Journal of Cellular Biochemistry 69:414–424 (1998)

# Purification of Guinea Pig YKL40 and Modulation of Its Secretion by Cultured Articular Chondrocytes

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**Abstract** The aim of this study was to purify, characterize, and study the regulation at the chondrocyte level of the guinea pig (gp) homologue of human (R) YKL40, a putative marker of arthritic disorders. Studying YKL40 in guinea pigs is of particular interest, as age-related osteoarthritis develops in this species spontaneously. Both N-terminal sequencing and total amino acid composition of gpYKL40 purified from the secretion medium of cultured articular chondrocytes indicate a high degree of identity with hYKL40. gpYKL40 was found to contain complex N-linked carbohydrate, as demonstrated by N-glycosidase F and endoglycosidase F digestion. Isoelectric focusing demonstrated the presence of a major band at pI 6.7. The secretion of gpYKL40 by confluent articular chondrocytes in the extracellular medium was studied by immunoblotting. gpYKL40 was released by chondrocytes continuously over a 7 day period and did not appear to be degraded by proteinases, as its signal intensity in cell-free medium at 37°C did not decrease with time. Thus, gpYKL40 displays high stability and accumulates in extracellular medium without reaching a steady-state level. Among the main factors known to regulate cartilage metabolism, IL-1β, TNF-α, bFGF, or $1,25(OH)_2D_3$ did not alter the basal level of gpYKL40, and retinoic acid had a slight inhibitory effect; TGF-β and IGF-I and -II dose-dependently and inversely modulated this basal level. TGF-β at 5 ng/ml decreased extracellular gpYKL40 2.9-fold, whereas IGF-I and IGF-II at 50 ng/ml increased extracellular gpYKL40 3.6- and 3.4-fold, respectively. The present biochemical and biological findings give new insights for studying the function of YKL40 in cartilage. J. Cell Biochem. 69:414-424, 1998. 

**Key words:** YKL40; purification; guinea pig; chondrocytes; biochemical characterization; regulation; insulin-like growth factors; osteoarthritis

YKL40 is a mammalian glycoprotein related in sequence to family 18 of bacterial and fungal chitinases [Henrissat and Bairoch, 1993], but it is devoid of glycosidase activity against chitinase substrates, as the glutamate in the active site Trp-Glu-Tyr-Pro of the latter is replaced by another amino acid. It may, however, use structural elements typical for chitinases to mediate binding to carbohydrates [Hakala et al., 1993], as also suggested for the related protein YKL39 [Hu et al., 1996]. YKL40 was initially discovered as a secretory protein in bovine mammary secretions from the nonlactating period [Rejman and Hurley, 1988]; it was subsequently isolated from the secretion media of human cultured synovial cells [Nyirkos and Golds, 1990] and osteosarcoma cells [Johansen et al., 1992]. Further studies have confirmed its importance in the pathophysiology of skeletal tissue. As observed by reverse transcription-polymerase chain reaction (RT-PCR), YKL40 is strongly expressed in the degrading cartilage of patients with rheumatoid arthritis [Hakala et al., 1993]. The serum YKL40 concentrations in osteoarthritic disorders and inflammatory joint diseases are significantly higher than those of controls [Johansen et al., 1993]; sera and synovial fluid levels of YKL40 correlate with the stage of osteoarthritis [Johansen et al., 1996], making of YKL40 a potential useful marker to

Abbreviations: gpYKL40, guinea pig YKL40; hYKL40, human YKL40; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; bFGF, fibroblast growth factor-basic; $1,25(OH)_2D_3$, dihydroxycholecalciferol; TGF-β, transforming growth factor-β; IGF-I and -II, insulin-like growth factor-I and -II; BSA, bovine serum albumin; FCS, fetal calf serum; PBS, phosphate-buffered saline; RP-HPLC, reversed-phase-high-performance liquid chromatography.

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Received 27 August 1997; Accepted 29 December 1997

assess the degradation of articular cartilage. No (or poor) expression is found in normal cartilage. By contrast, explants or chondrocytes isolated from normal cartilage secrete YKL40 in vitro, suggesting that the synthesis of this protein closely depends on its extracellular environment [Hakala et al., 1993]. Moreover, YKL40 accounts for 33% of the proteins in human chondrocyte-conditioned medium [Hu et al., 1996], and represents the major protein secreted by human cultured cartilage cells.

Despite these findings, which suggest an important role for YKL40 in cartilage, the number of data collected on the protein is relatively poor, and its biological function remains to be found. For such an investigation, the use of chondrocytes from non human species may be preferred, as human chondrocytes are not easily available and grow with difficulty in vitro. The guinea pig is a particularly attractive model, as this species develops age-related osteoarthritis spontaneously [Bendele and Hulman, 1988; De Bri et al., 1995], and thus may permit the investigation of the biological relevance of YKL40 in both normal and pathological cartilage. However it has not been ascertained that guinea pig cartilage produces YKL40 and more generally, no previous work has documented the presence of YKL40 in cartilage from non human mammals.

The present experiments were designed to purify and characterize biochemically YKL40 secreted by cultured guinea pig chondrocytes, to study its release kinetics and susceptibility to degradation in extracellular medium, and to determine which factors implicated in cartilage metabolism regulate gpYKL40 levels in the extracellular medium.

## MATERIALS AND METHODS

### Materials

Horizontal electrophoresis, immunoblotting, and isoelectric focusing (IEF) were performed on a Multiphor II apparatus using appropriate kits (Pharmacia Biotech, Uppsala, Sweden). Electrophoresis and immunoblotting reagents were purchased from Bio-Rad (Richmond, CA). HPLC purification was performed using a Perkin-Elmer high-performance liquid chromatography (HPLC) system consisting of a model 400 solvent delivery system and a model LC-95 absorbance detector. Chromatograms were registered and integrated using the PC Integration Pack from Kontron (St. Quentin-en-Yvelines, France). Ultragradient grade acetonitrile and trifluoroacetic acid (TFA) were from Baker (Deventer, Holland). Milli-Q plus grade water (Millipore, Bedford, MA) was used during the purification. Enzymes for cartilage digestion and N-glycosidase F were from Boehringer (Mannheim, Germany). Endoglycosidases F and H were from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) and culture media were from GIBCO (Grand Island, NY). Recombinant murine interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) and human transforming growth factor-β (TGF-β) were from R & D Systems (Minneapolis, MN); recombinant human basic fibroblast growth factor (GFGF) and insulin-like growth factors -I and -II (IGF-I, IGF-II) were from Genzyme (Boston, MA); BSA, retinoic acid and 1α,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) were from Sigma Chemical Co.

### Cell Cultures

Articular cartilage was obtained from tibial plateaus and femoral condyles of 14-day-old guinea pigs (Hartley strain). Cartilage slices were harvested under sterile conditions and cut into small pieces in a solution of Gey's salt balanced medium without Ca or Mg (Eurobio, Les Ulis, France) containing 0.05% hyaluronidase. Cartilage pieces were then treated with 0.25% trypsin for 30 min and twice with 0.2% clostridial collagenase for 60 min and 30 min, until dissolution of the fragments. Isolated chondrocytes (about $1 \times 10^6$ cells per guinea pig knee) were harvested by centrifugation at 500*g* for 3 min and plated in T225 flasks at a density of $5 \times 10^3$ cells/cm$^2$ in 30 ml Ham's F12 medium containing 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B. Cells were subcultured for 1–2 passages under similar conditions. At these stages, cells retained a differentiated polygonal shape. From passage 3, cells lengthened and rapidly dedifferentiated into fibroblastic cells. For the production of culture media intended for the purification of gpYKL40, confluent chondrocytes (passages 1 and 2) were washed three times in PBS-Dulbecco, incubated for 24 h in Ham's F12 medium without FCS containing 0.01% bovine serum albumin (BSA) and further incubated for 72 h in fresh Ham's F12 medium without FCS nor BSA. This latter medium was supplemented with 1 mM EDTA, 1 µM leupeptin, 1 µM pepstatin, 0.2 µg/ml sodium azide, and frozen at −30°C until used.

Human *MG 63* osteosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in minimum essential medium-α (MEM-α) containing 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B and $10^{-8}$ M vitamin K3. At confluency, the cell layers were washed and treated as described above.

### Immunoblotting Assay for YKL40

Guinea pig YKL40 immunoreactive protein was monitored before and during purification by an immunoblotting procedure using rabbit anti-human YKL40 primary antibodies kindly provided by Dr Justine Whaley (NovaDX, San Diego, CA). Aliquots from eluted fractions were either evaporated to dryness or desalted (Microcon 10 units, Amicon, Beverly, MA) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using laboratory-made 7.5–15% polyacrylamide gels. Proteins were electroblotted onto a nitrocellulose membrane. After blocking in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) and 5% powdered milk for 2 h at 22°C, each membrane was washed and incubated overnight at 4°C with primary antibodies diluted 1:2,500 in PBS-T containing 0.8% powdered milk. Membranes were washed and further incubated for 2 h at 22°C with anti-rabbit IgG HRP conjugate (1:2,000). Immunoreactive material was detected by enhanced chemiluminescence (ECL kit, Amersham, UK).

### Purification Scheme

Conditioned-medium from guinea pig chondrocytes was diafiltered in a 400 ml device fitted with a YM 10 membrane (Amicon). The concentrated and desalted medium was then loaded onto a 125 × 25-mm heparin HyperD column (Biosepra, Villeneuve-la-Garenne, France). Unretained material was washed from the column with a 10-column volume of 10 mM Tris-HCl pH 7.0, 50 mM NaCl, 0.02% $NaN_3$. Retained material was eluted with a linear gradient of 0.05 to 2 M NaCl for 4 h at a flow rate of 120 ml/h; 6-ml fractions were collected and monitored for YKL40 immunoreactivity. Immunoreactive fractions were pooled and further purified by reversed-phase HPLC (RP-HPLC), using a 250 × 4.5-mm Vydac 201TP $C_{18}$ column (The Separations Group, Hesperia, CA). Proteins were eluted in 0.1% TFA with a linear gradient of 0–70% acetonitrile for 70 min at a flow rate of 0.7 ml/min. The purity of immunoreactive fractions was assessed by the symetry of HPLC peaks and further confirmed by SDS-PAGE and silver staining (Silver stain plus kit, Bio-Rad). A similar procedure was used for the purification of human YKL40 from MG63 osteosarcoma cells.

### Biochemical Characterization

N-Terminal microsequencing was performed with a gas-phase protein sequencer (model 477A) from Applied Biosystems (Foster City, CA) connected to a Model 120 A phenylthiohydantoin aminoacid analyser (Applied Biosystems). Amino acid composition was determined using a model 420A derivatizer connected to a model 130 phenylthiocarbamyl amino acid analyser (Applied Biosystems).

The overall glycosylation was studied by use of the glycoprotein detection system from Amersham, following the instructions provided by the manufacturer. As proteins reacted positively in this system, glycosylation was further studied by glycosidase digestions. Protein aliquots were boiled for 1 min in 10 µl of SDS 1% and incubated for 24 h at 37°C with or without (control) 5 units N-glycosidase F, 1 unit endoglycosidase F, or 200 mU endoglycosidase H in a final volume of 100 µl of 20 mM sodium phosphate, pH 6.8 containing 0.5% Nonidet P-40. Then, samples were electrophoresed and analyzed by immunoblotting.

The isoelectric point (IEP) was determined by isoelectric focusing using a precast polyacrylamide gel (Pharmacia Biotech) containing a mixture of ampholines (pH range 3.5–9.5). Proteins were focused according to the manufacturer's instructions and detected by immunoblotting. The accurate isoelectric points were determined by comparison with pI standards (Sigma) run on the same gel and stained with R-250 Coomassie blue.

### Release and Degradation of gpYKL40 in Extracellular Medium

Studies were performed when chondrocytes had reached confluency. For release kinetics, chondrocytes in 24-well plates were changed for 1 ml of fresh serum-free medium at time 0 and further incubated at 37°C for different times. Then, a 100-µl aliquot was taken and analyzed by immunoblotting. For degradation studies in cell-free medium, chondrocytes in

T225 flasks were changed for 30 ml of fresh serum-free medium and further incubated at 37°C for 48 h. At the end of this incubation time, the secretion medium was distributed into cell-free 24-well plates (time 0) and further incubated at 37°C for different times. The degradation was studied by immunoblotting, starting from a 100-µl aliquot in each well.

### Regulation of gpYKL40 by Growth Factors and Cytokines

Chondrocytes grown to confluency in 24-well plates were transferred to serum-free medium containing 0.01% BSA for 16 h and then for 24 h in 1 ml of serum-free medium containing 0.01% BSA with or without various concentrations of IL-1β, TNF-α, FGFb, $1,25(OH)_2D_3$, retinoic acid, TGF-β, IGF-I, or IGF-II. Then, a 100-µl aliquot was taken and analyzed by immunoblotting. The bands corresponding to the gpYKL40 signals were quantified by densitometric analysis using a type Arcus II scanner (Agfa, France) with the 1D Image Analysis software (Eastman Kodak, Rochester, NY). In the range of densities measured in the regulation experiments, a linear relationship ($r = 0.98$; $P < 0.01$) was observed between the amount of gpYKL40 loaded on the gel and the x-ray film density.

### Statistical Analysis

In the regulation experiments, data are expressed as relative amounts of gpYKL40 released in extracellular medium, with controls assigned a value of 100. Results are expressed as the mean ± SEM of three independent experiments. The effect of treatments was assessed by one-way analysis of variance (ANOVA) and differences from the corresponding control assessed by Dunnett's t-test.

## RESULTS

### Purification of gpYKL40

In order to detect the presence of an YKL40 form secreted by the guinea pig chondrocytes, a 500-µl medium aliquot was electrophoresed by comparison with a 500-µl medium aliquot of human MG63 osteosarcoma cells cultured without serum for 24 h. A strong immunoreactive band was detected in the chondrocyte medium at an apparent molecular weight of 42 kDa similar to that of YKL40 secreted by MG63 cells, indicating that guinea pig chondrocytes produce in high amounts a protein related to human YKL40 (not shown). The purification was started from 300 ml of culture medium obtained from confluent chondrocytes incubated for 72 h without FCS and containing an amount of 4.31 mg of total protein. The diafiltered medium containing 3.97 mg of protein was loaded onto the heparin column. Immunoblot analysis of eluted fractions revealed a strong chemiluminescent signal in fractions 20-24 corresponding to 0.51–0.68 M NaCl, at an apparent molecular weight of 42 kDa. By comparison, hYKL40 had a slightly weaker affinity, eluting at 0.34–0.44 M NaCl (data not shown). The protein corresponding to the gpYKL40 signal was separated further by RP-HPLC (Fig. 1). It was eluted at 57–58% acetonitrile (as for hYKL40) as a sharp symmetric peak corresponding to an amount of 90 µg of protein based on area at 220 nm using BSA for calibration. Homogeneity was assessed by the presence of a single band after electrophoretic migration and silver staining (Fig. 1, inset).

### Specific Activities

Since no radioimmunoassay or ELISA is available for guinea pig YKL40, silver staining of total proteins was performed at each purification step (Fig. 2). Each well corresponds to 0.15% of the collected volume at either step. Thus, the recovery of gpYKL40 could be estimated directly by considering the intensity of the 42 kDa signal. This recovery was high, as only a low decrease in the intensity of the 42-kDa band was observed. After heparin chromatography, the ratio of gpYKL40 over total proteins was increased to an apparent specific activity close to 100%. However, the presence of minor contaminants that did not appear on the gel was shown by RP-HPLC (Fig. 1) and thus, this latter step was needed for homogeneity. The 82-kDa band observed in the two latter steps corresponds to a dimeric form of gpYKL40 that variably appears when the pure protein is dried and loaded onto a gel [Hu et al., 1996].

### Biochemical Characterization

N-Terminal microsequencing identified the purified protein as the guinea pig homologue of human YKL40. A strong degree of homology was found between the two N-terminal sequences, with only a single substitution of Leu 25 in hYKL40 by Ile in gpYKL40 (Table I). Since the signal intensities at cycles 24, 27, 31, and 32 were too low, the corresponding amino

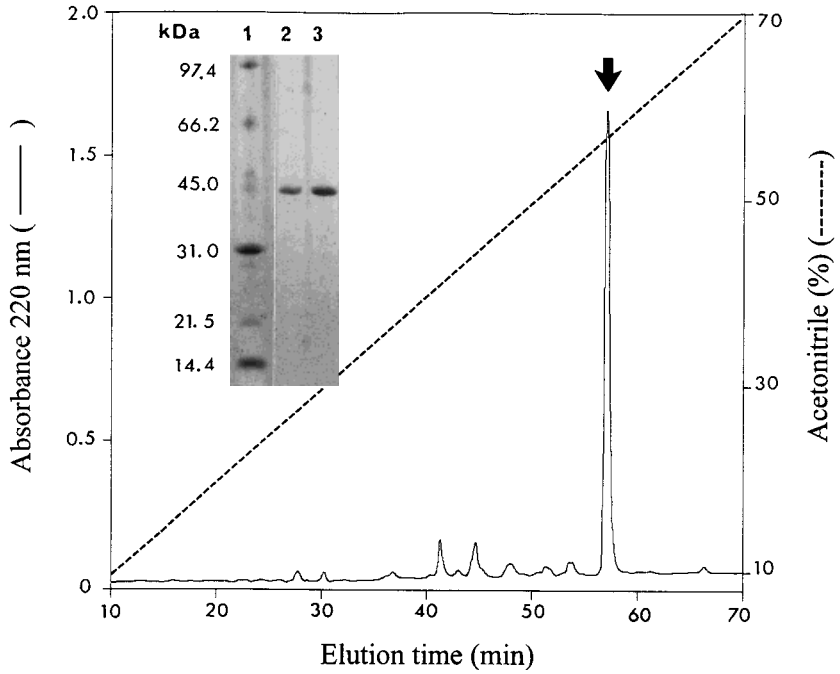


**Fig. 1.** Final purification of gpYKL40. Fractions 20-24 from the heparin chromatography were pooled and applied to a reversed-phase HPLC column. Arrow, peak corresponding to the YKL40 immunoreactive fractions. The purity of the two immunoreactive fractions is assessed by SDS-PAGE and silver staining (inset). **Lane 1**, molecular mass standards (Bio-Rad); **lane 2**, fraction eluting at 57 min; **lane 3**, fraction eluting at 58 min.

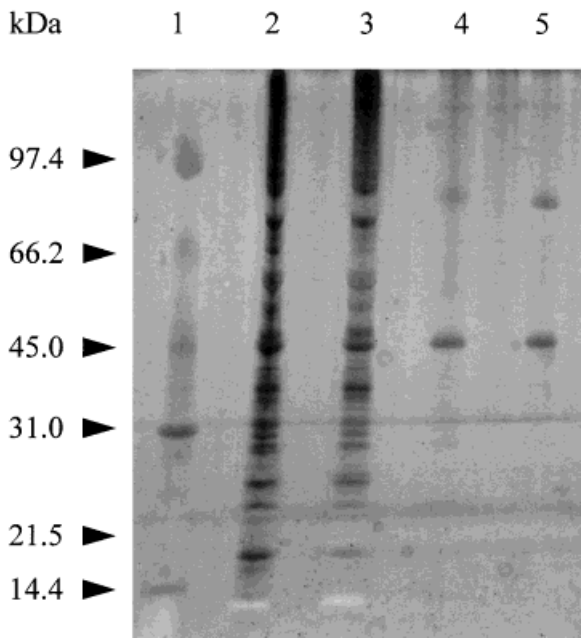


**Fig. 2.** Silver staining of total proteins at each purification step. An amount corresponding to 0.15% of total proteins at each purification step was electrophoresed and silver stained. **Lane 1**, molecular mass standards (Bio-Rad); **lane 2**, crude medium; **lane 3**, diafiltered medium; **lane 4**, pooled fractions 20–24 from heparin chromatography; **lane 5**, pooled fractions 57–58 from reversed-phase HPLC.

acids could not be identified. The total amino acid composition determined after acid hydrolysis confirmed the high degree of similarity between gpYKL40 and the human protein. However, a difference was found for residues Glx (Gln or Glu), Val, and Met (Table II).

Carbohydrate labeling identified the purified protein as a glycoprotein, as also observed for its human homologue (not shown). Therefore, carbohydrate was further studied by glycosidase digestions (Fig. 3). N-Glycosidase F treatment of gpYKL40 resulted in a 3 kDa reduction of its apparent molecular weight in SDS-PAGE, indicating the presence of N-linked oligosaccharides, as for hYKL40. Endoglycosidase F resulted in similar reduction of the molecular weight of gpYKL40, as for hYKL40, indicating the presence of N-linked complex oligosaccharides. No digestion of either protein was obtained with endoglycosidase H, suggesting that they do not contain high mannose oligosaccharides. It has to be noted that the dimeric glycosylated or deglycosylated forms of gpYKL40 and hYKL40 were also observed in these experiments.

IEF demonstrated the presence of a major band migrating at a pI 6.7 for both the guinea pig and human YKL40 (Fig. 4). In some experi-

**TABLE I. N-Terminal Sequence of Guinea Pig YKL40 Compared With Human YKL40**

| | 1 | 5 | 10 | 15 |
|---|---|---|---|---|
| hYKL40[a] | Tyr-Lys-Leu-Val-Cys-Tyr-Tyr-Thr-Ser-Trp-Ser-Gln-Tyr-Arg-Glu-Gly-Asp- | | | |
| gpYKL40 | Tyr-Lys-Leu-Val-Cys-Tyr-Tyr-Thr-Ser-Trp-Ser-Gln-Tyr-Arg-Glu-Gly-Asp- | | | |
| | 20 | 25 | 30 | 35 |
| hYKL40[a] (continued) | Gly-Ser-Cys-Phe-Pro-Asp-Ala-Leu-Asp-Arg-Phe-Leu-Cys-Thr-His-Ile-Ile-Tyr | | | |
| gpYKL40 (continued) | Gly-Ser-Cys-Phe-Pro-Asp-Xaa-Ile-Asp-Xaa-Phe-Leu-Cys-Xaa-Xaa-Ile-Ile-Tyr | | | |

[a]According to the sequence by Hakala et al. [1993].
Xaa, unidentified amino acid.

**TABLE II. Amino Acid Composition of Guinea Pig YKL40**

| Amino acid | hYKL40[a] (%) | gpYKL40 (%) | Similarity (%) |
|---|---|---|---|
| Asn + Asp | 10.22 | 10.40 | 98.1 |
| Gln + Glu | 7.18 | 9.06 | 78.6 |
| Ser | 7.73 | 8.00 | 96.6 |
| Gly | 8.84 | 8.48 | 95.9 |
| His | 2.49 | 2.77 | 89.9 |
| Arg | 5.25 | 4.67 | 89.0 |
| Thr | 7.18 | 7.02 | 97.8 |
| Ala | 7.73 | 8.43 | 91.7 |
| Pro | 4.70 | 4.33 | 92.1 |
| Tyr | 3.87 | 4.64 | 83.4 |
| Val | 3.87 | 5.01 | 77.2 |
| Met | 1.66 | 1.21 | 72.9 |
| Ile | 4.97 | 4.56 | 91.8 |
| Leu | 9.39 | 9.58 | 98.0 |
| Phe | 5.80 | 5.49 | 94.7 |
| Lys | 5.52 | 6.35 | 86.9 |
| Trp | 2.21 | n.d. | n.d. |
| Cys | 1.38 | n.d. | n.d. |

[a]According to the sequence by Hakala et al. [1993].
n.d., not determined.

ments, additional minor bands variably appeared. However, their relationship with YKL40 cannot be assessed with an absolute certainty.

### Release and Degradation of gpYKL40 in Extracellular Medium

The signal for gpYKL40 in the secretion medium of confluent chondrocytes increased day after day during the 7-day study, without reaching a plateau (Fig. 5A). When a 48-h secretion medium was replaced in cell-free conditions (Fig. 5B), no significant alteration of the gpYKL40 signal was observed up to 7 days of incubation at 37°C.

### Regulation of gpYKL40 by Growth Factors and Cytokines

A variety of growth factors or cytokines known to be implicated in the regulation of cartilage metabolism were tested for their ability to modulate the level of gpYKL40 in extracellular medium (Fig. 6). IL-1β, TNF-α, bFGF, and $1,25(OH)_2D_3$ used at their optimal effective concentrations in cartilage had no significant effect on the gpYKL40 level (Fig. 6A). Retinoic acid at $10^{-8}$ M (3 ng/ml) had a slight but significant ($P < 0.01$) inhibitory effect on gpYKL40 release. TGF-β exerted a dose-dependent inhibitory effect ($P < 0.01$) on the extracellular level of gpYKL40 in the range of 0.1–10 ng/ml, with a maximum of 2.9-fold at 5 ng/ml (Fig. 6B). Both IGF-I and -II increased gpYKL40 in extracellular medium in a dose-dependent manner ($P < 0.001$ for IGF-I and $P < 0.01$ for IGF-II) (Fig. 6C). IGF-I had a maximal effect of 3.6-fold at the concentration of 25 ng/ml and IGF-II had a maximal effect of 3.4-fold at the concentration of 50 ng/ml.

## DISCUSSION

The guinea pig homologue of human YKL40, gpYKL40, has been purified from the secretion medium of cultured chondrocytes obtained from articular cartilage. Polyclonal antibodies raised in rabbits against human YKL40 cross-react with guinea pig YKL40, showing that the two proteins share at least one common epitope. This cross-reactivity was used for monitoring gpYKL40 during purification.

The purification procedure developed in this study is based on the procedure by Nyirkos and Golds [1990], except that hyperdiffusion heparin was used, instead of heparin–Sepharose chromatography, together with a $C_{18}$ column previously shown to be highly resolutive for the

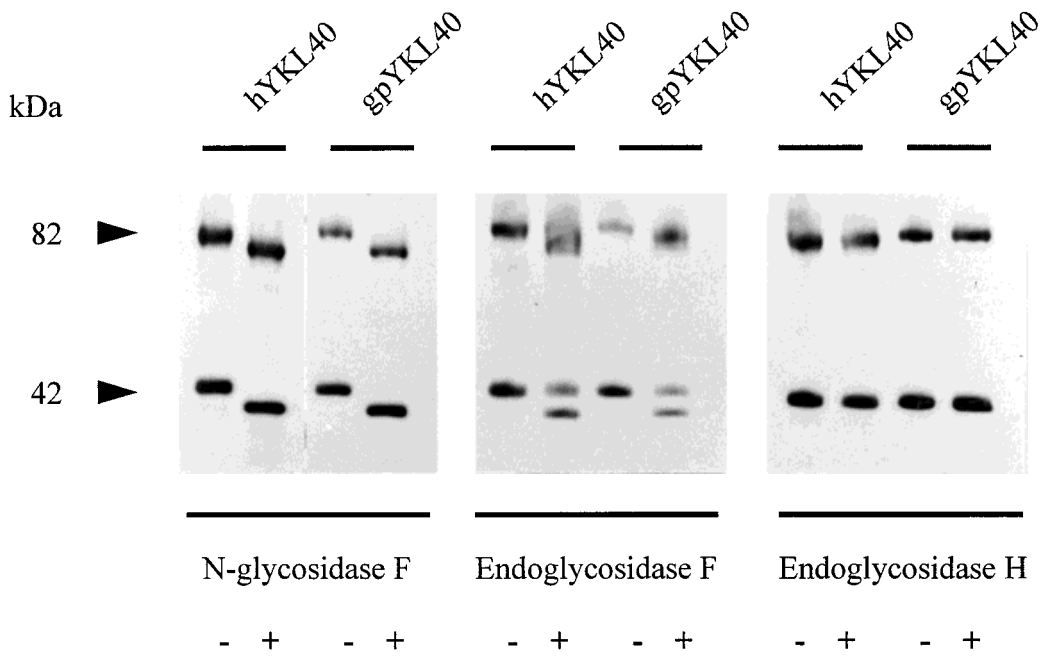


**Fig. 3.** Glycosylation pattern of gpYKL40. Pure gpYKL40 was subjected to glycosidase digestion as indicated, electrophoresed on a 7.5–15% gradient gel and immunoblotted. Pure hYKL40 was processed simultaneously for comparison with gpYKL40.

purification of complex protein mixtures [De Ceuninck et al., 1995a]. This procedure may also be applicable to the isolation of YKL40 from other cell types, as it was successfull for the purification of human YKL40 from MG63 osteosarcoma cells. As observed by silver staining, the most efficient purification step is heparin chromatography, as gpYKL40 was separated from most contaminating proteins in its elution range. However, RP-HPLC revealed the presence of minor remaining proteins and was thus retained as a final step for the purification of gpYKL40 to homogeneity. Whether or not gpYKL40 retains a functional activity after the purification procedure used remains to be determined.

The carbohydrate part of YKL40 may differ between species. Indeed, although bovine and human YKL40 both were shown to contain N-linked oligosaccharides [Rejman and Hurley, 1988; Hakala et al., 1993], only the bovine protein was efficiently deglycosylated by endoglycosidase H, indicating the presence of high mannose structures. The guinea pig YKL40 also contains N-linked glycans as demonstrated in this study by efficient N-glycosidase F digestion, but like its human homologue, it was resistant to endoglycosidase H digestion. Endoglycosidase F resulted in a reduction of the molecular weight of human YKL40, indicating the presence of complex oligosaccharides and this was also found for gpYKL40, in contrast to the results found for the bovine protein [Rejman and Hurley, 1988]. These results indicate that the glycosylation pattern of gpYKL40 more closely resembles the human protein than the bovine one. The isoelectric point of YKL40 had not been reported previously. Besides the major form at pI 6.7, some minor additional bands variably appeared depending on experiments. However, it cannot be concluded whether these faint bands reflect minor modifications in carbohydrate, phosphorylation or sequence of the protein, thus changing its pI. They may represent migration artifacts as well.

YKL40 accounts for 33% of the protein secreted by cultured chondrocytes obtained from healthy human donors [Hakala et al., 1993]. In the silver staining pattern of proteins secreted by guinea pig chondrocytes, it is obvious that gpYKL40 does not account for such a high ratio, although it is clearly one of the main components secreted in extracellular medium. This difference may be due to species or age specificities. In particular, chondrocytes derived from young animals have high anabolic activities compared to chondrocytes from older animals [Corvol et al., 1987], which may account for the lower relative abundance of

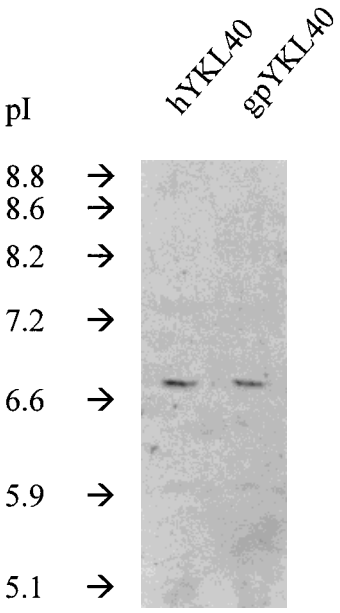


**Fig. 4.** Isoelectric focusing of gpYKL40. Pure gpYKL40 was loaded onto a precast gel containing 2.2% of ampholines (pH 3.5–9.5) (Pharmacia Biotech), and immunoblotted. Pure hYKL40 was processed simultaneously for comparison with gpYKL40. The migration position of pI standards is indicated. pI 8.8–8.6–8.2: lentil lectin; pI 7.2: horse myoglobin, basic; pI 6.6: human carbonic anhydrase B; pI 5.9: bovine carbonic anhydrase B; pI 5.1: β lactoglobulin A.

YKL40 secreted by chondrocytes from young guinea pigs.

Both N-terminal sequencing and determination of total amino acid composition indicate that guinea pig YKL40 retains a high degree of

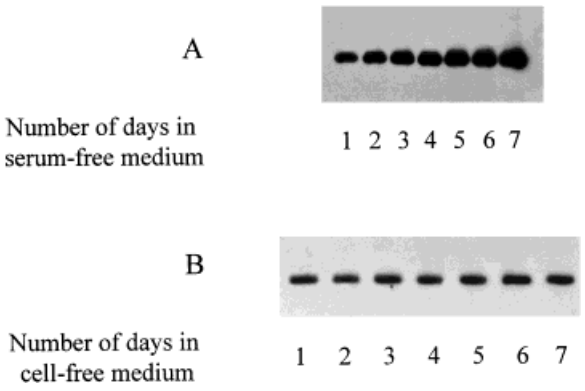


**Fig. 5.** Time course of the release (A) and degradation (B) of gpYKL40 in extracellular medium. **A:** At day 0, confluent chondrocytes were transferred to serum-free medium and further incubated at 37°C for the indicated period. Then, a 100 µl aliquot was taken and analyzed by immunoblotting. **B:** At day 0, the 48-h secretion medium of confluent chondrocytes cultured without serum was taken and further incubated at 37°C for the indicated period. A 100 µl aliquot was then taken and analyzed by immunoblotting.

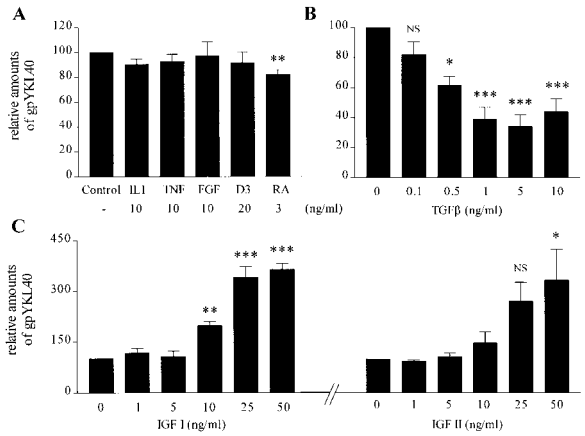


**Fig. 6.** Effect of growth factors and cytokines on the chondrocyte release of gpYKL40. At day 0, confluent chondrocytes were transferred in serum-free medium (0.01% BSA) for 16 h and then for 24 h in serum-free medium (0.01% BSA) with or without various factors at the concentrations indicated. Then, 100-ml aliquots were taken and analyzed by immunoblotting. Results were obtained from densitometric scan analyses of the corresponding immunoblots and expressed as percentages of the control value. Data presented are means ±SEM of three independent experiments. Differences from controls were determined by Dunnett's t-test: NS, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. **A:** Effect of IL-1β, TNF-α, bFGF, 1,25$(OH)_2D_3$ ($D_3$), and retinoic acid (RA). **B:** Dose-dependent effect of TGF-β. **C:** Dose-dependent effect of IGF-I and IGF-II.

similarity with human YKL40. This is also confirmed by the glycosylation pattern and IEF. The high similarity between human and guinea pig YKL40 throughout phylogenetic evolution suggests an important metabolic role for YKL40 in cartilage. It has been suggested that YKL40 may use some of the structural elements of the chitinases to mediate its own function, such as binding to specific carbohydrates [Hakala et al., 1993]. If so, such an association is likely to stabilize the protein. In regard to this point, we here demonstrate that gpYKL40 is a highly stable protein. Indeed, its level in the extracellular medium continuously increased throughout the extensive secretion time studied, suggesting that little degradation occurred during this period of time. This was further confirmed by the observation that the initial level of YKL40 secreted by chondrocytes over a 48-h period did not significantly decrease with time in cell-free medium. This observation is particularly striking, as chondrocytes are known to secrete proteinases, including cysteine proteinases [Maciewicz et al., 1990; De Ceuninck et al., 1995b] and metalloproteinases [Lohmander, 1994], which degrade matrix macromolecules but also smaller proteins [Enghild et al., 1989;

Mitchell et al., 1993; Fowlkes et al., 1994; Ito et al., 1996]. Moreover, IL-1β or TNF-α, known to induce expression of metalloproteinases by a large variety of cells, including chondrocytes [Lefebvre et al., 1990], did not modulate the level of gpYKL40 in the extracellular medium, as also observed in human synovial cells [Nyirkos and Golds, 1990].

The modulation of extracellular gpYKL40 by various factors was studied on confluent chondrocytes synchronized by an incubation of 16 h in serum-free medium. bFGF, and $1,25(OH)_2D_3$ did not affect the secretion of gpYKL40, although the latter was demonstrated to increase synthesis of hYKL40 in osteosarcoma cells [Johansen et al., 1992]. This is not surprising, as $1,25(OH)_2D_3$, which is one of the most potent anabolic factors for osteoblastic cells, may influence cartilage metabolism only during chondrogenesis [Akiyama et al., 1996] or during the terminal events of chondrocyte differentiation [Kato et al., 1990]. We noticed that chondrocytes on the way to dedifferentiation (above passage 3) exhibit a dramatic loss of ability to secrete immunoreactive YKL40 material. Thus, it is likely that the slight but significant decreasing effect of retinoic acid on gpYKL40 secretion may be due to its potent dedifferentiating action on chondrocytes. TGF-β or insulin-like growth factors modulated the release of gpYKL40 in a dose-dependent manner. In the serum-free conditions used, confluent chondrocytes remain in a quiescent state, and a 24-h incubation with IGF-I or IGF-II is unable to promote DNA synthesis [Demarquay et al., 1992; De Ceuninck et al., 1995b]. In addition, TGF-β-treated confluent chondrocytes enter mitosis after 48 h only if fetal calf serum is present to sufficient level [Vivien et al., 1993]. Thus, modulation of extracellular YKL40 by TGF-β or IGFs does reflect not a change in the number of cells, but rather a transcriptional or post-transcriptional effect of these factors on YKL40 secretion. The decreased secretion of gpYKL40 by TGF-β agrees with the observations in explants of human cartilage [Hakala et al., 1993], where TGF-β at a single concentration of 5 ng/ml also decreased the extracellular level of YKL40. However, this result must be interpreted with caution, as TGF-β has opposite effects in cultured chondrocytes depending on its concentration, the phase of the cell cycle [Trippel, 1995], or the mode of administration [Van den Berg, 1995]. IGF-I and, to a lesser extent, IGF-II, is known as the most potent growth factor to stimulate type II collagen and proteoglycans synthesis by chondrocytes [Guenther et al., 1982; MacQuillan et al., 1986]. IGF peptides also stimulate the chondrocyte release of their own regulatory binding proteins [Froger-Gaillard et al., 1989; Olney et al., 1993]. IGF-I and IGF-II dose-dependently increased the secretion of YKL40. The apparent controversy with the reported uneffectiveness of IGF-I [Hakala et al., 1993] may rest on differences in the species studied (guinea pig versus human), or the age of donors. However, IGF-I-mediated stimulation of proteoglycan synthesis in mice cartilage is not age-dependent [Van Beuningen et al., 1993].

In human arthritic disorders, where a two- to threefold increase in YKL40 sera levels is observed [Johansen et al., 1993; Johansen et al., 1996], the stable equilibrium between synthesis and degradation of chondrocyte matrix components is disrupted, so that cartilage suffers progressive destruction. In the lesions of osteoarthritic cartilage, IGF-I gene expression by chondrocytes is upregulated [Middleton and Tyler, 1992], resulting in increased production of IGF-I peptide and IGF-binding proteins, all of which may be involved in the pathological process [Olney et al., 1996; Tardif et al., 1996]. However, it is not clear whether these modifications are in favour of a repairing effect of IGF-I in damaged cartilage [Middleton and Tyler, 1992; Middleton et al., 1996], or result in an altered anabolic response of this tissue to IGF-I [Olney et al., 1996; Tardif et al., 1996]. The finding that the production of YKL40 by chondrocytes is increased by IGF-I suggests that YKL40 may mediate or reflect some effects of the IGF axis in arthritic disease. In regard to these findings, the guinea pig, which develops age-related osteoarthritis spontaneously, offers a convenient model to elucidate the significance of YKL40 in normal and pathological cartilage and its interaction with the IGF system as a function of age, both in vitro and in vivo.

## ACKNOWLEDGMENTS

The authors are grateful to Dr Jean-Luc Fauchère for critical reading of this manuscript and for helpful comments and suggestions.

## REFERENCES


Akiyama H, Hiraki Y, Shigeno C, Kohno H, Shukunami C, Tsuboyama T, Kasai R, Suzuki F, Konishi J, Nakamura T (1996): 1α,25-Dihydroxyvitamin $D_3$ inhibits cell growth and chondrogenesis of a clonal mouse EC cell line, ATDC5. J Bone Miner Res 11:22–28.

Bendele AM, Hulman JF (1988): Spontaneous cartilage degeneration in guinea pigs. Arthritis Rheum 31:561–565.

Corvol MT, Carrascosa A, Tsagris L, Blanchard O, Rappaport R (1987): Evidence for a direct in vitro action of sex steroids on rabbit cartilage cells during skeletal growth: Influence of age and sex. Endocrinology 120:1422–1429.

De Bri E, Reinholt FP, Svensson O (1995): Primary osteoarthrosis in guinea pigs: A stereological study. J Orthop Res 13:769–776.

De Ceuninck F, Willeput J, Corvol M (1995a): Purification and characterization of insulin-like growth factor II (IGF II) and an IGF II variant from human placenta. J Chromatogr B 666:203–214.

De Ceuninck F, Poiraudeau S, Pagano M, Tsagris L, Blanchard O, Willeput J, Corvol M (1995b): Inhibition of chondrocyte cathepsin B and L activities by insulin-like growth factor-II (IGF-II) and its $Ser^{29}$ variant in vitro: Possible role of the mannose 6-phosphate/IGF-II receptor. Mol Cell Endocrinol 113:205–213.

Demarquay D, Dumontier MF, Bourguignon J, Hintz RL, Corvol M (1992): Stimulation by GH of IGF1 proforms synthesized by rabbit chondrocytes cultured with bFGF serum-free medium. Exp Cell Res 202:412–422.

Enghild JJ, Salvesen G, Nagase H (1989): Interaction of human rheumatoid synovial collagenase (matrix metalloproteinase 1) and stromelysin (matrix metalloproteinase 3) with human $\alpha_2$-macroglobulin and chicken ovostatin. J Biol Chem 264:8779–8785.

Fowlkes JL, Enghild JJ, Suzuki K, Nagase H (1994): Matrix metalloproteinases degrade insulin-like growth factor-binding protein-3 in dermal fibroblast cultures. J Biol Chem 269:25742–25746.

Froger-Gaillard B, Hossenlopp P, Adolphe M, Binoux M (1989): Production of insulin-like growth factors and their binding proteins by rabbit articular chondrocytes: Relationships with cell multiplication. Endocrinology 124: 2365–2372.

Guenther HL, Hennelore E, Guenther E, Froesch ER, Fleisch H (1982): Effect of insulin-like growth factor on collagen and glycosaminoglycan synthesis by rabbit articular chondrocytes in culture. Experientia 38:979–986.

Hakala BE, White C, Recklies AD (1993): Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. J Biol Chem 268:25803–25810.

Henrissat B, Bairoch A. (1993): New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 293:781–788.

Hu B, Trinh K, Figueira WF, Price PA (1996): Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. J Biol Chem 271:19415–19420.

Ito A, Mukaiyama A, Itoh Y, Nagase H, Thogersen IB, Enghild JJ, Sasaguri Y, Mori Y (1996): Degradation of interleukin 1β by matrix metalloproteinases. J Biol Chem 271:14657–14660.

Johansen JS, Williamson MK, Rice JS, Price PA (1992): Identification of proteins secreted by human osteoblastic cells in culture. J Bone Miner Res 7:501–512.

Johansen JS, Jensen HS, Price PA (1993): A new biochemical marker for joint injury. Analysis of YKL-40 in serum and synovial fluid. Br J Rheumatol 32:949–955.

Johansen JS, Hvolris J, Hansen M, Backer V, Lorenzen I, Price PA (1996): Serum YKL-40 levels in healthy children and adults. Comparison with serum and synovial fluid levels of YKL-40 in patients with osteoarthritis or trauma of the knee joint. Br J Rheumatol 35:553–559.

Kato Y, Shimazu A, Iwamoto M, Nakashima K, Koike T, Suzuki F, Nishii Y, Sato K (1990): Role of 1,25-dihydroxycholecalciferol in growth-plate cartilage: Inhibition of terminal differentiation of chondrocytes in vitro and in vivo. Proc Natl Acad Sci USA 87:6522–6526.

Lefebvre V, Peeters-Joris C, Vaes G (1990): Modulation by interleukin 1 and tumor necrosis factor α of production of collagenase, tissue inhibitor of metalloproteinases and collagen types in differentiated and dedifferentiated articular chondrocytes. Biochim Biophys Acta 1052:366–378.

Lohmander LS (1994): Articular cartilage and osteoarthrosis. The role of molecular markers to monitor breakdown, repair and disease. J Anat 184:477–492.

Maciewicz RA, Wotton SF, Etherington DJ, Duance VC (1990): Susceptibility of the cartilage collagens types II, IX and XI to degradation by the cysteine proteinases, cathepsins B and L. FEBS Lett 269:189–193.

MacQuillan DJ, Handley CJ, Campbell MA, Bolis S, Milway VE, Herington AC (1986): Stimulation of proteoglycan biosynthesis by serum and insulin-like growth factor-I in cultured bovine articular cartilage. Biochem J 240:423–430.

Middleton J, Tyler JA (1992): Upregulation of insulin-like growth factor I gene expression in the lesions of osteoarthritic human articular cartilage. Ann Rheum Dis 51:440–447.

Middleton J, Manthey A, Tyler J (1996): Insulin-like growth factor (IGF) receptor, IGF-I, interleukin-1β (IL-1β), and IL-6 mRNA expression in osteoarthritic and normal human cartilage. J Histochem Cytochem 44:133–141.

Mitchell TI, Jeffrey JJ, Palmiter RD, Brinckerhoff CE (1993): The acute phase reactant serum amiloid A (SAA3) is a novel substrate for degradation by the metalloproteinases collagenase and stromelysin. Biochim Biophys Acta 1156:245–254.

Nyirkos P, Golds EE (1990): Human synovial cells secrete a 39 kDa protein similar to a bovine mammary protein expressed during the non-lactating period. Biochem J 268:265–268.

Olney RC, Smith RL, Kee Y, Wilson DM (1993): Production and hormonal regulation of insulin-like growth factor binding proteins in bovine chondrocytes. Endocrinology 133:563–570.

Olney RC, Tsuchiya K, Wilson DM, Mohtai M, Maloney WJ, Schurman DJ, Smith RL (1996): Chondrocytes from osteoarthritic cartilage have increased expression of insulin-like growth factor I (IGF-I) and IGF-binding protein-3 (IGFBP-3) and -5, but not IGF-II or IGFBP-4. J Clin Endocr Metab 81:1096–1103.

Rejman JJ, Hurley WL (1988): Isolation and characterization of a novel 39 kilodalton whey protein from bovine mammary secretions collected during the nonlactating period. Biochem Biophys Res Commun 150:329–334.

Tardif G, Reboul P, Pelletier JP, Geng C, Cloutier JM, Martel-Pelletier J (1996): Normal expression of type 1 insulin-like growth factor receptor by human osteoarthritic chondrocytes with increased expression and synthesis of insulin-like growth factor binding proteins. Arthritis Rheum 39:968–978.

Trippel SB (1995): Growth factor actions on articular cartilage. J Rheumatol 22(suppl 43):129–132.

Van Beuningen HM, Arntz OJ, van den Berg WB (1993): Insulin-like growth factor stimulation of articular chondrocyte proteoglycan synthesis. Availability and responses at different ages. Br J Rheumatol 32:1037–1043.

Van den Berg WM (1995): Growth factors in experimental osteoarthritis: Transforming growth factor β pathogenic? J Rheumatol 22(suppl 43):143–145.

Vivien D, Petitfrère E, Martiny L, Sartelet H, Galéra P, Haye B, Pujol JP (1993): IPG (inositolphosphate glycan) as a cellular signal for TGF-β1 modulation of chondrocyte cell cycle. J Cell Physiol 155:437–444.