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Regulation of YKL-40 Production by Human Articular Chondrocytes

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Objective. **YKL-40 (human cartilage glycoprotein 39) is one of the most abundant proteins secreted by cultured chondrocytes. The objectives of the present study were to identify regulators of YKL-40 production in cartilage and chondrocytes and to map the localization of YKL-40 in chondrocytes.**

Methods. **Human articular chondrocytes and cartilage explants (obtained from subjects at autopsy, from a tissue bank, and from osteoarthritis [OA] patients undergoing total joint replacement surgery) were stimulated with cytokines, growth factors, and other agents. YKL-40 expression was analyzed by Northern blot and polymerase chain reaction. YKL-40 secretion into the media was determined by enzyme-linked immunosorbent assay.**

Results. **YKL-40 production increased to very high levels during the early phase of chondrocyte monolayer culture and in normal cartilage explant cultures as a response to tissue injury. Spontaneous YKL-40 release was higher in OA than in normal cartilage explant cultures. In chondrocyte monolayer cultures, interleukin-1β (IL-1β) and transforming growth factor β (TGFβ) decreased the levels of secreted YKL-40, and this was associated with a reduction in YKL-40 messenger RNA levels. IL-1β, but not TGFβ, reduced YKL-40 production in cartilage explant cultures. Media from explants treated with cycloheximide had no detectable YKL-40, suggesting that the released protein was newly synthesized. Immunofluorescence microscopy showed YKL-40 staining in the Golgi system of the chondrocytes, but YKL-40 could not be detected in the extracellular matrix.**

Conclusion. **The spontaneous increase in the production of YKL-40 in the early phase of culture appears to represent a cellular response to changes in the extracellular matrix environment. This, coupled with the profound suppressive effects of IL-1β and TGFβ on YKL-40 production, identifies a novel regulatory pattern for this major chondrocyte-derived protein.**

YKL-40, also called human cartilage glycoprotein 39, belongs to a family (1–4) of mammalian genes (family 18 of glycosyl hydrolases) that are homologous to the chitinases from lower organisms. The protein's name is derived from its 40-kd MW and the 1-letter code for its 3 N-terminal amino acids (tyrosine, lysine, and leucine). The biologic function of YKL-40 and the other mammalian members of this protein family is unknown, except for chitotriosidase, which has chitinase activity (3).

YKL-40 is synthesized and secreted in environments in which substantial remodeling of extracellular matrix occurs, and it is thought that the protein may function in the remodeling process through binding to carbohydrate polymers. YKL-40 binds heparin (5) and chitin (3), but the protein has no chitinase activity due to the lack of glutamate residue in position 141 (1–3), which is known from mutagenesis studies to be essential for the activity of bacterial chitinases (6). Recently, a vertebrate chitin synthase has been identified (7–9) that is thought to create short chitin stretches that are essential to initiate hyaluronan synthesis (8).

It is possible that YKL-40 recognizes hyaluronan precursor as a substrate and interferes with its synthesis, which could affect local hyaluronan concentrations. In

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disease states with elevated levels of YKL-40, such as arthritis (1,10,11), liver fibrosis (12), or cancer (13,14), increased levels of hyaluronan have also been reported. It is also possible that the lectin binding activity of YKL-40 may target carbohydrate moieties on the cell surface or in the extracellular matrix for processing during tissue remodeling. One physiologic ligand for the heparin binding site in YKL-40 could be perlecan, which is the major heparan sulfate proteoglycan of basement membranes, but which is also expressed in the extracellular matrix of many tissues (15), including cartilage (16). Perlecan is known to be involved in cell migration and proliferation and in cell adhesion. Studies have shown that perlecan can store, activate, or inactivate growth factors and cytokines (15,16) that play important roles in cartilage degradation.

YKL-40 is synthesized by articular chondrocytes (1,2) and synovial cells (17,18) from patients with rheumatoid arthritis (RA) or osteoarthritis (OA). Immunohistochemical analysis of articular cartilage biopsy samples from the hip joints of patients with OA has shown staining of YKL-40 in chondrocytes mainly in the superficial and middle zones and particularly in chondrocytes in areas of the femoral head with a considerable biomechanical load. YKL-40 expression in chondrocytes from normal cartilage was low or not detectable (11). YKL-40 levels are elevated in synovial fluid and serum from patients with active RA or severe knee OA compared with normal subjects (10,19–21), and a correlation exists between the levels of YKL-40 in serum and in synovial fluid, with ~15-fold higher values in synovial fluid. Continuously elevated serum levels of YKL-40 in patients with early RA are related to progression in the Larsen score (10).

YKL-40 contains several HLA–DR4 peptide binding motifs, and YKL-40–derived peptides can elicit T cell proliferative responses in peripheral blood mononuclear cells from patients with RA, indicating that YKL-40 could be a target of the immune response in RA (22,23). Furthermore, injection of YKL-40 into BALB/c mice initiated arthritis, and intranasal administration of YKL-40 induced antigen-specific immunotolerance and suppression of the YKL-40–induced arthritis (22). It has recently been shown that intranasal application of YKL-40 also interferes with the expression of disease in murine type II collagen–induced arthritis (23).

The gene for YKL-40 has been sequenced (24), but little is known about the regulation of its production. Transforming growth factor β (TGFβ) reduces the level of YKL-40 messenger RNA (mRNA) in human chondrocytes (1), as well as the level of secreted YKL-40 from guinea pig chondrocytes (25), while the expression does not seem to be influenced by interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) in human synovial cells (17) and guinea pig articular chondrocytes (25). Insulin-like growth factor 1 (IGF-1) has been reported to have no effect on the expression of YKL-40 mRNA in human chondrocytes (1), but IGF-1 increased the level of YKL-40 in conditioned media from guinea pig articular chondrocytes (25).

The purpose of the present study was to define stimuli and mechanisms that regulate YKL-40 production by human chondrocytes.

MATERIALS AND METHODS

Human cartilage procedure and assessment. Human knee cartilage from 37 donors with no history of arthritis (median age 46 years, range 18–91 years) was obtained at autopsy or from tissue banks. Specimens of OA knee cartilage from femoral condyles and tibial plateaus were obtained from 21 donors with OA (median age 69 years, range 50–84 years) at autopsy or at the time of total knee joint replacement surgery. The degree of histologic/histochemical changes in OA and normal cartilage was graded according to gross morphologic changes assessed by established criteria (26) following the application of India ink: grade 1 (intact surface, does not retain any ink, n = 23 donors), grade 2 (minimal fibrillation, site appears normal before staining but retains India ink as elongated specks or light gray patches, n = 14 donors), grade 3 (overt fibrillation, the cartilage is velvety in appearance and retains ink as intense black patches, n = 8 donors), and grade 4 (erosion, loss of cartilage exposing the underlying bone, n = 13 donors).

Chondrocyte isolation. Chondrocytes were isolated as previously described (27). Briefly, cartilage slices were removed from the femoral condyles and tibial plateaus and cut into pieces (2–3 mm^3), washed with Dulbecco's modified Eagle's medium (DMEM)/high glucose (BioWhittaker, Walkersville, MD), and treated for 15 minutes with trypsin (10% [volume/volume]) in a 37°C water bath. The tissues were transferred to a digestion buffer containing DMEM/high glucose, 5% fetal bovine serum (FBS), L-glutamine, antibiotics (penicillin, streptomycin, and fungizone), and 2 mg/ml clostridial type IV collagenase (C-5138; Sigma, St. Louis, MO), and digested overnight on a gyratory shaker at 37°C. Residual, multicellular aggregates were removed by sedimentation (1*g*), and the cells were washed 3 times in DMEM containing 5% FBS before use.

Monolayer culture. Following cell isolation, aliquots of the cells were plated (5×10^6/plate) in T175 tissue culture flasks. Following initial adherence, the cells were allowed to reach confluence. At this point, the cultures were trypsinized. Cells were used for experiments or plated for further monolayer culture at 1/3 confluence. Experiments reported here were performed with freshly isolated chondrocytes and first-passage, second-passage, third-passage, and ninth-passage cells, as noted below. In each experiment, chondrocytes were plated (50,000/well) in 96-well plates with 200 μl complete

medium (DMEM/high glucose, 10% FBS, L-glutamine, penicillin, and streptomycin) and incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Three-dimensional chondrocyte cultures. *Alginate.* The procedure was performed as originally described by Häuselmann et al (28), with minor modifications. Briefly, chondrocytes were collected by trypsinization from monolayer cultures, then washed and resuspended in 0.15*M* NaCl. A suspension of low-viscosity alginate (1.2%) (Keltome LV; Kelco/Merck, Chicago, IL) was prepared in 0.15*M* NaCl and passed through 0.45-μm filters. Cells (passages 1 and 2) were resuspended in alginate solution at 3–4 $\times$ 10^6/ml. Drops of the cell suspension were expressed slowly from a syringe through a 20-gauge needle into a gently agitated 102 m*M* $CaCl_2$ solution. The alginate beads gelled immediately and were allowed to stabilize for an additional 10 minutes in the $CaCl_2$ solution. The beads were then washed twice in NaCl and once in complete medium. Culture was performed in complete medium in 6-well plates.

Chondrocyte clusters. Poly-2-hydroxyethyl-methacrylate (poly-HEMA) (H8633; Sigma) was prepared as a 10% solution in 95% ethanol. Poly-HEMA (10 gm) in 100 ml of 95% ethanol was stirred overnight at 37°C (solution is highly viscous). Ninety-six–well plates were coated by adding 50 μl of the solution to each well and drying it overnight under a laminar flow hood to allow complete evaporation of ethanol. The plates were then stored for up to 2 weeks sealed with plastic wrap at 4°C. Cells (passages 1 and 2) were plated (50,000/well) in 200 μl complete medium and incubated at 37°C.

Cartilage explant cultures. Cartilage tissues were cut into small pieces and cultured in 6-well plates in 2 ml complete medium. All of the conditioned media were collected and replaced with fresh media every second day or otherwise, as noted. The conditioned media were stored at −20°C until YKL-40 analysis by enzyme-linked immunosorbent assay (ELISA). At the end of the study, the weight of each explant was determined and the YKL-40 level in the media was corrected for the weight of the cartilage explants.

Effects of cytokines and growth factors on YKL-40 secretion by chondrocytes cultured in monolayer or explant cultures. Chondrocytes in monolayer cultures (cultured in DMEM with 1% FBS) or explant cultures (cultured in DMEM with 10% FBS) were stimulated with IL-1β (1 ng/ml or 5 ng/ml), TGFβ1 (10 ng/ml), TNFα (10 ng/ml), IL-18 (10 ng/ml or 20 ng/ml), IL-6 (10 ng/ml), IL-17 (20 ng/ml), dexamethasone (100 n*M*), platelet-derived growth factor (PDGF; 20 ng/ml), IGF-1 (50 ng/ml), and basic fibroblast growth factor (bFGF; 20 ng/ml), and media were collected at different time points as noted and stored at −20°C until YKL-40 analysis by ELISA. Cartilage explant cultures were also treated with cycloheximide (50 μg/ml). TGFβ1, IL-1β, IL-6, IL-17, IL-18, TNFα, PDGF, bFGF, IGF-1, dexamethasone, and cycloheximide were obtained from R&D Systems (Minneapolis, MN).

YKL-40 assay. YKL-40 concentrations in conditioned media from the monolayer chondrocyte cultures and the cartilage explant cultures were determined by a 2-site, sandwich-type ELISA (Quidel [formerly Metra Biosystems], Mountain View, CA) (21) using streptavidin-coated microplate wells, a biotinylated-Fab monoclonal capture antibody, and an alkaline phosphatase–labeled polyclonal detection antibody. The detection limit was 20 ng/ml and the intraassay and interassay variations were <4.3% and <7.0%, respectively. No detectable YKL-40 was measured in DMEM/high glucose medium containing 10% FBS.

Immunofluorescence microscopy of monolayer chondrocytes. Human chondrocytes (passage 1) isolated from normal cartilage were grown overnight on glass coverslips placed in 6-well plates. The monolayer chondrocytes were fixed for 20 minutes at room temperature with 2% formaldehyde (Polysciences, Warrington, PA) buffered with phosphate buffered saline (PBS), rinsed several times with PBS, and permeabilized with 100% acetone for 3 minutes at −20°C. Fixed and permeabilized cells were drained off of PBS and then incubated for 1 hour at room temperature with a monoclonal mouse antibody against YKL-40 and with a polyclonal rabbit antibody against giantin (kindly donated by E. K. L. Chan, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA), a protein located specifically in the Golgi system (29,30). After incubation with primary antibodies, coverslips were rinsed 3 times with PBS and then incubated for 1 hour at room temperature with a 1:100 dilution of rhodamine-labeled goat anti-mouse IgG (Caltag Laboratories, Burlingame, CA) and fluorescein isothiocyanate (FITC)–labeled goat anti-rabbit IgG (Caltag Laboratories). The coverslips were rinsed 3 times with PBS and then mounted in Vectashield (Vector, Burlingame, CA). For double-label immunofluorescence, both primary and secondary antibodies were mixed and added together. Fluorescence images were recorded on Ektachrome 400 slide film (Eastman Kodak, Rochester, NY).

Immunohistochemical analysis of cartilage explants. Normal cartilage explants were cultured for 10 days in complete medium. The cartilage explants were then fixed in 2% formaldehyde, embedded in paraffin, and cut into 4-μm sections. A conventional peroxidase staining technique for monoclonal antibodies was used (Envision System/HR K1390; Dako, Glostrup, Denmark). The monoclonal antibody against human YKL-40 was used in a concentration of 40 μg/ml. A solution of 3,3′-diaminobenzidine chromogen was used as color substrate (positive staining was recognized as a brown color).

Northern blot analysis. Total RNA was isolated from chondrocytes (passages 0–2) with TRIzol reagent (Gibco BRL/Life Technologies, Gaithersburg, MD). The cells were plated (1 million/well) in 6-well plates with 1 ml medium (DMEM/high glucose, L-glutamine, penicillin, and streptomycin). Total RNA was isolated after 24 hours of no stimulation or stimulation with IL-1β (5 ng/ml) or TGFβ1 (10 ng/ml). Ten micrograms of the total RNA was fractionated on 1% formaldehyde–containing agarose gels and transferred onto a 0.45-micron Hybond-N membrane (Amersham, Piscataway, NJ) by the capillary method.

Prehybridization was carried out for 2 hours at 65°C in 5× saline–sodium citrate (SSC; 1× SSC is 150 m*M* NaCl [pH 7.0]), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 1 m*M* EDTA. A specific YKL-40 complementary DNA (cDNA) probe was labeled with ^{32}P-dATP using High prime labeling kit (Boehringer Mannheim, Indianapolis, IN). Hybridization was performed for 18 hours under the same conditions described for prehybridization after adding the probe and 20 μg/ml salmon sperm DNA (Gibco BRL/Life Technologies). The blot was washed twice in 2× SSC, 0.1% SDS at room temperature for 2 minutes; twice in 2× SSC,

0.1% SDS at 60°C for 20 minutes; and once in 0.5× SSC, 0.1% SDS for 20 minutes at 65°C. Autoradiography was performed at −70°C for 15 hours with XAR-5 film (Eastman Kodak) and Cronex Lightning Plus screens (DuPont NEN Research Products, Boston, MA). The blot was then stripped by boiling in H_2O containing 0.1× SSC and 0.1% SDS and rehybridized with a cDNA probe for GAPDH.

Reverse transcriptase—polymerase chain reaction (RT-PCR). Complementary DNA was synthesized from 2–5 μg of total RNA using SuperScript II and random primer (Gibco BRL/Life Technologies). RT-PCR was performed with the following conditions for YKL-40: after a 2-minute denaturation step at 94°C, the reaction proceeded in 16 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C, followed finally by 1 cycle of 7 minutes at 72°C. RT-PCR for GAPDH was performed with the same conditions as those for YKL-40, but with 22 cycles. The following PCR primers were prepared: for YKL-40 (493-bp product), sense 5′-CCT-GCT-CAG-CGC-AGC-ACT-GT-3′ and antisense 5′-GCT-TTT-GAC-GCT-TTC-CTG-GTC-3′; for GAPDH (190-bp product), sense 5′-TGG-TAT-CGT-GGA-AGG-ACT-CAT-G-3′ and antisense 5′-ATG-CCA-GTG-AGC-TTC-CCG-TTC-AGC-3′.

Statistical analysis. Statistical analysis was performed with Sigma Stat (SPSS, Chicago, IL). Results are given as the mean ± SEM. For all results shown, at least 3 separate experiments with cells from different donors were performed, if not otherwise noted. Within experiments with monolayer chondrocyte cultures, each individual measurement was performed in triplicate. Statistical analysis was performed with the unpaired or paired 2-tailed *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Increased spontaneous secretion of YKL-40 by normal articular chondrocytes during monolayer culture. Directly following collagenase digestion of normal human articular cartilage, the isolated chondrocytes were cultured in 96-well plates. These primary cells secreted low levels of YKL-40 (19 ng/ml per 50,000 cells) after 1 day in culture and 2,002 ng/ml per 50,000 cells after 7 days of culture (cumulated production). This basal production of YKL-40 increased >300-fold in first- and second-passage culture. The cells produced 7,017 ng/ml of YKL-40 during the initial 24 hours of culture in second passage and 43,067 ng/ml after 7 days of culture (Figure 1). There was no significant further increase in the basal secretion of YKL-40 after passage 2. Cells were analyzed up to passage 9, when they continued to secrete high levels of YKL-40 (5,752 ng/ml during the initial 24 hours of culture and 27,232 ng/ml after 4 days of culture in ninth passage).

Effects of serum and extracellular matrix on YKL-40 production. The influence of serum components on YKL-40 production was analyzed by changing adherent first- and second-passage cells from DMEM containing 10% FBS to serum-free medium. There was no significant difference in the YKL-40 levels in the conditioned media of monolayer chondrocytes cultured with or without 10% FBS for 2 or 3 days (3,707 ng/ml per 50,000 cells with 10% FBS versus 3,638 ng/ml per 50,000 cells without serum; $P = 0.898$; n = 11 samples analyzed from 5 different donors with normal cartilage). In cartilage explant cultures (n = 12 samples analyzed from 2 different donors), there were no differences in YKL-40 concentrations in the conditioned media during the first 7 days of culture between explants cultured with 10% FBS (224 ng YKL-40 per 24 hours per 100 mg cartilage) or without serum (214 ng YKL-40 per 24 hours per 100 mg cartilage). From 8 to 20 days of culture, YKL-40 levels were significantly higher in the conditioned media from explants cultured with serum (184 ng YKL-40 per 24 hours per 100 mg cartilage) than in that from explants cultured without serum (103 ng YKL-40 per 24 hours per 100 mg cartilage) ($P = 0.008$).

Three-dimensional culture systems have been reported either to maintain the differentiation status of articular chondrocytes or to induce reexpression of chondrocyte markers that are reduced or lost during monolayer culture (28). To evaluate whether the loss of extracellular matrix after enzymatic digestion of cartilage was responsible for the induction of spontaneous

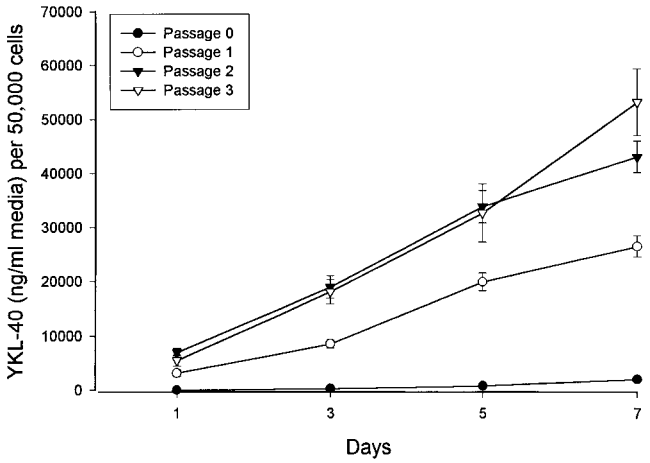

Figure 1. Increase in spontaneous secretion of YKL-40 by normal articular chondrocytes during monolayer culture. Chondrocytes were tested as freshly isolated cells (Passage 0) and during the initial 3 passages. Conditioned media were collected after 1, 3, 5, and 7 days for quantification of YKL-40 release (cumulative production) determined by enzyme-linked immunosorbent assay. Results are the mean ± SEM of 6 experiments. Chondrocytes originated from 2 donors with normal cartilage.

YKL-40 production by normal chondrocytes, we studied YKL-40 production in monolayer chondrocytes versus that in 3-dimensional chondrocyte culture. Cells (passages 1 and 2) were cultured either in alginate suspension to create a 3-dimensional culture system (31) or on plates coated with methyl methacrylate, which prevents the chondrocytes from adhering to the well; the cells will adhere to each other instead, form clusters, and produce a cartilage-like matrix (32). The YKL-40 levels in the conditioned media from alginate cultures (using passage 2 chondrocytes) were significantly lower ($P < 0.001$) than those in media from monolayer passage 2 chondrocytes. This difference was not found using passage 1 chondrocytes. During the first week in clusters on methyl methacrylate–coated plates, the cells continued to produce amounts of YKL-40 similar to those in monolayer (Figure 2).

YKL-40 production in explant culture. Full-thickness explants were resected from normal human articular cartilage and placed in serum-containing culture (i.e., with 10% FBS). Media were changed every second day during 10 days of culture. These explant cultures secreted low levels of YKL-40 (333 ng/ml media per 100 mg cartilage) during the first 2 days in culture. YKL-40 production increased >6-fold by day 6 and significantly declined during the following 4 days (Figure 3). To determine whether the YKL-40 detected in the media of the cultured explants was newly synthesized, the protein synthesis inhibitor cycloheximide was introduced. Cycloheximide completely suppressed YKL-40 release. Transfer of cartilage that had been cultured with cycloheximide for 10 days to culture media without this drug led to a resumption of YKL-40 production after 8 days (Figure 3). These results suggested that the early release of YKL-40 in explant cultures was dependent on new protein synthesis.

Next, we determined whether the trauma of resecting the explants from cartilage was a stimulus for YKL-40 production. Explants were cultured for 10 days (with media change every second day), the point at which YKL-40 release had declined to <50% of maximal levels. On day 10, the media were removed and the full-thickness cartilage was cut into smaller pieces. These pieces were cultured for an additional 10 days. The dissection of the cartilage triggered a second peak in YKL-40 expression (Figure 3).

Collectively, these findings show that in normal cartilage, YKL-40 is newly synthesized and released from cells through cartilage into the space outside the cartilage extracellular matrix in response to the trauma of tissue resection.

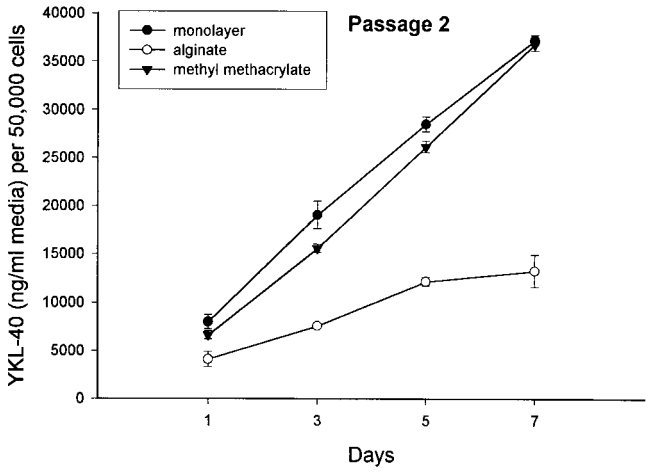

Figure 2. YKL-40 production in 3-dimensional chondrocyte cultures. Normal articular chondrocytes (Passage 2) were cultured as monolayers, in alginate beads, or on methyl methacrylate–coated plates. Chondrocytes from the same donor were used in all experiments. Conditioned media were collected after 1, 3, 5, and 7 days for quantification of YKL-40 release (cumulative production) determined by enzyme-linked immunosorbent assay. Results are the mean ± SEM of triplicate experiments.

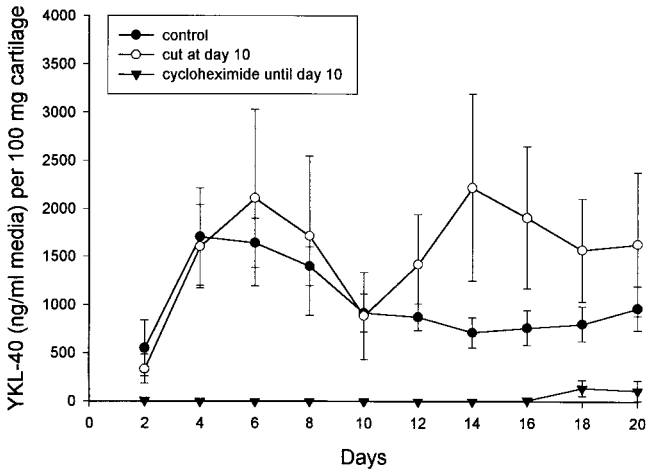

Figure 3. YKL-40 production in cartilage explant culture. Full-thickness explants were resected from normal human articular cartilage and placed in serum-containing culture. Conditioned media were changed every second day for quantification of YKL-40 release (determined by enzyme-linked immunosorbent assay) during 20 days of culture. Explants were treated with media only (control) or with cycloheximide (50 μg/ml) every second day until day 10. On day 10, some of the full-thickness explants were cut into 8 smaller pieces, and these pieces were then cultured for an additional 10 days with change of media every second day (○). Results are the mean ± SEM YKL-40 levels from 5 donors with osteoarthritis grades 1 and 2. For each donor, the control explant treatments were performed in duplicate.

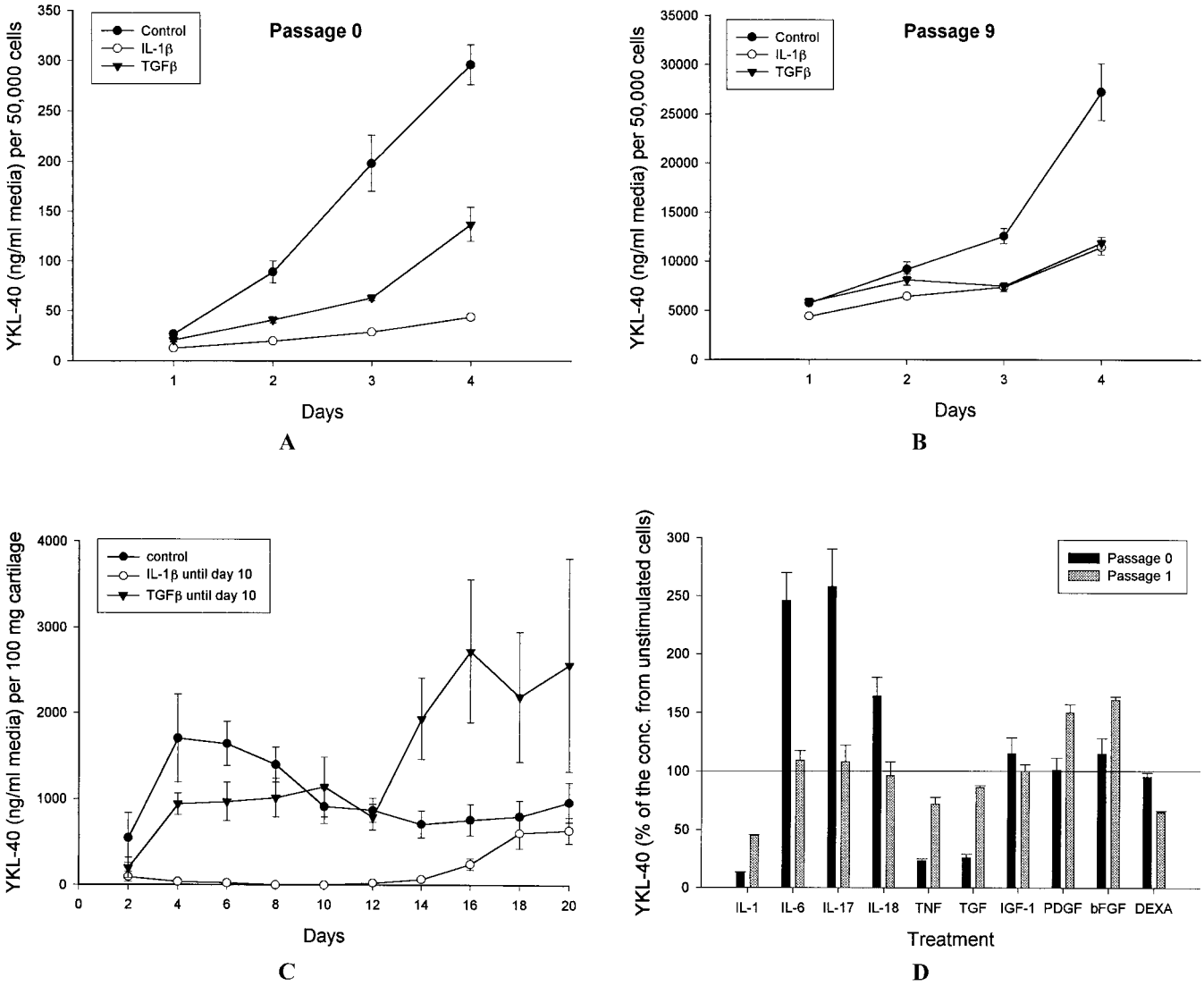

Figure 4. Cytokine regulation of YKL-40 production in monolayer and cartilage explant cultures of normal cartilage. Shown are the effects of interleukin-1β (IL-1β) and transforming growth factor β (TGFβ) on the secretion of YKL-40 by **A**, normal freshly isolated articular chondrocytes (Passage 0), **B**, late-passage (Passage 9) chondrocytes, and **C**, normal cartilage explants. **A** and **B,** Conditioned media were collected from the monolayer cultures at 24, 48, 72, and 96 hours after stimulation with IL-1β (5 ng/ml), TGFβ (10 ng/ml), or media only (Control). Results are the mean ± SEM YKL-40 levels from 3 donors, each tested in triplicate. **C,** Cartilage explants were treated with media only (control), IL-1β (5 ng/ml), or TGFβ (10 ng/ml) every second day for 10 days. On day 10, the treatment with IL-1β and TGFβ was stopped and the explants were then cultured without treatment for an additional 10 days with change of media every second day. Results are the mean ± SEM YKL-40 levels from 5 donors with osteoarthritis grades 1 and 2. For each donor, the control explant treatments were performed in duplicate. **D,** Influence on YKL-40 production of 72 hours of stimulation of monolayer chondrocytes from normal cartilage (Passages 0 and 1; 50,000 cells per condition) with IL-1β (IL-1; 1 ng/ml), IL-6 (10 ng/ml), IL-17 (20 ng/ml), IL-18 (20 ng/ml), tumor necrosis factor α (TNF; 10 ng/ml), TGFβ (TGF; 10 ng/ml), insulin-like growth factor 1 (IGF-1; 50 ng/ml), platelet-derived growth factor (PDGF; 20 ng/ml), basic fibroblast growth factor (bFGF; 20 ng/ml), or dexamethasone (DEXA; 100 n*M*). Results are the mean and SEM percentage of the concentration from unstimulated chondrocytes (triplicate experiments).

YKL-40 regulation by cytokines and growth factors. IL-1β and TGFβ are major chondrocyte regulators and induce expression of distinct patterns of genes. Both IL-1β (5 ng/ml) and TGFβ (10 ng/ml) significantly suppressed YKL-40 levels in conditioned media from freshly isolated chondrocytes from normal cartilage ($P < 0.001$) (Figure 4A). This effect of IL-1β and TGFβ was also found in passage 1 and passage 2 chondrocytes and

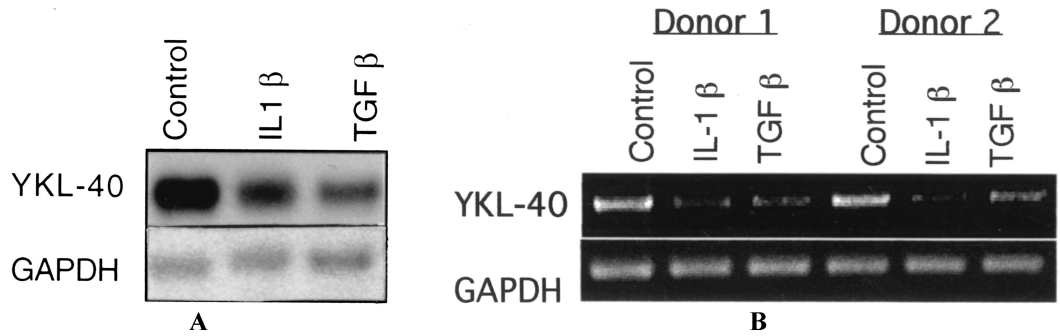

Figure 5. Suppression of YKL-40 messenger RNA (mRNA) expression by IL-1β and TGFβ. **A,** Northern blot analysis of YKL-40 mRNA expression by normal human monolayer chondrocytes (passage 1; representative of 3 different donors) after 24 hours of stimulation with media alone (Control), IL-1β (5 ng/ml), or TGFβ (10 ng/ml). Total RNA (10 μg) was isolated from the chondrocytes and subjected to Northern blot analysis with the YKL-40 complementary DNA (cDNA) probe and the GAPDH cDNA probe as described in Materials and Methods. **B,** The effect on YKL-40 message and corresponding GAPDH message is shown by reverse transcriptase–polymerase chain reaction (see Materials and Methods) performed on cDNA isolated from human chondrocytes (passage 1 chondrocytes from Donor 1, passage 2 chondrocytes from Donor 2) after 24 hours of stimulation with Control, IL-1β (5 ng/ml), or TGFβ (10 ng/ml). See Figure 4 for other definitions.

in late-passage chondrocytes (passage 9), although the difference was first significant in the late-passage cells after 3 days of stimulation (Figure 4B). YKL-40 secretion from freshly isolated chondrocytes from OA cartilage was also inhibited by IL-1β and TGFβ (data not shown). IL-1β inhibited YKL-40 release from explant cultures of normal cartilage to undetectable or very low levels (Figure 4C). In cartilage explants treated with TGFβ, no difference was found in the YKL-40 level in the conditioned media compared with that in the nonstimulated explants, but when stimulation with TGFβ was stopped at day 10, the YKL-40 level in the conditioned media increased significantly ($P = 0.008$ at day 14) compared with that in the nonstimulated explants (Figure 4C).

Among a series of other cytokines analyzed, TNFα also suppressed YKL-40 release from monolayer chondrocytes (passages 0 and 1). IL-6, IL-17, and IL-18 stimulated the YKL-40 production in passage 0 chondrocytes, but not in passage 1 cells. The growth factor IGF-1 had no effect on YKL-40 release; however, PDGF and bFGF stimulated YKL-40 production by passage 1 chondrocytes, but not by passage 0 chondrocytes (Figure 4D).

Articular chondrocytes (passages 0 and 1) isolated from normal cartilage were tested for the expression of YKL-40 mRNA by Northern blot. Stimulation of chondrocytes for 24 hours with IL-1β (5 ng/ml) or TGFβ (10 ng/ml) inhibited YKL-40 mRNA (Figure 5A) (the result shown is representative of 3 different donors). PCR analysis of cDNA confirmed the inhibition of YKL-40 expression from chondrocytes after 24 hours of stimulation with IL-1β (5 ng/ml) or TGFβ (10 ng/ml) (Figure 5B) (the result shown is representative of 5 different donors). No inhibition of YKL-40 mRNA was found after 12 hours of stimulation with IL-1β or TGFβ (data not shown).

Regulation of YKL-40 in OA cartilage. Cartilage with different degrees of OA was subjected to explant culture. The basal release of YKL-40 after 1 day in culture was significantly higher in OA cartilage explant cultures than in normal cartilage explant cultures (413 ng YKL-40 per 100 mg cartilage in OA explant culture [OA grades 3 and 4] versus 82 ng YKL-40 per 100 mg normal cartilage [OA grade 1]; $P = 0.0016$).

Cartilage with various degrees of OA (from 28 donors, ages 18–91 years), including OA grades 1 (intact surface, n = 5 donors), 2 (minimal fibrillation, n = 7 donors), 3 (overt fibrillation, n = 5 donors), and 4 (erosion, n = 11 donors), was subjected to explant culture. After 3 days of culture, the explants were stimulated for 48 hours with IL-1β (5 ng/ml), TNFα (10 ng/ml), or IL-18 (10 ng/ml). YKL-40 levels in the conditioned media were determined and corrected for the weight of the cartilage explants (Figure 6). The secretion of YKL-40 from nonstimulated explants was significantly higher ($P < 0.001$) in explants with OA grade 4 (4,800 ng per 100 mg cartilage) than in those with OA grades 1 (2,500 ng per 100 mg cartilage), 2 (2,700 ng per 100 mg cartilage), and 3 (2,700 ng per 100 mg cartilage). IL-1β inhibited the release of YKL-40 in all types of OA samples by a mean of 62% ($P < 0.002$). YKL-40 production after 48 hours of stimulation with TNFα or IL-18 was lower (mean decreases of 31% and

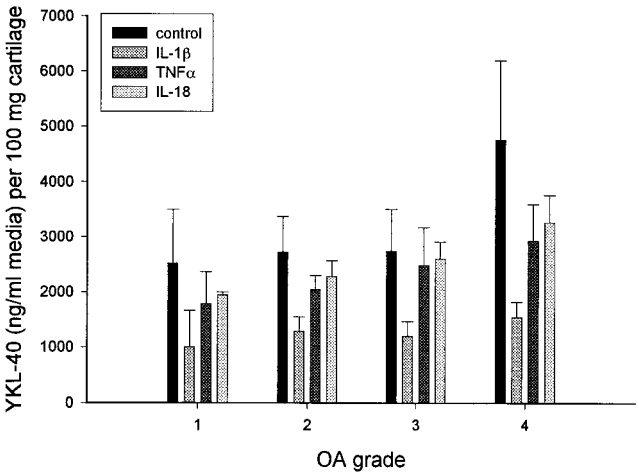

Figure 6. Cytokine regulation of YKL-40 production in cartilage explant cultures of normal and osteoarthritic (OA) cartilage. Shown is the relationship between the basal secretion of YKL-40 from cartilage explant cultures and the effect of 48 hours of stimulation with interleukin-1β (IL-1β; 5 ng/ml), tumor necrosis factor α (TNFα; 10 ng/ml), and IL-18 (10 ng/ml) according to different OA grades. A total of 28 different cartilage specimens were analyzed, including OA grades 1 (intact surface, n = 5 donors), 2 (minimal fibrillation, n = 7 donors), 3 (overt fibrillation, n = 5 donors), and 4 (erosion, n = 11 donors). The YKL-40 level in the media was corrected for the weight of the cartilage explant. Results are the mean and SEM.

21%, respectively), but was not significantly different from that in nonstimulated explants.

Distribution of YKL-40 in monolayer chondrocytes and cartilage explants. Immunofluorescence microscopy was performed on monolayer chondrocytes. The staining for YKL-40 antigen (Figure 7A) colocalized with the staining for giantin (Figure 7B), a protein secreted and stored in the Golgi system (29,30). Double staining using the monoclonal mouse antibody against YKL-40 and the polyclonal rabbit antibody against giantin demonstrated that YKL-40 was located in the Golgi system of the chondrocytes (the Golgi system in a cell only positive for giantin is marked with an arrow, while all the other cells are positive for both YKL-40 and giantin). Figure 7A shows that YKL-40 was distributed more widely than giantin, indicating that YKL-40 was found in both the Golgi system and the endoplasmic reticulum. Figures 7C and D illustrate immunohistochemical staining of YKL-40 in normal cartilage explant after 10 days in culture using the same monoclonal YKL-40 antibody employed for the immunofluorescence procedure. YKL-40 staining was found in the cytoplasm of all chondrocytes in the superficial and middle layers of the cartilage explant, but in only a few chondrocytes in the deep layer. Extracellular staining of YKL-40 in the cartilage matrix was not detected.

DISCUSSION

YKL-40 was originally identified as one of the most abundant proteins secreted by cultured chondrocytes (1,2). It is overexpressed in arthritis (1,11) and has been proposed to be of value as a marker of disease activity in inflammatory joint diseases and OA (10,19–21). However, stimuli that induce YKL-40 production had not been identified prior to this study. The purpose of the present study was to define conditions that lead to increased production of YKL-40 by normal human articular cartilage and isolated chondrocytes.

Immunohistochemistry studies of normal human articular cartilage that had been fixed immediately after resection from the joint have shown no or very low numbers of YKL-40–positive chondrocytes and no detectable YKL-40 in the extracellular matrix (11). The present study demonstrated that normal cartilage explants produce low levels of YKL-40 during the first days of culture. YKL-40 production was increased severalfold by day 6, then declined by ~50% by day 10 and remained at this level of production over the remaining 10 days of culture. The release of YKL-40 during explant culture was dependent on new protein synthesis, since it was abrogated by cycloheximide. Freshly isolated chondrocytes from cartilage did not release YKL-40 until they were cultured for at least 2 days, the time at which low levels of YKL-40 were detectable. When these chondrocytes were cultured to confluence, then trypsinized and replated as first-passage cells, YKL-40 was already being released during the first 24 hours.

After 7 days in first-passage culture, the cells produced >300-fold levels of YKL-40 compared with freshly isolated chondrocytes. This profound increase in the spontaneous production of a major secreted protein has not yet been reported for any other chondrocyte-derived protein. Serum was considered as a potential stimulus for this increased production, but it did not influence YKL-40 levels either in conditioned media from chondrocyte monolayers or in cartilage explants during the first week of culture.

The relatively slow onset of increased YKL-40 production during monolayer culture of primary chondrocytes implies that regulators of YKL-40 production change slowly during this process. This could represent either the disappearance of an inhibitor of YKL-40 production or the slow emergence of a stimulator. One possible explanation is that the presence of pericellular

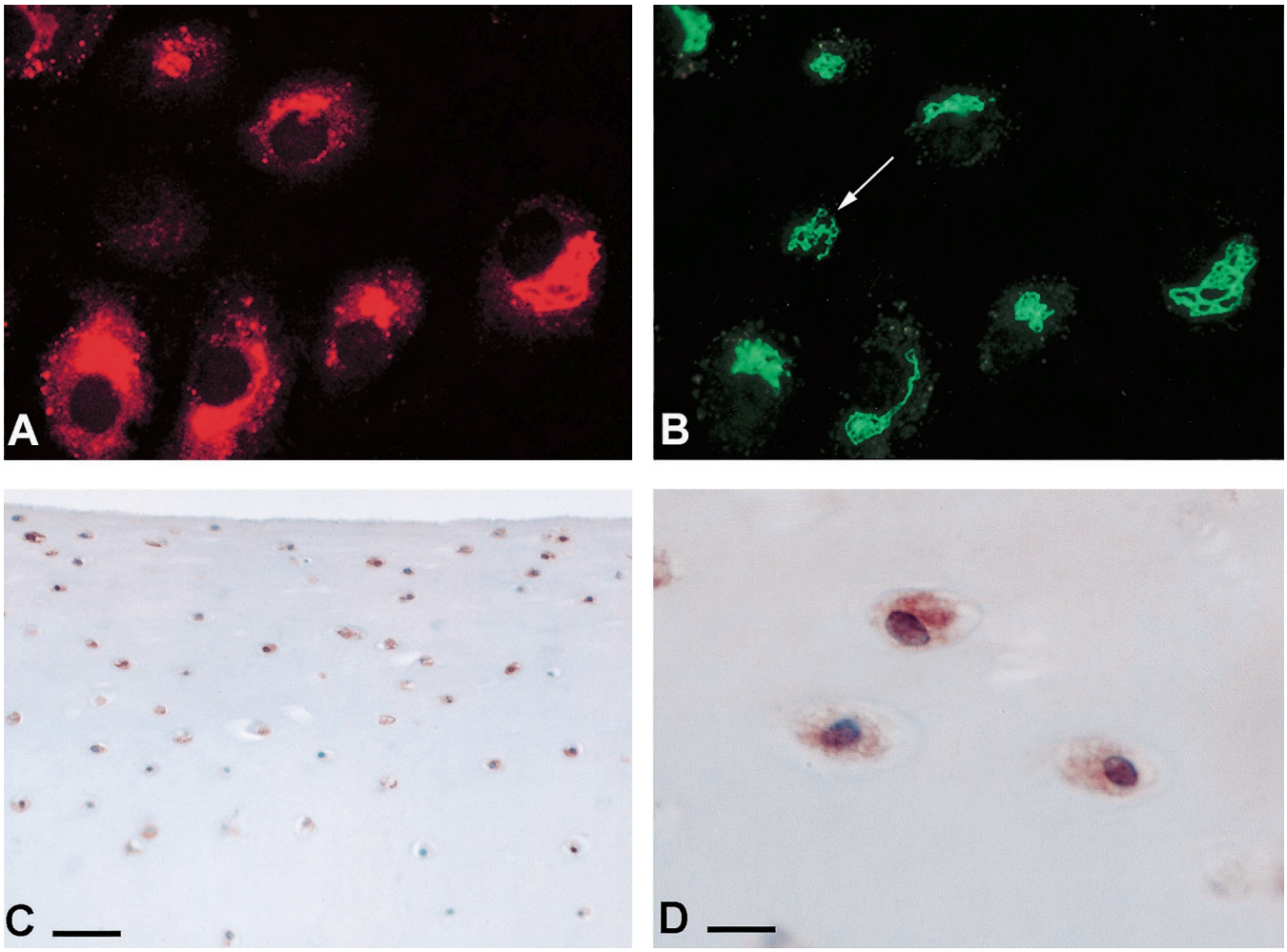

Figure 7. Distribution of YKL-40 in chondrocyte monolayer cultures and cartilage explant cultures. **A,** Fluorescence micrographs of immunofluorescence staining of YKL-40 in normal articular chondrocytes (passage 1) cultured as monolayer show positive YKL-40 intracellular staining in many cells (recognized as a red color, secondary antibody coupled to rhodamine). **B,** Similar staining is found for the Golgi protein giantin (recognized as a green color, secondary antibody coupled to fluorescein isothiocyanate). **Arrow** indicates a cell with no staining for YKL-40. **C,** YKL-40 staining in normal cartilage explant after 10 days of culture. YKL-40–positive chondrocytes were found in the superficial and middle layers of the cartilage and in only a few cells in the deep layer (positive chondrocytes are recognized as a brown color). Bar = 50 μm. **D,** Granular distribution of YKL-40 in the cytoplasm of the chondrocytes. No staining is detectable in the extracellular matrix of the cartilage. Bar = 10 μm.

matrix inhibits YKL-40 production. This interpretation is consistent with the requirement of several days of primary chondrocyte culture until the cells adhere to the culture plate and spread out.

To determine whether this change in the extracellular environment contributes to the increase in YKL-40 production during monolayer culture, cells were transferred to different 3-dimensional culture systems. After transfer to alginate culture, the levels of YKL-40 in the culture media were significantly reduced. In contrast, during the time interval of 7 days, the cell clusters in methyl methacrylate–coated plates still continued to release levels of YKL-40 similar to those released by the monolayer cultures. The difference in results obtained with these 2-dimensional culture systems may relate to the ability of the cells to directly attach to the alginate matrix, while in the methyl methacrylate cultures, the cells first have to produce a matrix to which they can subsequently adhere. A possible alternative explanation is that chondrocytes in monolayer and suspension culture make cell-to-cell contact, which is prevented in the alginate. Cells produce extracellular matrix in monolayer culture and even higher levels in alginate culture. However, YKL-40 production

increased with time in either system. This suggests that the type of matrix that is produced in these culture systems does not exactly replicate the influence of native extracellular matrix in cartilage explants.

An alternative explanation for the increase in YKL-40 production during monolayer culture is chondrocyte dedifferentiation. Chondrocytes are known to undergo phenotypic changes in monolayer culture. This process is characterized by a decrease in the levels of type II collagen expression (33). However, this mechanism is not likely to be operative, since the differentiated cells in cartilage explant cultures can produce YKL-40. The stimulus for YKL-40 production in the cartilage explants appears to be generated by tissue injury. This was demonstrated in single explants that produced higher levels of YKL-40 after being cut into several smaller pieces. Collectively, the increase in spontaneous YKL-40 production during the early phases of chondrocyte monolayer culture, the reduction in the levels produced after transfer to alginate, and the production of YKL-40 by cells in injured cartilage are all consistent with the hypothesis that changes in the extracellular matrix environment are responsible for the induction of YKL-40 production.

We reported earlier that YKL-40–positive chondrocytes are found in the superficial and middle layers of OA cartilage from the hip (11) or knee joint (34). These tissues are characterized by degradation of the various components of the extracellular matrix, and this provides further support for the concept that matrix injury triggers production of YKL-40.

In the present study, the basal secretion of YKL-40 from OA cartilage explant cultures during the first 24 hours of culture was significantly higher compared with normal cartilage explant cultures, and after 5 days in culture, the highest level of YKL-40 secretion was still found in OA cartilage explant cultures. This observation of increased YKL-40 production by chondrocytes in diseased tissue is in accordance with the findings of Hakala et al (1), who demonstrated no YKL-40 mRNA in normal cartilage but found high expression of YKL-40 in arthritic cartilage, and is consistent with the findings of several studies showing that YKL-40 levels are elevated in synovial fluid and serum from patients with active RA or severe OA of the knee joint compared with normal subjects (10,19–21).

The secretion of most proteins in chondrocytes can be regulated by cytokines and growth factors. In particular, it is well established that IL-1β and TGFβ alter the levels of many proteins in cartilage, often in qualitatively distinct patterns (35,36). Catabolic cytokines, such as IL-1β and TNFα, have been shown to be responsible for increased cartilage degradation and inhibition of synthesis of the extracellular cartilage matrix (36,37). In contrast, the growth factors TGFβ, IGF-1, and PDGF stimulate new cartilage formation (36,37).

In this study, IL-1β was characterized as a potent inhibitor of YKL-40 production. In more than 30 cell preparations and cartilage explant cultures from different donors, IL-1β inhibited YKL-40 release, often to undetectable levels. TGFβ also inhibited YKL-40 production from monolayer chondrocytes, although it was less potent than IL-1β. The inhibition of YKL-40 release from chondrocytes in monolayer was associated with a suppression of YKL-40 mRNA levels, and this was shown for both IL-1β and TGFβ. The observation that IL-1β inhibits YKL-40 expression is in apparent contrast to the finding that it stimulates extracellular matrix degradation. However, the finding that IL-1β inhibits YKL-40 mRNA expression suggests that it directly suppresses YKL-40 gene expression. In cartilage explant cultures, no inhibitory effects of TGFβ were found during treatment, but when stimulation with TGFβ was stopped, YKL-40 production increased significantly compared with that in nonstimulated explants.

TNFα also inhibited YKL-40 production from cells in monolayer, but not in cartilage explant cultures. IL-6 (a cytokine that up-regulates tissue inhibitors of metalloproteinases [38]), IL-17 (a cytokine that induces nitric oxide production and up-regulates inducible nitric oxide synthase and cyclooxygenase 2 genes [39]), and IL-18 (a cytokine that contributes to cartilage degradation [40]) stimulated YKL-40 production from freshly isolated chondrocytes. The increases were relatively small compared with the spontaneous increase during monolayer culture. IL-18 had no significant effect on YKL-40 production by cartilage explants. The growth factors IGF-1, bFGF, and PDGF had no significant effects on the production of YKL-40. The regulation of YKL-40 by cytokines and growth factors is therefore relatively rare, since YKL-40 is suppressed by both IL-1β and TGFβ and not stimulated by a large number of qualitatively distinct factors.

This study also defined the subcellular distribution of YKL-40. The intracellular presence of YKL-40 was demonstrated in monolayer chondrocyte cultures. Its precise localization was shown in the Golgi system, as indicated by double staining with the Golgi-specific marker giantin. Similar intracellular distribution was seen in cartilage explant cultures. The localization of YKL-40 in the Golgi system is in accordance with the observation that YKL-40 is a secreted protein. Interest-

ingly, YKL-40 was not detectable by immunohistochemistry on the surface of cells in monolayer or explant culture, and was also not detectable in the pericellular or extracellular spaces of cartilage, in accordance with findings of earlier studies (11,34). However, the presence of YKL-40 in the pericellular matrix or extracellular matrix can not be excluded, since YKL-40 epitopes recognized by antibodies may be masked by interaction with other matrix components.

In conclusion, the present study demonstrates that YKL-40 is produced in response to removal of chondrocytes from their native extracellular matrix environment and after injury to the cartilage extracellular matrix. In contrast, cytokines and growth factors that are known to be important regulators of chondrocyte function have only minor stimulatory effects on YKL-40 production. The major effect of cytokines and growth factors is the suppression of YKL-40 production. This was observed in response to IL-1β and TGFβ. These findings identify a unique regulatory network that controls YKL-40 production in cartilage.

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