standard probes were gel-purified using the QIAquick Gel Extraction Kit (Qiagen). Purified probes (fragments) were quantified using a fluorometric assay (Picogreen; Molecular Probes, Eugene, OR). Concentrations were confirmed by measuring the absorbance at 260 nm in a spectrophotometer and by comparison with DNA bands of known concentration (MassRulerDNA Ladder; MBI Fermentas, St. Leon-Rot, Germany) in an ethidium bromide-stained agarose gel. For the standard curves, concentrations of 10, 100, 1000, 10,000, and 100,000, as well as 1,000,000 molecules per assay were used (all in triplicate).

For the analyses of the different genes, a separate master mix was made up for each of the primer pairs, which contained a final concentration of 200 μ mol/L nucleotide triphosphates, 600 nmol/L Roxbuffer, and 100 nmol/L TaqMan probe. For all genes, the final reaction mix contained cDNA, 1 U of polymerase (Eurogentec), forward and reverse primers, the corre-

sponding probes, and MgCl_2 at concentrations given in Table 2. All experiments were performed in triplicate.

Statistical Evaluation

For correlation analysis a nonparametric correlation test (Kendall's tau) was used. For the *in vitro* investigations, statistical evaluation of significant differences in levels of expression was performed using the *t*-test for pairwise comparison. For the evaluation of significant differences in staining intensities a nonparametric Wilcoxon test was used. *P* values less than 0.05 were considered significant.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting

Articular chondrocytes were isolated from two normal donors (54 and 67 years) at autopsy no later than 48 hours after death as well as from two patients (64 and 76 years) with advanced osteoarthritis during endoprosthetic surgery as described above. For lysis, media were removed, and $100\ \mu\text{l}$ of $1\times$ Laemmli buffer²⁷ per 1.5×10^6 of cells was added. Gel running procedure was also according to Laemmli using self-prepared 12.5% acrylamide gels. Lysates of 0.5×10^6 cells were loaded per lane. For Western blotting, proteins were transferred to polyvinylidene difluoride membranes (Amersham, Freiburg, Germany) for 1 hour at $0.8\ \text{mA}/\text{cm}^2$. Membranes were blocked in 2% bovine serum albumin (Sigma). Primary antibodies (Table 1) and anti-rabbit secondary antibody conjugated to alkaline phosphatase (Santa Cruz Biotechnology, Heidelberg, Germany) were used in recommended concentrations. For detection, the NBT/BCIP system (Roche Applied Science, Mannheim, Germany) was used according to the manufacturer's protocol.

Results

Expression Analysis of IL-1 β and IL-6 in Articular Chondrocytes *In Vivo* by Quantitative Real-Time PCR

First, we attempted to evaluate mRNA expression levels of IL-1 β in normal and osteoarthritic cartilage samples. Very little expression of IL-1 β was found in any of the samples investigated. No significant difference was found between normal and osteoarthritic cartilage samples (Figure 1A). Next, we were interested in investigating one of the primary target genes of IL-1 β activity in articular chondrocytes, IL-6. Again, quantitative PCR analysis showed very low expression of IL-6 in normal articular cartilage with no significant up-regulation found in osteoarthritic cartilage (Figure 1A; $P > 0.1$). Finally, we were interested in whether the expression of IL-6 was directly correlated with IL-1 β expression *in vivo*, as this would be expected given the strong induction of IL-6 by IL-1 β in articular chondrocytes (see below). Interestingly, a correlation blot

Figure 1. A: Real-time PCR analysis for expression of mRNA levels of IL-1 β and IL-6 in normal, early degenerative (early deg.), and late-stage osteoarthritic (late OA) cartilage. Bars show the means and standard deviations (expression levels standardized to glyceraldehyde-3-phosphate dehydrogenase; GAPDH). B: Correlation blot analysis in between IL-1 and IL-6 expression in cartilage samples investigated (normal, early degenerated, and late-stage osteoarthritic).

showed a significant correlation of the expression of both cytokines within all cartilage ($P < 0.005$) (Figure 1B).

Expression Analysis of IL-1 β and IL-6 in Articular Chondrocytes *In Vitro* by Quantitative Real-Time PCR

Isolated normal adult human articular chondrocytes also expressed very low amounts of IL-1 β and IL-6. Of note, IL-1 β was strongly induced by itself ($P < 0.001$) independently whether serum was added to the culture medium or not (Figure 2, A and C). A very strong up-regulation of IL-6 expression was found by IL-1 β (both with and without the addition of serum to the culture medium) (Figure 2, B and C), which confirmed the correct performance of the PCR assay used.

Presence of ERK, JNK, p38, and NF- κ B in Normal and Osteoarthritic Articular Chondrocytes

Next, our interest was to investigate whether the four main intracellular IL-1 signal transduction pathways were active

C

IL-1 [ng/ml]	IL-1/GAPDH	IL-6/GAPDH
0	0.0002 $\pm$ 0.0003	0.05 $\pm$ 0.06
0.01	0.0002 $\pm$ 0.0003	0.20 $\pm$ 0.17
0.1	0.0036 $\pm$ 0.0040	1.21 $\pm$ 0.74
1	0.0215 $\pm$ 0.0264	2.83 $\pm$ 1.39
10	0.0308 $\pm$ 0.0278	3.33 $\pm$ 1.45

Figure 2. Quantitative TaqMan analysis for mRNA expression levels of IL-1 β (A) and IL-6 (B) in normal articular chondrocytes with and without stimulation with IL-1 β (in Dulbecco's modified Eagle's medium/Ham's F-12 media supplemented with 10% fetal calf serum) for 96 hours (given are the log ratios of stimulation/control of six independent experiments each). * $P < 0.05$ versus control; ** $P < 0.01$ versus control; *** $P < 0.001$ versus control. C: Table showing the absolute values (per glyceraldehyde-3-phosphate dehydrogenase; GAPDH) with standard deviations.

Figure 3. A–L: Immunostaining of total ERK (A and C), JNK (E and G), and p38 (I and K) as well as activated P-ERK (B and D), P-JNK (F and H), and P-p38 (J and L) in normal (A, B, E, F, I, and J) and osteoarthritic (C, D, G, H, K, and L) cartilage. M–P: Immunostaining of NF-κB in normal (M and N) and osteoarthritic (O and P) cartilage (N and P, confocal imaging micrographs: red, antigen staining; green, nuclear DNA counterstain). Scale bars: 50 μ m (A–M, O); 10 μ m (N and P).

in articular chondrocytes and whether there were zonal or focal alterations in these signaling cascades during the disease process. For this, we chose to localize key molecules involved in all four IL-1 signaling pathways (ERK1/2, JNK1/2, p38, and NF-κB) by immunostaining. In these experiments, staining for all four pathway molecules was found in the chondrocytes from both normal and osteoarthritic cartilage (Figure 3). The staining in the lower deep zone was weaker compared with the middle and upper deep zone (Figure 4). However, very few cells in any of the tissue zones displayed negative staining. A significant portion of the lacunae within the calcified zone did not show intact cells²⁸ and was therefore negative for these cellular mediators.

Demonstration of IL-1 Pathway Activation in Tissue Cultures of Normal Articular Chondrocytes

To check whether chondrocytes are responsive in their native environment to IL-1 β and whether this involves all four potential IL-1 signaling pathways, we took full-depth cartilage explants (three independent experiments) and stimulated them with 10 ng of IL-1 β for 5, 30, or 60 minutes. Subsequent positive immunostaining for the different phosphorylated mediators was found in most chondrocytes (Figure 5). In addition, translocation of NF-κB to the nucleus was observed (Figure 5H). The fact

Figure 4. A–C: Schematic representation of semiquantitative evaluation of the activated/phosphorylated MAPKs in normal and osteoarthritic articular cartilage sections depending on the location of the chondrocytes within the different cartilage zones. Note that the superficial zone is missing in osteoarthritic cartilage. sup, superficial zone; inter, intermediate cartilage zone; up-d, upper deep cartilage zone; lo-d, lower deep cartilage zone.

that the ERK pathway seems to be most activated, in terms of absolute staining, should be interpreted with some caution, as our immunostaining protocol was not quantitative and results may also reflect differences in antibody quality.

Confirmation of the Presence of the Intracellular Mediators and Their Potential Activation by Western Blot Analysis

Western blot analysis confirmed the presence of ERK1/2, JNK1/2, p38, and NF- κ B in lysates of freshly isolated articular chondrocytes (Figure 6A). In addition, IL-1 β was found to be able to induce phosphorylation of all three MAPKs (Figure 6B).

Analysis of Activated MAP Kinase Pathways in Normal Articular Chondrocytes

In most superficial and middle zone chondrocytes of normal articular cartilages, a weak to moderate staining for phosphorylated isoforms of all three MAPKs (P-JNK, P-p38, and P-ERK) was detectable (Figures 3, B, F, and J, and 4). In contrast, significantly less positive-staining cells were observed in the deeper zones for all three molecules. A gradient of staining was observed in all specimens investigated. In other words, none of the specimens showed an inverse pattern of staining (ie, more staining in the deeper zone compared with upper zones) or an even distribution of staining throughout all zones of the cartilage. Of all antigens analyzed, P-JNK was by far the most highly expressed mediator. However, this may be related to antibody staining properties rather than the presence of molecules in absolute amounts.

Analysis of Activated MAP Kinase Pathways in Osteoarthritic Articular Chondrocytes

Compared with the normal specimens, late-stage osteoarthritic cartilage specimens showed limited activation of P-MAPKs (Figures 3, D, H, and L, and 4). Again, a clear gradient from the upper to the lower zones was observed with the deepest zones showing the lowest levels of positive staining.

Analysis of the NF- κ B Pathway in Normal and Osteoarthritic Cartilage

NF- κ B was found to be present in nearly all cells of normal and osteoarthritic cartilage samples similar to ERK1/2, JNK1/2, and p38, with no difference detectable between normal and diseased cells (Figure 3, M and O). The staining was largely restricted to the cytoplasm as shown by conventional immunohistochemistry. To evaluate NF- κ B activation, which correlates to its translocation to the nuclear cell compartment, sections were stained with immunofluorescence and evaluated by laser scanning confocal microscopy. This confirmed, both in normal and osteoarthritic cells, nearly exclusive staining within the cytoplasm (ie, inactivated NF- κ B) (Figure 3, N and P).

Discussion

One important finding from the present study was that IL-1 β is not significantly up-regulated in osteoarthritic cartilage. Thus, our quantitative results have clarified previous contradictory reports claiming either a down-regulation or an up-regulation of IL-1 β in osteoarthritic cartilage.^{5,9,10} In line with our IL-1 expression data, no significant induction of IL-6 was observed in osteoarthritic chondrocytes *in situ*, though this gene is strongly induced by IL-1 β in articular chondrocytes.^{16,18} The observed minor changes in cytokine expression levels might be due to a selective zonal induction of IL-1 β , which, as discussed below, could be important in mediating matrix

Figure 5. Immunostaining of phosphorylated ERK1/2 (**A** and **B**), p38 (**C** and **D**), JNK1/2 (**E** and **F**), and NF- κ B (**G** and **H**; confocal analysis: red, antigen staining; green, nuclear DNA counterstain) in normal cartilage unstimulated (**A**, **C**, **E**, and **G**) and after treatment with 10 ng/ml IL-1 β for 60 minutes (**B**, **D**, **F**, and **H**).

degradation as well as normal matrix homeostasis. The presence of low IL-1 β bioactivity was, however, documented by the significant coexpression patterns of IL-6 with IL-1 β in osteoarthritic cartilage. This was most likely due to the influx of external IL-1 from synovial fluid, leading to an up-regulation of IL-6 and, to a lesser extent, IL-1 itself.

The second important new result of our study is that all major cellular signaling pathways of IL-1 β are seemingly active in articular chondrocytes *in vivo* as indicated by the ubiquitous expression of ERK1/2, JNK1/2, p38, and NF- κ B in all normal and osteoarthritic tissue samples. This was confirmed by Western blotting in isolated normal and osteoarthritic chondrocytes and is in agreement with previous reports.^{16,20} The few cells that did not stain positive for the investigated molecules, particularly in the osteoarthritic samples, might indicate individual damaged cells with aberrant gene expression pattern, which we have described for other genes as well.^{24,28} Tissue culture studies confirmed that the ERK, JNK, p38, and NF- κ B pathways can be activated in articular chondrocytes as indicated by the presence of the phosphorylated

isoforms or nuclear translocation of NF- κ B after IL-1 β stimulation. These data confirm on the tissue level *in vitro* experiments using isolated chondrocytes as those performed in this and other studies.^{16,20} These results are in agreement with the *in vitro* experiments performed in the present study as well with published data.^{16,20}

The third important result of our study is that IL-1-pathway activation was detectable both in osteoarthritic and, to a lesser extent, in normal adult articular chondrocytes. However, these signaling cascades are not specific for IL-1 (ie, they are also activated by other factors such as tumor necrosis factor- α or by overexpression of IL-1 receptors and/or accessory proteins); our data as a whole suggest that the IL-1 β pathway (or more specifically, the MAPK signaling pathway) is active in these cells.

One important question arising from the distinct distribution pattern of activated MAPKs is whether IL-1 synthesized by synovial cells diffuses from the surrounding synovial fluid²⁹ or is IL-1 locally expressed by the chondrocytes to function in an auto-/paracrine fashion. From our data, both scenarios may in fact be true. The localization of activated signaling pathways concentrated in the uppermost zones of both normal and osteoarthritic cartilage suggests that diffusion of IL-1 β from the synovial space into the articular cartilage is one likely path. Such diffusion might be enhanced in osteoarthritis due to higher levels of IL-1 β present in synovial fluid³⁰ in addition to the presence of a more damaged extracellular matrix that may permit better diffusion. Alternatively, the expression of IL-1 β might be concentrated in the upper zone (maybe even stimulated via external IL-1 β) and thus might be locally secreted and active in an auto-/paracrine fashion.^{5,10} In fact, IL-1 β and caspase 1 (or interleukin-1-converting enzyme), which is essential for IL-1 β pro-

Figure 6. **A:** Western blot analysis of cell lysates of chondrocytes isolated enzymatically from normal (N; $n = 2$) and osteoarthritic (OA; $n = 2$) cartilage showing the presence of all JNK, p38, and ERK1/2 MAPKs as well as NF- κ B. **B:** Chondrocytes were stimulated *in vitro* with 10 ng/ml IL-1 β for 10 and 20 minutes (lanes 2 and 3; lane 1, control) and probed for the three activated MAPKs. For control, PC-12 cells were treated with (lane 4) and without (lane 5) IL-1 β as positive and negative controls.

cessing, have been shown to be present mainly in the upper zones in osteoarthritic cartilage and, to a lesser degree, in normal articular cartilage.^{5,10} This localization resembles the distribution pattern of the activated mediators of the IL-1-signaling cascades found in our analysis. In addition, expression of many IL-1 effector genes in the upper zones of osteoarthritic cartilage is consistent with IL-1 activation, which results in a down-regulation of anabolic genes such as aggrecan and collagen type II³¹ and an up-regulation in matrix-degrading proteases matrix metalloproteinase-1 and matrix metalloproteinase-13.^{5,13,15} An auto-/paracrine stimulation might be supportive in this context to activate fully IL-1 β signaling, as IL-1 β concentrations found in osteoarthritic synovial fluid alone (about 28 pg/ml³⁰) would likely not be sufficient to activate the three kinase pathways in chondrocytes (unpublished data).

However, this may be different for superficial zone chondrocytes, which were reported to be more susceptible to IL-1 than the cells in the deeper zones.³² Interestingly, some activation of the IL-1 β pathways was observed in normal cartilage. Presumably, this reflects a constant baseline level of exposure of IL-1 β in the joint system. This may be due to a normal response of the synoviocytes to a continual exposure of molecular debris derived from the articular cartilage surface, even in the normal joint. In addition, there is good evidence that IL-1 β is expressed and physiologically active in some cells of all zones of normal articular cartilage, although only at a very minor level.³³ These findings suggest that IL-1 β might play a supportive role in joint tissue maintenance, which is also indicated by data obtained from IL-1 β knockout animals, since these mice display enhanced osteoarthritic cartilage degeneration.³⁴ One explanation for this might be that at least some stimulation of matrix catabolism is needed for proper matrix turnover, which is obviously essential for matrix integrity where damaged matrix molecules need to be removed and replaced by new matrix constituents. Alternatively, IL-1 β itself may, directly or indirectly, be involved in stimulation of anabolic chondrocyte activity. Such complex regulatory mechanisms of cartilage matrix homeostasis was recently proposed by Fukui and colleagues,³³ who showed that IL-1 β was able to induce anabolic activity in cartilage tissue culture, contradicting much of the previously reported *in vitro* data on isolated chondrocytes.^{15,26,35–37} This “anabolic” activity of IL-1 β seems to be mediated mostly via the induction of known anabolic factors such as bone morphogenetic protein (BMP)-2 and, to a lesser extent, BMP-7 (unpublished results).

In summary, our data suggest very low expression levels of IL-1 β both in normal and osteoarthritic chondrocytes *in vivo*. However, IL-1 may still be of major relevance for cartilage tissue integrity in two ways. First, with respect to the suppression of anabolic activity³¹ and induction of catabolic gene expression pattern^{38,39} found primarily in the progression zone of articular cartilage, this may be induced by IL-1 β diffusing in from the synovial space and then further enhanced by auto-/paracrine stimulation of IL-1 β expression and synthesis in chondrocytes of the upper zones. Second, IL-1 also seems to be

involved in normal tissue homeostasis because we observed low, baseline IL-1 expression and signaling cascade activation in normal cartilage tissue. These results suggest the presence of a complex interwoven network of cytokines and growth factors responsible for tissue homeostasis and pathology.^{33,40} The importance of IL-1 in joint and cartilage homeostasis is clearly documented by the arthritic and degenerative changes that occur in knockout mice of IL-1 and its processing molecules^{34,41} in addition to the genetic association of the *IL-1/IL-1 receptor* gene cluster with osteoarthritis development.⁴²

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