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IL-18 Is Produced by Articular Chondrocytes and Induces Proinflammatory and Catabolic Responses

Tsaiwei Olee, Sanshiro Hashimoto, Jacqueline Quach, and Martin Lotz

IL-18, a cytokine originally identified as IFN-γ-inducing factor, is a member of the IL-1 family of proteins. Because IL-1α and IL-1β are important mediators in the pathogenesis of arthritis, the present study addresses the expression of IL-18 and its role in regulating in articular chondrocytes. IL-18 mRNA was induced by IL-1β in chondrocytes. Chondrocytes produced the IL-18 precursor and in response to IL-1 stimulation secreted the mature form of IL-18. Studies on IL-18 effects on chondrocytes showed that it inhibits TGF-β-induced proliferation and enhances nitric oxide production. IL-18 stimulated the expression of several genes in normal human articular chondrocytes including inducible nitric oxide synthase, inducible cyclooxygenase, IL-6, and stromelysin. Gene expression was associated with the synthesis of the corresponding proteins. Treatment of normal human articular cartilage with IL-18 increased the release of glycosaminoglycans. These finding identify IL-18 as a cytokine that regulates chondrocyte responses and contributes to cartilage degradation. The Journal of Immunology, 1999, 162: 1096–1100.

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ynovial inflammation, hyperplasia of the synovial membrane, and degradation of articular cartilage are major components in the pathogenesis of arthritis. This is in part the consequence of activation of synoviocytes and chondrocytes by cytokines and growth factors. IL-1 and TNF-α are principal mediators in the pathogenesis of arthritis (1). Neutralization of TNF-α is a promising intervention in human rheumatoid arthritis (2), and intra-articular expression of IL-1β in rabbits can produce the major pathologies of human rheumatoid arthritis (3).

In chondrocytes two qualitatively distinct functional programs can be distinguished. The catabolic program is induced by proinflammatory stimuli and characterized by the secretion of proteases, suppression of matrix synthesis, and inhibition of chondrocyte proliferation. The anabolic program is associated with the production of extracellular matrix, protease inhibitors, and cell replication. IL-1 is the prototypical inducer of catabolic responses in chondrocytes (4). IL-1 stimulates the expression of proteases including stromelysin, collagenase (5, 6), and tissue plasminogen activator (7). IL-1 suppresses relaxin (II) procollagen mRNA expression (8) and type II collagen and proteoglycan synthesis (9). IL-1 is also a potent inducer of PG synthesis, and this is related to the induction of cyclooxygenase II (COXII) gene expression (10). Nitric oxide (NO) production is part of the catabolic program in chondrocytes. Inducible nitric oxide synthase (iNOS) is induced by IL-1 or TNF (11), but not by the growth factors TGF-β, platelet-derived growth factor, or insulin-like growth factor (12). The IL-1 inhibition of proteoglycan synthesis (9) and of chondrocyte proliferation (13) depends at least in part on endogenous NO. Exogenous NO donors can also induce apoptosis in chondrocytes (14, 15).

The formation of cartilage extracellular matrix is primarily stimulated by growth factors such as TGF-β, insulin-like growth factor, and fibroblast growth factor (16, 17). IL-1 suppresses many of the anabolic effects of these growth factors (18, 19).

In synoviocytes, IL-1 induces a similar spectrum of genes as in chondrocytes, and this includes a large number of cytokines, metalloproteases, and adhesion molecules. In contrast with chondrocytes, IL-1 stimulates the proliferation of synoviocytes and may contribute to pannus formation (20).

IL-18 is a cytokine that was originally identified as IFN-γ-inducing factor. IL-18 has structural similarities with the IL-1 family of proteins (21). IL-18 is also synthesized as a biologically inactive precursor and cleaved by the IL-1 converting enzyme (ICE, or also termed caspase 1) (22, 23). IL-18 gene expression has been demonstrated in murine macrophages, Kupffer cells (21), osteoblasts (24), and in the adrenal cortex (25). The human IL-18 cDNA was isolated from a liver cDNA library. Human rIL-18 induced IFN-γ production by mitogen-stimulated PBMC, augmented granulocyte-macrophage CSF production, and inhibited IL-10 production (26). IL-18 also stimulated the proliferation of activated T cells and inhibited the formation of osteoclast-like cells (27). The human IL-18R was purified (27), and its sequence was found to be identical to the previously known IL-1R-related protein (28).

In view of the structural homology with IL-1, the present study examined whether joint tissue cells express IL-18 and determined IL-18 effects on connective tissue metabolism. The results show that IL-18 can be produced by chondrocytes and activates responses in chondrocytes that contribute to the pathogenesis of arthritis.

Materials and Methods
Isolation and culture of chondrocytes

Cartilage from the femoral condyles and tibial plateaus of the knee joints was obtained at autopsy from donors without known history of joint disease or from healthy organ donors from the University of California at San Diego tissue bank. Cartilage slices were cut into pieces (2–3 mm²), washed with DMEM (BioWhittaker, Walkerville, MD), and treated for 15 min with trypsin (10% v/v) in a 37°C waterbath. The tissues were transferred to DMEM, 5% FBS, penicillin-streptomycin-fungizone, and 2 mg/ml cistriatal collagenase type IV (Sigma, St. Louis, MO) and digested overnight on a gyratory shaker. The cells were washed three times with DMEM and cultured in DMEM and 5% FBS.
**Chondrocyte proliferation studies**

All experiments were performed with chondrocytes in primary culture. Cells were distributed into 96-well plates (5000/well) in a total volume of 100 or 200 µl of DMEM supplemented with l-glutamine, penicillin, streptomycin, and dialyzed FBS as indicated in each experiment with the various stimuli in triplicate. After 3–5 days, the cultures were pulsed with [³H]Tdr (1 µCi/well) for 12 h and the cells were harvested on glass fiber filter paper by a multiwell automated-PHD cell harvester. Total radioactivity was quantified by liquid scintillation counting.

**RNA isolation and RT-PCR**

Total RNA was isolated by a single step guanidinium thiocyanate-phenol-chloroform method. Cells were lysed directly in the flasks using RNA Stat60 (Tel-Test, Friendswood, TX), and the samples were processed following the manufacturer’s protocol.

cDNA was prepared from RNA using random hexamers and SuperScriptII (Life Technologies, Gaithersburg, MD) according to the manufacturer’s suggestion. PCR was performed with the following primers: IL-18, 5'-GCT TGA ATC TAA ATT ATC AGT C-3' and 5'-GAA GAT TCA AAT TGC ATC TTA T-3' (26); IL-6, 5'-CAC ACA CAG CCA CTC ACC TCT TC-3' and 5'-GCT GCG CAG AAT AGT AGT TGT-3'; stromelysin, 5'-TGG ACA AAG CAT ACA AGA GG-3' and 5'-AGC TCG TAC CTC ATT TCC TCT G-3'; COXII, 5'-TTG AGA AGC AGG C-3' and 5'-CAT TCC TAC CAG CAA CC-3'; iNOS, 5'-ACA TTG ATC AGA AGC TGT CCC AC-3' and 5'-CAA AGG CTC GGA TGC TGT CAC-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TTG TAT CGT CGA AGG ACT CAT GAC-3' and 5'-ATG CCA GTG AGC TTC CCG TTC AGC-3'.

**Quantitation of nitrates**

Chondrocytes were plated at 50,000 cells per well in 96-well plates and cultured for 48 h when culture supernatants were collected for nitrite measurements.

NO formation was detected by NO₂⁻ accumulation in the culture supernatants by the Griess reaction, using sodium nitrite as standard. Briefly, 50 µl of culture supernatant were incubated with 50 µl 1% sulfanilamide and 0.1% N-1-naphthylethylendiamine dihydrochloride in 25% H₃PO₄ at room temperature for 5 min. Optical density was measured at 570 nm.

**IL-6 ELISA**

Half-area ELISA plates (Costar, Cambridge, MA) were coated with 25 µl of 2 µg/ml monoclonal mouse anti-human IL-6 Ab (R&D Systems, Minneapolis, MN) for 3 h and blocked with 3% BSA. Twenty-five microliters of samples or human IL-6 standard were added to the plate and incubated for 2 h followed by 1 µl/mg rabbit anti-human IL-6 (R&D Systems). Biotin-labeled goat anti-rabbit Ig (Sigma) was then added (1:2000 dilution). Bound IL-6 was detected with 0.16 ng/ml polyhorseradish peroxidase labeled streptavidin (Accurate Chemicals and Scientific, Westbury, NY) and TMB plus hydrogen peroxide substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The sensitivity of the assay is 1–1200 pg/ml.

**Immunoprecipitations**

Protein synthesis was analyzed by metabolic labeling and immunoprecipitation. Primary chondrocytes were washed in PBS and incubated in cysteine- and methionine-free RPMI 1640 (ICN Biomedicals, Costa Mesa, CA) supplemented with 10% dialyzed FBS, and [³⁵S]methionine (ICN Biomedicals; 100 µCi/ml) for 24 h. Caspase-1 inhibitor (10 μM; Calbiochem, San Diego, CA) was added with IL-1 in one experiment. The supernatants were collected and incubated with 4 µg rabbit anti-human IL-18 (Peprotech) overnight on a rotor (4°C) and 1.5 h later with goat anti-rabbit Ab coated-agarose beads (Sigma). The precipitates were washed five times with RIPA-M buffer (50 mM Tris (pH 7.4), 50 mM NaCl, 0.5% Nonidet P-40, 1 mM EGTA, 1 mM Na₂VO₄, 1 mM NaF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM PMSF). The beads were boiled for 3 min in Laemmli buffer and the proteins were separated on 15% SDS-polyacrylamide gels under reducing conditions. Gels were fixed in 10% acetic acid, treated with Amplify (Amersham, Arlington Heights, IL) for 30 min, dried, and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY) at ~70°C for 2–4 days.

**Western blot analysis**

Cell lysates were prepared for Western blot analysis of iNOS and COXII. Passage 1 chondrocytes were plated and cultured in serum-free media for 24 h. Cells were then cultured with or without IL-1β (1 ng/ml) for 24 h. After they were washed with cold PBS, the cells were lysed with RIPA-M buffer. The lysates of chondrocytes and standard of iNOS were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Western blot analysis was performed with primary Abs to iNOS and COXII (Cayman Chemical, Ann Arbor, MI).

**Proteoglycan release from articular cartilage**

Pieces of full thickness articular cartilage were incubated for 72 h in the presence of cytokines. Conditioned media were collected and the concentration of sulfated glycosaminoglycans (GAG) was measured using 1,9-dimethylene blue as a monitor of the spectrophotometric changes that occur during the formation of the GAG-SO₂ dye complex as described by Goldberg and Kollias (29). To generate a standard curve, chondroitin sulfate (Sigma) was used at concentrations between 1 and 20 µg/ml.

The statistical significance of the differences in GAG levels was analyzed by paired Student’s t test.

**Cytokines**

Human rIL-18 was purchased from Peprotech (Rocky Hill, NY). The ED₅₀ was determined by the stimulation of IFN-γ production by human PBMC stimulated with either Con A or PHA is 15 ng/ml. The ED₅₀ of human rIL-1β (Peprotech) as determined by the stimulation of [³H]thymidine incorporation by murine C3H/HeJ thymocytes is 0.1 ng/ml. Endotoxin levels of IL-1 and IL-18 were less than 0.1 ng per µg of cytokine preparation. In our experiments, IL-1 was used at 1 ng/ml and IL-18 at 10 ng/ml, thus the endotoxin contamination level for IL-1 was less than 0.1 pg/ml, whereas for IL-18 it was less than 1 pg/ml. These levels of endotoxin when using lipopolysaccharide from Salmonella minnesota do not increase NO production in chondrocytes.

**Statistical methods**

The significance of difference between group means was determined by a Student’s t test in the program StatView.

**Results**

**IL-18 expression in chondrocytes**

Analysis for IL-18 mRNA expression was performed by RT-PCR on a total of 11 different chondrocyte isolates. Without in vitro stimulation, IL-18 mRNA was detected in 8 of 11 chondrocyte preparations. After stimulation with IL-1, all chondrocyte samples exhibited increased IL-18 mRNA expression; the results from four donors are shown in Fig. 1. The time course of IL-18 mRNA induction by IL-1 varied among donors with some showing peak expression after 1–2 h whereas this was observed in donor 3 only after 16 h (Fig. 1).

IL-18 protein synthesis in chondrocytes was tested by [³⁵S]methionine labeling and immunoprecipitation of secreted proteins.
from the culture media after 24 h stimulation with IL-1. Immunoprecipitates from unstimulated cells contained a prominent band at ~24 kDa, the molecular mass of pro-IL-18. After IL-1 stimulation, the intensity of this band increased and a newly synthesized protein was detected at 18 kDa (Fig. 2), the molecular mass of secreted IL-18. The IL-18 precursor is processed by caspase-1 to the mature secreted form (22, 23). To further ascertain that the protein detected was IL-18, a caspase-1 inhibitor (Ac-Tyr-Val-Ala-Asp-aldehyde) was added to IL-1-stimulated chondrocytes. The expression of the 18-kDa mature form of IL-18 was completely inhibited by an inhibitor of caspase-1 that did not alter the levels of the IL-18 precursor (data not shown).

**IL-18 inhibits chondrocyte proliferation stimulated by TGF-β**

IL-18 was tested in primary cultures of human articular chondrocytes for effects on cell proliferation. IL-18 did not stimulate chondrocyte proliferation. However, IL-18 significantly reduced chondrocyte proliferation induced by TGF-β (p ≤ 0.03), with inhibition ranging from ~30 to 100% (Fig. 3).

**IL-18 induces NO production**

NO is induced by proinflammatory stimuli such as IL-1, TNF-α, or LPS in chondrocytes (11, 12). At the concentration of 10 ng/ml, IL-18 increased NO production in chondrocyte cultures. This was of borderline significance (p ≤ 0.06) when compared with the control. IL-18 was less potent for the induction of NO when compared with IL-1β (Fig. 4). Chondrocytes from five donors were tested. The results shown represent average values of NO production from three donors each tested in duplicate.

**IL-18 stimulates gene expression and protein synthesis in chondrocytes**

The next series of experiments analyzed potential effects of IL-18 on selected genes that are expressed as part of the known chondrocyte response to catabolic stimuli such as IL-1 or TNF. RT-PCR was used to obtain a qualitative assessment of gene expression. IL-18 stimulation increased the steady-state mRNA levels for iNOS, COXII, IL-6, and stromelysin (STML) in chondrocytes (Fig. 5). Western blot analysis for iNOS and COXII showed that IL-18 also increased intracellular levels of these proteins in chondrocytes (Fig. 6). Using ELISA, increased levels of IL-6 protein were detected in the supernatants of chondrocytes stimulated with IL-18 for 24 h (Fig. 7).

**IL-18 effects on proteoglycan degradation**

The proteoglycan content in articular cartilage is determined by the balance between synthesis and degradation. Fresh human articular cartilage was stimulated in organ culture with IL-18. As shown in Fig. 8, IL-18 at 20 ng/ml significantly increased the release of proteoglycan from cartilage.
Discussion

IL-18 is a new member of the IL-1 family of cytokines (21). Originally identified as an IFN-γ-inducing factor with functional similarities to IL-12, IL-18 is predominantly known for its role in polarizing T cells to a Th1 response (26) and in activating NK cells. Production of IL-18 has been demonstrated for mononuclear phagocytes (21), the adrenal cortex, and the neurohypophysis (25). A recent study suggested a role of IL-18 in mesenchymal cells by demonstrating that the cytokine is expressed in osteoblast-like stromal cell lines and suppresses the formation of osteoclasts (24). As IL-1α and IL-1β are among the principal catabolic and proinflammatory cytokines in arthritis (30), the present study analyzed expression and functional properties of IL-18 in joint tissue cells.

The first part of the study examined the regulation of IL-18 expression by chondrocytes. Human articular chondrocytes were found to express IL-18 mRNA. Expression of IL-18 mRNA in chondrocytes isolated from normal articular cartilage in the absence of in vitro stimulation was variable. After in vitro stimulation with IL-1β, IL-18 mRNA was expressed in all chondrocyte preparations analyzed. The cells also produced IL-18 protein. In immunoprecipitates of chondrocytes, two forms of IL-18 were detected, the precursor with a molecular mass of 24 kDa and the mature form of 18 kDa. The synthesis of both forms was increased by IL-1 stimulation and processing of the precursor to the mature form was prevented by an inhibitor of caspase-1, previously known as IL-1 converting enzyme. Thus, in addition to mononuclear phagocytes, chondrocytes are potential sources of IL-18 in the joint. The present study did not address expression of IL-18 protein or mRNA directly in noncultured tissues from arthritis patients, but a preliminary report indicates that rheumatoid arthritis synovial tissue cells express IL-18 mRNA and spontaneously produce IL-18 protein in vitro (31).

The second part of the present study examined the role of IL-18 in the regulation of cell proliferation and secretory function of human articular chondrocytes. In chondrocytes, IL-18 inhibited cell proliferation induced by TGF-β, one of the major growth factors for these cells (18). IL-18 thus shares at least one effect with IL-1 on chondrocytes; it antagonized the action of TGF-β. IL-18 was found to be a regulator of gene expression and secretory function in chondrocytes. Representative examples of genes whose products are involved in the pathogenesis of different aspects of arthritis were analyzed. This demonstrated that IL-18 induces or increases the expression of mRNAs for iNOS and COXII, the enzymes responsible for the production of NO and PG. Expression of these mRNAs was associated with the increased synthesis of intracellular iNOS and COXII proteins. We also demonstrated increased release of NO with a borderline significance (p < 0.06) in IL-18-stimulated chondrocyte and cartilage cultures. NO is thought to be involved in the pathogenesis of arthritis through the suppression of proteoglycan synthesis (32), disruption of integrin signaling, inhibition of chondrocyte migration (33, 34), and the induction of chondrocyte apoptosis (14). PG are thought to be involved in the pathogenesis of arthritis by increasing local blood flow and vascular permeability (35). IL-18 also stimulated cytokine gene expression and protein secretion in chondrocytes as demonstrated in this study for IL-6.

The potential of IL-18 to contribute to the degradation of cartilage extracellular matrix was demonstrated by the induction of mRNA for stromelysin in articular chondrocytes. Additional evidence for catabolic effects of IL-18 was obtained in cultures of cartilage that released GAG after IL-18 stimulation. As IL-18 could induce the expression of IL-1β and TNF-α mRNA and the latter two cytokines have qualitatively similar effects on chondrocytes as IL-18, it was possible that the IL-18 effects were dependent on or mediated by the release of IL-1 or TNF. To address this, chondrocytes were treated with IL-18 in the presence of neutralizing Abs to IL-1β or TNF-α. The TNF neutralizing Ab caused a modest decrease in the IL-18-induced NO production.
whereas the IL-1 neutralizing Ab had no detectable inhibitory effect (data not shown). These findings suggest that the IL-18 effects are not IL-1 dependent and pertain to a modest degree on TNF-α. Additional support for a direct IL-18 effect on chondrocytes was obtained with kinase assays. IL-18 caused an increase in the phosphorylation of the MAP kinases p38 and JNK within 15 min (T.O., unpublished observations).

The IL-18R is a member of the IL-1R family but IL-1 does not compete binding of IL-18 to its receptor (27). Furthermore, transfection of the IL-18R was sufficient to induce intracellular signals as indicated by the activation of NF-κB, suggesting that the IL-1R accessory protein that is required for IL-1 action is not part of the IL-18R. Thus, at least at the receptor level and at the very proximal events of intracellular signaling, there are differences between IL-1 and IL-18.

In conclusion, this study indicates that human articular chondrocytes produce IL-18 mRNA and protein. IL-18 activates chondrocytes in a pattern qualitatively similar to IL-1, but IL-18 appears to be less potent as compared with IL-1. These findings characterize IL-18 as a new cytokine that can contribute to cartilage degradation.

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References


