Inflammatory Cytokines Induce Production of CHI3L1 by Articular Chondrocytes*

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Anneliese D. Recklies1, Hua Ling‡, Chantal White‡, and Suzanne M. Bernier‡

From the ‡Joint Diseases Laboratory, Shriners Hospital for Children, and the Department of Surgery, McGill University, Montreal, Quebec H3G 1A6, Canada and the ‡Department of Anatomy and Cell Biology, Canadian Institutes of Health Research Group in Skeletal Development and Remodeling, University of Western Ontario, London, Ontario N6A 5C1, Canada

Elevated levels of CHI3L1 (chitinase-3-like protein 1) are associated with disorders exhibiting increased connective tissue turnover, such as rheumatoid arthritis, osteoarthritis, scleroderma, and cirrhosis of the liver. This secreted protein is not synthesized in young healthy cartilage, but is produced in cartilage from old donors or patients with osteoarthritis. The molecular processes governing the induction of CHI3L1 are currently unknown. To elucidate the molecular events involved in CHI3L1 synthesis, we investigated two models of articular chondrocytes: neonatal rat chondrocytes, which do not express CHI3L1, and human chondrocytes, which express CHI3L1 constitutively. In neonatal rat chondrocytes, the inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) potently induced steady-state levels of CHI3L1 mRNA and protein secretion. Treatment of chondrocytes with TNF-α for as little as 1 h was sufficient for sustained induction up to 72 h afterward. Using inhibitors selective for the major signaling pathways implicated in mediating the effects of TNF-α and interleukin-1, only inhibition of NF-κB activation was effective in curtailing cytokine-induced expression, including after removal of the cytokine, indicating that induction and continued production of CHI3L1 are controlled mainly by this transcription factor. Inhibition of NF-κB signaling also abolished constitutive expression by human chondrocytes. Thus, induction and continued secretion of CHI3L1 in chondrocytes require sustained activation of NF-κB. Selective induction of CHI3L1 by cytokines acting through NF-κB coupled with the known reduction of the catabolic responses by CHI3L1 in response to these inflammatory cytokines represents a key regulatory feedback process in controlling connective tissue turnover.

CHI3L1 (chitinase-3-like protein 1; also known as HC-gp39 and YKL40) has been linked to both rheumatoid arthritis and osteoarthritis (1). Elevated levels of this protein are present in the sera and synovial fluids of patients with these diseases, and some association with disease progression has been observed (1–3). In addition, increased serum levels of CHI3L1 have been linked to other disease states associated with increased tissue fibrosis, such as cirrhosis of the liver (4) and scleroderma (5). Although CHI3L1 is often found in inflammatory environments, the factors triggering its production in these pathological conditions are currently unknown.

CHI3L1 is a 39-kDa glycoprotein secreted by articular chondrocytes (6), synoviocytes (7), and differentiated macrophages (8). It is a member of a family of mammalian proteins belonging structurally to glycohydrolase family 18 (9), which includes bacterial and vertebrate as well as invertebrate chitinases. The mammalian group of this family consists of catalytically active members (chitinase-1 or chitotriosidase and acidic mammalian chitinase) and several inactive ones (CHI3L1 and CHI3L2 in humans and YM1 and YM2 in mice). The structures of the human CHI3L1 (10, 11) and murine YM1 (12, 13) proteins have been solved, demonstrating the conservation of the catabolic structural framework in the inactive members of this protein family. However, although a lectin-like function has been predicted, no physiological ligands have as yet been identified.

CHI3L1 is synthesized constitutively by isolated human articular chondrocytes or in cartilage explants (6), whereas its secretion from macrophages in vitro is associated with differentiation preceding induction and secretion of chitotriosidase (8). CHI3L1 is secreted from human articular cartilage explants or isolated human chondrocytes in culture, although its expression in vivo is restricted to older and osteoarthritic cartilage (14). Secretion of CHI3L1 from cartilage explants diminishes with time in culture, but renewed cutting of the explants appears to restore secretion levels (15), suggesting that CHI3L1 production is an injury response of the tissue. However, the factors that induce this response or how production is maintained for a relatively long time period is not known. Synthesis of CHI3L1 was reported to be enhanced by insulin-like growth factor 1 in isolated guinea pig chondrocytes (16). However, like human chondrocytes, these cells produce CHI3L1 constitutively in culture; and thus, the effect may be pleiotropic.

Some insight into possible physiological roles for CHI3L1 has been gained by the observation that this protein stimulates growth of connective tissue cells such as chondrocytes, synoviocytes, and skin fibroblasts (17, 18). CHI3L1 was reported to promote adhesion and migration of vascular endothelial cells (19, 20), suggesting a role in angiogenesis. De Ceuninck et al. (18) also reported that CHI3L1 increases proteoglycan synthesis in guinea pig chondrocytes. In addition, CHI3L1 dampens the response of chondrocytes and synovial cells to the inflammatory cytokines tumor necrosis factor-α (TNF-α)2 and interleukin-1 (IL-1), decreasing the production of matrix metalloproteases and chemokines (21). These observations suggest that CHI3L1 may play a protective role in inflammatory environments, limiting degradation of the extracellular matrix and thus controlling tissue damage. However, increased levels of CHI3L1 may also contribute to the development of tissue fibrosis.

In contrast to human articular chondrocytes, neonatal rat articular chondrocytes do not express CHI3L1 in primary culture. These cells

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1 To whom correspondence should be addressed: Shriners Hospital for Children, 1529 Cedar Ave., Montreal, Quebec H3G 1A6, Canada. Tel.: 514-282-7165; Fax: 514-849-9684; E-mail: arecklies@shriners.mcgill.ca.

2 The abbreviations used are: TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; DMEM, Dulbecco’s modified Eagle’s medium; RT, reverse transcription; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; JAK, Janus kinase.
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therefore provide an ideal system to study the regulation of CHI3L1 expression. We report here that synthesis of CHI3L1 is induced in rat articular chondrocytes by the inflammatory cytokines TNF-α and IL-1 and that this process is uniquely dependent on the activity of the transcription factor NF-κB. These results indicate that production of CHI3L1 is a component of the inflammatory response of articular chondrocytes. By feeding back to modulate the extent of the response of cells to such inflammatory cytokines, the regulated expression of CHI3L1 functions to limit the degradative response in connective tissue.

EXPERIMENTAL PROCEDURES

Exogenous Factors, Plasmids, and Inhibitors—Cells and explants were treated with factors in serum-free RPMI 1640 medium (Invitrogen, Burlington, Ontario, Canada) containing 100 units/ml penicillin, 100 units/ml streptomycin, and 10 μg/ml HEPEs. Recombinant human TNF-α, recombinant human IL-6, and its soluble receptor, and recombinant mouse epidermal growth factor were obtained from Sigma (Misisssauga, Ontario). Recombinant rat IL-1β, recombinant human IL-1β, and human parathyroid hormone-(1–34) were purchased from Pierce, R&D Systems (Minneapolis, MN), and Peptide Institute, Inc. (Louisville, KY), respectively. The vehicle for the cytokines and factors was phosphate-buffered saline and 1 mg/ml bovine serum albumin (Roche Diagnostics, Laval, Quebec, Canada). A constitutively expressed construct of wild-type IkB (pSVK3-IκB) and a dominant-negative form of IkB (pSVK3-IκB-2NA4) were a generous gift from Dr. J. Hiscott (Lady Davis Institute for Medical Research, McGill University) (22). The specific antibody for IkBα was purchased from New England Biolabs Inc. (Beverly, MA). Mouse anti-human NF-κB p65 and p50 monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Radiolabeled [32P]CTP was obtained from ICN Biomedicals (Aurora, OH), and the 3′-end labeling kits were obtained from Amersham Biosciences (Montreal).

In some experiments, pharmacologic inhibitors were used to selectively block individual signaling pathways. AG490, BAY 11-7085, BAY 11-7082, bisindolylmaleimide I, cyclosporin A, FK506, lactacystin, LY294002, PD98059, wortmannin, SN50, and inactive analogs of bisindolylmaleimide I (bisindolylmaleimide V) and U0126 (U0124) were purchased from EMD Biosciences (San Diego, CA). U0126 was obtained from Promega Corp. (Madison, WI).

Cell Cultures—Chondrocytes were harvested from the distal femoral condyles of 1-day-old Sprague-Dawley rats as described previously (23). Typically, 8–12 x 10^5 cells were obtained per condyle. Cells were cultured in RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 10 μg/ml HEPEs, and 5% fetal bovine serum (Invitrogen). Cells were plated at 450–550 cells/mm^2 in 60-mm dishes (BD Biosciences, Mississauga). The medium was changed every 3 days and was replaced with serum-free medium 1 day prior to experiments except where indicated. All experiments were carried out on either primary or first passage cultures that retained expression of chondrocytic phenotypic markers. Fibroblast cultures were established by placing minced fragments of neonatal rat skin in culture dishes to allow outgrowth of fibroblasts, which occurs within a couple of days. Osteoblasts were prepared by timed sequential collagenase digestions of neonatal rat calvaria (24). Fractions 2/3 and 4/5 represent early and late stage osteoblasts, respectively. Skin fibroblasts and osteoblasts were seeded at 500 cells/mm^2 and allowed to expand to near confluence before exposure to cytokines.

Rat femoral heads were used for explant culture of intact articular cartilage. They were dissected clear of the ligamentum teres, washed three times with phosphate-buffered saline containing 100 units/ml penicillin and 100 units/ml streptomycin, and cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum.

Human juvenile chondrocytes had been prepared previously from knee cartilage obtained at autopsy (6) and maintained as frozen stocks after one passage. The cells were revived and expanded in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. For expression studies, trypsinized cells were plated at 500 cells/mm^2 and allowed to adhere for 48 h in serum-containing medium unless indicated otherwise. The human chondrocytes used in this study were between passages 3 and 5. Although type I collagen mRNA transcripts could be detected by reverse transcription (RT)-PCR, the cells still expressed mRNA for type II collagen and aggrecan, indicating that they retained a chondrocytic phenotype.

RNA Extraction and RT-PCR—Confluent cultures were serum-deprived for 24 h prior to addition of exogenous factors in the presence or absence of pharmacologic inhibitors. Total RNA was collected from cells after 24 or 48 h using the acid/guanidium/phenol/chloroform extraction method (TRizol, Invitrogen) according to the manufacturer’s instructions. Levels of CHI3L1 transcripts were analyzed by RT-PCR using oligonucleotides GCCCCTGACCATTTCCCTGTGACC (upstream primer) and TGTTCTGCTGCCTCGAGAGG (downstream primer), giving rise to a 550-bp amplification product. Levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts were evaluated as housekeeping gene using upstream primer GACGCCCA- GAACATCATCCTCCGCA and downstream primer CATTTTCAT- CCAGGAATGAGCCTT, resulting in a 372-bp amplification product. For analysis of rat MMP-13, oligonucleotides CTGACACCTCAGCA-GTTGA and CTCATAGACAGCATCATTGTTC were used for upstream and downstream priming, respectively, resulting in a 205-bp amplification product. Expression of GRO-1 was assessed using upstream primer GCGAGGATTCCTCAAGA and downstream primer GCCATCGGCAATCATTGTTC, giving rise to a 205-bp product. All primers were synthesized in the Biotechnology Core Facility of the Shriners Hospital for Children. All primers were designed for a melting temperature of ~60 °C to allow parallel amplification of different primer sets. RNA prepared from involuting rat mammary gland was used as a positive control for RT-PCR amplification of CHI3L1 because it is expressed at high levels in corresponding mouse tissue (25). Mammary glands from lactating rats were harvested from dams with litters, used by Dr. Lee (Shriners Hospital for Children, Montreal, Canada) in a study requiring harvesting of neonatal rat bones. Mammary gland tissue was collected when no pups remained, and the dams were killed. RNA was prepared from the tissue as described above.

Collection and Analysis of Conditioned Culture Media—Confluent cultures of chondrocytes, fibroblasts, and osteoblasts or explant cultures of three femoral heads/well were serum-deprived for 24 h prior to addition of exogenous factors in the presence or absence of pharmacologic inhibitors. Culture media were collected after 24 or 48 h, and proteins were precipitated overnight by addition of 2 volumes of cold acetone. The precipitate was recovered by centrifugation. Pellets were redissolved in 0.1 volume of SDS sample buffer unless indicated otherwise, separated by 12% SDS-PAGE, and analyzed by Western blotting using a polyclonal antibody recognizing rat CHI3L1 prepared as described below. For detection of human CHI3L1, a polyclonal antibody raised against the purified protein was used as described previously (6). Antibody dilution was 1:1000 unless stated otherwise.

For detection of rat CHI3L1, a polyclonal antibody was prepared using the C-terminal peptide sequence CGGKEALAVA. The N-terminal sequence CGG was added to provide a linker for coupling to ovalbu-
min for immunization. All peptides were synthesized and purified in the Biotechnology Core Facility at the Shriners Hospital for Children. The antiserum was shown by Western blotting to be specific for rat CHI3L1; it did not react with the human or murine protein. The reactivity could be absorbed with the unconjugated peptide used for immunization, but not with C-terminal peptides based on the human and murine sequences. This antiserum was used for all Western blot analysis at 1000-fold dilution. Bound immunoglobulin was visualized by enhanced chemiluminescence (ECL, Amersham Biosciences, Baie d’Urfe, Quebec). To identify the protein species, bands were excised from the SDS-polyacrylamide gels and subjected to peptide fragmentation and analysis by mass spectrometry, performed at Génome Québec (Montreal).

Transfection of Human Chondrocytes with IκB Constructs—The plasmids pSVK3-IκB, pSVK3-IκB-2-NA4, and pCMVS-EGFP were transfected into human articular chondrocytes using Magnetofection™ (OZ Biosciences, Marseilles, France) to enhance transfection efficiency. Chondrocytes (5 × 10^5 cells/well) were seeded into 6-well plates and allowed to attach for 48 h in the presence of DMEM supplemented with 10% fetal bovine serum. The cell layers were washed three times with serum-free DMEM to remove residual serum components. 2.5 μg of freshly purified DNA (using a plasmid purification kit, Promega Corp.) was added to 0.2 ml of DMEM, followed by 2 μl of PolyMag II transfection reagent (OZ Biosciences). This mixture was added to 0.5 ml of DMEM covering the cell layer, and the plates were exposed to a magnetic field for 10 min at room temperature, followed by an additional 6-h incubation at 37°C. The medium was replaced with fresh DMEM containing 10% fetal calf serum, and the cells were allowed to recover overnight. To determine levels of synthesis and secretion of CHI3L1, the cells were exposed to serum-free medium for 24 h. Responsiveness to TNF-α was determined by addition of 30 ng/ml TNF-α during the last 30 min of the culture period. Cell layers were harvested and analyzed for the presence of CHI3L1 and its secretion of CHI3L1 during the last 30 min of the culture period. Cell layers were harvested and analyzed for the presence of CHI3L1 and IκB by SDS-PAGE and Western blotting. Stimulation with TNF-α is expected to result in decreased cytoplasmic levels of IκB. The culture medium was collected at the end of the incubation period and prepared as described above for analysis of secreted CHI3L1.

Analysis of NF-κB Binding to the Human CHI3L1 Promoter—Confluent human chondrocytes were treated with TNF-α (50 ng/ml) or IL-1β (10 ng/ml) in serum-free medium for 30 min, and nuclear extracts were prepared and analyzed for NF-κB binding by electrophoretic mobility shift assay as described by Sakai et al. (26). Cells were washed once with Ca^2+ - and Mg^2+ -free phosphate-buffered saline and then harvested with lysis buffer A (10 mM HEPES-KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 0.25% (v/v) Nonidet P-40, 1 mM dithiothreitol, and protease inhibitors (5 mM phenylmethylsulfonyl fluoride, 5 μM pepstatin, 10 μM leupeptin, and 1 mM sodium vanadate)). After centrifugation at 1200 rpm for 30 min at 4°C, the nuclear pellets were washed once with 0.5 ml of Ca^2+ - and Mg^2+ -free phosphate-buffered saline and resuspended in an equal volume of lysis buffer B (50 mM HEPES-KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM MgCl2, and 2% (v/v) glycerol). Protein content was determined using the Bradford protein assay (Bio-Rad, Mississauga). Aliquots containing 10 μg of protein were incubated in the presence of 1 μM dithiothreitol and protease inhibitors as described above with 32P-labeled DNA fragments corresponding to positions −571 to −702 of the human CHI3L1 promoter (27). The fragments were generated by PCR and 3′-end-labeled following the manufacturer’s instructions. In control experiments, a 100-fold excess of unlabeled DNA fragment was added. Binding specificity was also investigated using a DNA fragment with a C-to-G substitution in the consensus sequence GGGAATTTCCC of the NF-κB/Rel DNA-binding motif at the underlined position. This fragment was generated by site-directed mutagenesis using the overlap extension method (28). To identify NF-κB subunits, a supershift analysis was performed by adding 1 μg of anti-human NF-κB p65 or p50 antibody or an unrelated IgG to the incubation mixtures. All samples were incubated for 16 h at 4°C and analyzed by electrophoresis on 6% (w/v) native polyacrylamide gels. The gels were dried and exposed overnight to Hyperfilm (Amersham Biosciences) at −80°C.

RESULTS

Induction and Secretion of CHI3L1 from Isolated Rat Articular Chondrocytes—Synthesis and secretion of CHI3L1 are detectable almost immediately following isolation of human articular chondrocytes (6). However, upon screening a large number of cellular RNA preparations from primary neonatal rat chondrocytes, no evidence for its presence was detected. However, neonatal rat chondrocytes are responsive to various pro-inflammatory cytokines. As CHI3L1 is often found in inflammatory environments, neonatal rat chondrocytes therefore provide an excellent model system in which to characterize induction of CHI3L1 production by cytokines. Rat chondrocytes were treated with factors associated with inflammation (IL-1β, IL-6, TNF-α, and epidermal growth factor) or with a factor involved in cartilage development (parathyroid hormone). Of the cytokines tested, only TNF-α and IL-1β induced mRNA expression for CHI3L1 (Fig. 1A). IL-6 is in the presence of its soluble receptor (parathyroid hormone) and epidermal growth factor concentration known to elicit cellular responses in these cells was not effective in increasing CHI3L1 transcript levels (data not shown). CHI3L1 mRNA was clearly detectable 24 h after addition of TNF-α, and levels remained elevated at 48 h, indicating that this is not a transient phenomenon. Low levels of CHI3L1 transcripts were detectable in cultures maintained for 48 h in serum-free medium. TNF-α and IL-1β also induced CHI3L1 expression in osteoblast cultures (prepared from neonatal rat calvaria) at both early and late stages of differentiation (Fig. 1B), but not in neonatal rat skin fibroblasts (Fig. 1C). Levels of transcripts for both MMP-13 and the chemokine GRO-1 (the rodent equivalent of IL-8), known targets of TNF-α and IL-1β, were
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FIGURE 2. Secretion of CHI3L1 from neonatal rat chondrocytes. Confluent monolayers of primary cells were exposed to control medium, TNF-α (30 ng/ml), or IL-1β (1 ng/ml). Culture media were harvested after 48 h, concentrated 10-fold, and analyzed for the presence of CHI3L1 by Western blotting using an antiserum raised against the C-terminal peptide of rat CHI3L1 (A). Bound immunoglobulin was visualized by enhanced chemiluminescence. The total protein composition of the harvested media, demonstrated by SDS-PAGE with identical loading and staining with Coomassie Brilliant Blue, is shown in B. The arrows indicate the migration position of the CHI3L1 doublet, identified on the Western blots. Media from cultures of intact femoral heads exposed to cytokines as described for A and analyzed by Western blotting are shown in C.

A short exposure to TNF-α is sufficient for CHI3L1 induction. Primary cultures of neonatal rat chondrocytes were treated with TNF-α (30 ng/ml) for 4 h, followed by removal of the cytokine and subsequent culture in control medium. At the times indicated, the cell layers and culture media were harvested and analyzed for CHI3L1 mRNA levels by RT-PCR (A) or for protein by Western blotting (B). No CHI3L1 protein was detectable in control cultures (not shown). Similar induction and maintenance of CHI3L1 protein secretion were observed in rat chondrocytes exposed for 1 or 4 h to either 30 ng/ml TNF-α or 1 ng/ml IL-1β, followed by culture in the absence of cytokines for 24 or 48 h (C). Culture media were concentrated 20-fold for analysis by Western blotting as described for B.

increased following exposure to the cytokines, indicating that the lack of CHI3L1 induction is not a consequence of a lack of response of the fibroblasts to either TNF-α or IL-1β.

To determine whether the stimulatory effect of TNF-α and IL-1 at the gene level translates to increased secretion of CHI3L1 protein, an antibody was raised against the C-terminal peptide of rat CHI3L1. This antibody detected a protein doublet migrating at ~39 kDa in the culture medium from cells stimulated with TNF-α or IL-1β, but not in control medium (Fig. 2, A and B). The immune reactivity could be absorbed with the immunizing peptide, but not with similar peptides from the C terminus of either mouse or human CHI3L1 (data not shown). In addition, analysis by peptide fragmentation and mass spectrometry indicated that both bands were indeed rat CHI3L1. The lower molecular mass band may represent a glycosylation variant or an unglycosylated form of the protein.

Based on these observations, the question arose as to whether or not CHI3L1 secretion could be induced in intact cartilage. Femoral heads from neonatal rats consisting mainly of epiphyseal and articular cartilage were harvested and cultured in the presence or absence of cytokines. Culture media were analyzed for secreted CHI3L1 by Western blotting (Fig. 2C). As was observed for the isolated cells, no protein was detectable at 24 or 48 h in control cultures. Both TNF-α and IL-1β induced production of the protein in femoral heads, and secretion was maintained over a 48-h culture period. Thus, the capacity of cytokines to initiate synthesis of CHI3L1 did not result from removal of the chondrocytes from their intact tissue environment, but is an inherent response of these cells in their native environment.

Short Exposure to Cytokines Is Sufficient for Prolonged CHI3L1 Production—As we have previously found that short exposures of chondrocytes to TNF-α (1–4 h) result in persistent activation of NF-κB signaling pathways and reduction of type II collagen and link protein synthesis in rat chondrocytes (23), the requirement for the continued presence of cytokines with respect to CHI3L1 production was investigated. Isolated rat chondrocytes were exposed to TNF-α for 4 h, followed by removal of the cytokine and continuation of culture in unsupplemented medium. Cells were harvested at 24, 48, or 72 h and analyzed for the presence of CHI3L1 mRNA by RT-PCR (Fig. 3A). Elevated transcript levels were evident up to 72 h after removal of the cytokine. Similarly, CHI3L1 protein levels increased between 48 and 72 h (Fig. 3B), suggesting that the cells continued to secrete the protein. CHI3L1 synthesis was induced after only 1 h of exposure to cytokines, as reflected by the presence of the protein in the culture medium harvested 24 h after cytokine removal, and the increased production was maintained for at least 48 h (Fig. 3C). To facilitate detection of CHI3L1 protein, media were concentrated 20-fold for this series of experiments. Similar results were obtained for IL-1β, although this cytokine appeared less efficient after a 1-h exposure compared with TNF-α, with which the amount of secreted protein appeared similar after 1- and 4-h exposures (given the quantitative limitations of the method of analysis). Low levels of CHI3L1 protein were detectable in the control culture medium from untreated cells at 48 h, but not at 24 h, consistent with induction of mRNA (Fig. 3A). These results demonstrate that a short pulse of cytokine exposure induces a long-term effect with respect to CHI3L1 production.

Activation of NF-κB Is Essential for CHI3L1 Induction in Rat Chondrocytes—TNF-α and IL-1β can activate a plethora of signaling mediators, including the MAPKs ERK1/2 and p38, protein kinase C, phosphatidylinositol 3-kinase (PI3K), and NF-κB (29). To analyze which pathways are involved in the induction of CHI3L1 by TNF-α, monolayer cultures of rat chondrocytes were pretreated for 30 min with selective inhibitors for the above pathways, followed by exposure to TNF-α. Cells were harvested at 24 h, and RNA was screened for CHI3L1 mRNA by RT-PCR (Fig. 4A). Low intensity PCR products were observed in some of the control cultures, and addition of some of the inhibitors appeared to decrease their intensity. However, this inhibition was inconsistent; for example, background expression was reduced by
the PI3K inhibitor wortmannin, but not by the equally effective inhibitor LY294002 (lanes 15 and 14, respectively). These differences could be attributable to slight differences in PCR efficiencies or in the quality of the RNA preparations. However, inhibition of ERK1/2, JAK2, protein kinase C, or PI3K had no discernible effect on TNF-α-induced CHI3L1 mRNA expression, as there was no major decrease in the intensity of the PCR products, suggesting that there is no major contribution to the regulation of CHI3L1 induction by TNF-α from these pathways. Similarly, inhibition of calcineurin-mediated signaling pathways by cyclosporin A or FK506 did not alter induction of mRNA or accumulation of CHI3L1 protein in the culture medium from chondrocytes stimulated with either TNF-α or IL-1β (Fig. 4B). It is thus unlikely that these pathways contribute significantly to transmission of the TNF-α or IL-1 signal with respect to CHI3L1 induction. In contrast, inhibitors of the NF-κB signaling pathway strongly inhibited the induction of CHI3L1 by both TNF-α and IL-1 (Fig. 5). When cells were treated with the proteasome inhibitor lactacystin, no induction of CHI3L1 mRNA was detectable following co-incubation with TNF-α for 24 h (Fig. 5A). The inhibitor did not affect mRNA levels for GAPDH, determined in parallel (data not shown), indicating that viability of the cells was not severely affected by this treatment. No immune reactive protein was detectable in the culture medium from cells treated with either TNF-α or IL-1 in the presence of lactacystin (Fig. 5B). Inhibition of CHI3L1 synthesis and secretion was also observed when intact femoral heads from neonatal rats were exposed to these cytokines in the presence of lactacystin (Fig. 5C). The inhibitor did not affect mRNA levels for G3PDH, determined in parallel (data not shown), indicating that viability of the cells was not severely affected by this treatment. Thus, NF-κB needs to be maintained in an active state to permit continued production of CHI3L1. These observations indicate that both the induction and continued expression of CHI3L1 are primarily mediated by signaling through NF-κB and that a short exposure to cytokines is sufficient to activate this pathway for extended periods of time. Secretion of CHI3L1 from Human Articular Chondrocytes Depends on NF-κB—Although CHI3L1 secretion was easily detectable in monolayer cultures of human articular chondrocytes, the levels eventually decreased after 5–7 days in the absence of serum.3 Following serum starvation of the cells for 3 days to reduce the level of CHI3L1 secretion,
addition of IL-1β or TNF-α increased levels of CHI3L1 protein in the culture medium within 48 h (Fig. 7A). The effects observed at the protein level were mirrored by similar reductions or increases in mRNA levels determined by RT-PCR (data not shown). Thus, as described for neonatal rat chondrocytes, production of CHI3L1 in human chondrocytes is up-regulated by these two cytokines.

As the prolonged secretion of CHI3L1 from rat articular chondrocytes was investigated. Monolayer cultures were maintained in unsupplemented culture medium in the absence or presence of the IkB kinase inhibitor BAY 11-7085. Culture media and cells were harvested at various time points from 6 to 24 h, and culture media were analyzed for the presence of CHI3L1 by Western blotting (Fig. 7). CHI3L1 protein was detectable in media collected at 6 h, and levels increased steadily over the 24-h period. In the presence of the inhibitor, no CHI3L1 protein was detectable at any of these time points, suggesting that this inhibitor suppressed CHI3L1 synthesis effectively. These results were mirrored by decreases in CHI3L1 mRNA levels, whereas GAPDH mRNA levels remained constant (data not shown). Similar results were obtained with the IkB kinase inhibitor BAY 11-7082; the proteasome inhibitor lactacystin; and the peptide inhibitor SN50, which prevents nuclear translocation of NF-κB. The MAPK inhibitors U0126 and PD98059 and the PI3K inhibitor LY29004 had no effect on CHI3L1 production (data not shown). These results support the requirement for activated NF-κB for the constitutive expression of CHI3L1 observed in human chondrocytes.

To further support the pivotal role of NF-κB in the control of CHI3L1 production in human articular chondrocytes, cells were transiently transfected with a plasmid containing either IkB or a dominant-negative form of this protein (IkB-2N), which cannot be phosphorylated and hence prevents the release of NF-κB from the inhibitory complex and its translocation into the nucleus (Fig. 8). Cells were serum-deprived 24 h after transfection with the plasmids, and IkB levels were analyzed in cell lysates by Western blotting with an antisera specific for this protein (Fig. 8A). IkB was easily detected in all untreated cells. As expected, the IkB levels in transfected cells appeared to be somewhat higher compared with those in non-transfected cells. Following exposure to TNF-α for 30 min, IkB was undetectable in control cells and in cells transfected with the empty vector or IkB, suggesting that the level of expression of the transfected construct may not be high enough to counteract the efficient TNF-α-stimulated removal of IkB. Residual IkB was detectable, however, in cells transfected with the dominant-negative construct, and these levels may be sufficient to interfere with the traffic of NF-κB to the nucleus. CHI3L1 secretion over the ensuing 24-h period was analyzed by Western blotting (Fig. 8B). The levels of synthesis of CHI3L1 by human articular chondrocytes were sufficiently high to allow detection of the protein in cell lysates as well as in the culture medium. This analysis provides a more precise measurement of the rate of synthesis of the protein at a given time point. Transfection of the control or wild-type IkB vector had no effect on the level of synthesis or secretion of CHI3L1 compared with non-transfected controls. However, the dominant-negative construct reduced both constitutive and TNF-α-stimulated intracellular levels of CHI3L1 and that secreted into the medium, further supporting the importance of NF-κB in the regulation of CHI3L1 expression and synthesis.

The CHI3L1 Promoter Binds NF-κB—A putative NF-κB-binding motif was identified in the promoter region of the human gene using the
DISCUSSION

The pivotal role of the inflammatory cytokines IL-1 and TNF-α in the development and progression of rheumatoid arthritis is well recognized (29). These pro-inflammatory cytokines direct the behavior of cells of the immune system as well as connective tissue cells, including articular chondrocytes, toward an inflammatory and degradative phenotype. Synthesis and secretion of a large variety of matrix metalloproteases (31) and chemokines (32) are the most well characterized responses of connective tissue cells, mediating damage to the extracellular matrix and, in the case of articular cartilage, leading to the loss of cartilage matrix and joint function. The data presented here indicate that CHI3L1 is part of the response repertoire of chondrocytes to the inflammatory cytokines TNF-α and IL-1.

We have demonstrated that not only NF-κB is essential for induction of CHI3L1, but also that it contributes to a large extent to constitutive secretion of CHI3L1 observed in some connective tissue cells. In addition to chondrocytes, both early and late stage osteoblasts responded to TNF-α with induction of CHI3L1 expression, whereas skin fibroblasts did not respond, as has been reported for human skin fibroblasts (6). Thus, this response suggests a more generalized capacity of cells of the skeletal system to produce CHI3L1, which differentiates them from other connective tissue fibroblasts. The production of CHI3L1 had also been demonstrated in osteocytes (bony outgrowths associated with degenerating cartilage) and in fetal osteoblasts by in situ hybridization (33), suggesting a link between the skeletal response to inflammation and tissue remodeling or repair.

We previously demonstrated that CHI3L1 abrogates catabolic responses induced by inflammatory cytokines (21); thus, induction of CHI3L1 in chondrocytes would likely represent a survival response. Chondrocyte function is highly dependent on signals initiated by the local microenvironment such as the matrix. CHI3L1 secretion by articular chondrocytes was induced in both isolated cell cultures and intact femoral heads, revealing that CHI3L1 expression in response to cytokines is not a consequence of isolation of the cells from their native environment. With the exception of modulators of NF-κB signaling (proteasome inhibitors, IkB kinase inhibitors, and IkB dominant-negative constructs), selective inhibitors for a variety of signaling pathways were ineffective in affecting induction of CHI3L1. In human chondrocytes, the apparent constitutive secretion of CHI3L1 was not altered by inhibition of p38, PI3K, or ERK1/2 4; and thus, the mechanisms controlling the production of this protein appear very similar in both species. The promoter regions of the rat and human CHI3L1 genes are rich in putative response elements for regulatory factors (27, 34), although only NF-κB activity appears to drive the transcriptional complex sufficiently to generate expression. The analysis of the human CHI3L1 promoter indicated that the NF-κB-binding motif indeed binds this transcription factor, supporting the suggested role for NF-κB. Additional studies will be necessary to establish the other signals that regulate this NF-κB-mediated expression.

Although expression of CHI3L1 is regulated mainly by NF-κB in chondrocytes from both species, there are some significant differences between the two systems. First, CHI3L1 expression in isolated human chondrocytes and cartilage explants appears constitutive, i.e. it occurs in the absence of added agonists such as cytokines. This is not the case in neonatal rat cartilage, where expression in both isolated cells and intact cartilage requires exogenous stimulation. Several factors could account for these differences. The production of CHI3L1 in human articular cartilage explants is not evenly distributed, but is restricted to the more superficial cells of the tissue, 5 indicating that these cells exhibit a specific phenotype that is different from the phenotype of the cells in the deeper

4 H. Ling and A. D. Recklies, manuscript in preparation.
5 A. D. Recklies, unpublished data.
layer, which allows them to respond to changes in their physiological or metabolic environment. Tissue injury due to cutting of the cartilage explants could be such an inducing factor, as recently suggested by Gruber et al. (35). The lack of spontaneous production of CHI3L1 in isolated rat femoral heads could be due to the facts that this process requires minimal tissue injury and that the heads are cultured intact. It remains to be established whether or not increased tissue injury induces CHI3L1 production in these neonatal tissues and whether adult tissue responds in a similar fashion.

Although, in contrast to isolated human chondrocytes, neonatal rat cells require an inducing stimulus, there appears to be a drift toward spontaneous production of the protein after prolonged serum deprivation for 48 h or more. This could represent the appearance of a response to a stress-inducing environment. The role of NF-κB in cell survival particularly in response to extra- or intracellular stress is well recognized (36, 37), and it is conceivable that this occurs in chondrocytes exposed to serum-free conditions.

Once NF-κB is activated, there seems to be a prolonged memory effect, as suggested previously by Séguin and Bernier (23), who observed residual activation of NF-κB following transient exposure of rat chondrocytes to TNF-α. This incomplete return of NF-κB to starting levels could lead to accumulation and alteration of signals initiated in response to subsequent exposure to inflammatory cytokines. Furthermore, induction of such constitutive levels of active NF-κB is associated with physiological activation of B cells (i.e. an alteration of phenotype) (38) and with neoplastic transformation as a result of chromosomal rearrangement (39, 40). In cartilage, such residual activation of NF-κB is likely to occur normally over time following acute damage or inflammation. Thus, residual activation of NF-κB may account for the continued secretion of CHI3L1, even after removal of the initiating stimulus. Accumulation of NF-κB could be due to either insufficient production of IκB to assist in the clearance of NF-κB from the nucleus (41) or sustained IκB kinase activity, resulting in increased basal levels of NF-κB. This may account for the lack of effect of transfection of wild-type IκB on CHI3L1 production in human chondrocytes and the effectiveness of the non-phosphorylated mutant to inhibit this process.

Macrophages are a major source of CHI3L1; secretion of this protein in macrophages is a marker for late stage differentiation (8), preceding non-phosphorylated mutant to inhibit this process. The transcription factor B could be due to either insufficient production of IκB to assist in the clearance of NF-κB in cell survival or in isolated culture. Thus, the observed production of CHI3L1 in cartilage explants may be the result of a brief pulse of TNF and/or IL-1 stimulation as a response to the mechanical trauma of tissue harvesting, which results in an activated NF-κB state of the cells. Such cell selectivity in induced expression of CHI3L1 may represent an important biomarker for monitoring the progression of connective tissue disorders such as cartilage degeneration and tissue fibrosis. Data published by Johansen et al. (4) suggest that CHI3L1 is elevated in patients with cirrhosis of the liver. Correlations with disease activity have been reported in patients with rheumatoid arthritis (1), and elevated serum levels of CHI3L1 appear to correlate with the presence of arthritis in patients with inflammatory bowel disease (43).

Induction of CHI3L1 in response to inflammatory cytokines provides a negative feedback loop for the control of cytokines. The regulation feedback loop is fundamental to connective tissue turnover, with CHI3L1 functioning to limit catabolic and inflammatory responses locally and imparting protection to connective tissue cells. In contrast, overproduction of this regulator may in fact lead to increased matrix accumulation and promote tissue fibrosis. Identifying NF-κB as a critical signal in the expression of CHI3L1 is important in the understanding of cytokine-mediated pathogenesis.

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