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Up-regulation of Cartilage Oligomeric Matrix Protein Gene Expression by Insulin-like Growth Factor-I Revealed by Real Time Reverse Transcription-Polymerase Chain Reaction

Hua TIAN[1,2]* and Ioannis STOGIANNIDIS[2]

[1] *Department of Orthopaedics, Peking University Third Hospital, Beijing 100083, China;*
[2] *Coxa Hospital for Joint Replacement, Biokatu 6 b, Tampere 33101, Finland*

Abstract Cartilage oligomeric matrix protein (COMP) strengthens cartilage by binding to type II and type IX collagen-forming bridges between collagen fibrils. It was hypothesized that perhaps one or more anabolic growth factors such as insulin-like growth factor-I (IGF-I), fibroblast growth factor-1 (FGF-1) or platelet derived growth factor-BB (PDGF-BB) increase *COMP* gene expression. Their effects on primary human chondrocytes and the chondrogenic cell line ATDC5 were studied using real time reverse transcript-polymerase chain reaction (RT-PCR) for quantification. IGF-I, but not the FGF-1 or PDGF-BB, up-regulated *COMP* gene expression by approximate 5-fold in human adult chondrocytes in a dose- and time-dependent manner. IGF-I exerted similar effects on ATDC5 cells. Results from these real time RT-PCR experiments were confirmed by transfecting into ATDC5 cells a full-length mouse *COMP* promoter cloned upstream of a luciferase reporter gene. On stimulation with IGF-I, the luciferase reporter activity increased by about eight times. In conclusion, IGF-I seems to be an important positive regulator of COMP, which may play an important role in an attempted repair of either traumatized or degenerated cartilage.

Key words cartilage oligomeric matrix protein (COMP); insulin-like growth factor-I (IGF-I); gene expression

Articular cartilage consists of an abundant extracellular matrix of proteoglycans, cartilage oligomeric matrix protein (COMP) and type II, IX and XI collagens, in which relatively few matrix maintaining chondrocytes are embedded.

The role of the hydrophilic proteoglycan matrix with a high swelling pressure controlled by the collagen network is already well recognized. More recent findings have provided interesting insights into the role of COMP, which is one of the main non-collagenous proteins of articular cartilage [1–4]. It was found that the disproportionate dwarfism, ligamentous laxity and early-onset osteoarthritis typical for patients with pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) are in the great majority in cases caused by mutations in the *COMP* gene, more specifically in its calcium-binding motifs [5]. These sites are highly conserved in COMP protein among different species and seem to be essential for its ability to strengthen cartilage by binding to type II and type IX collagen-forming bridges between collagen fibrils. In addition, COMP might also be able to regulate the chondrocyte cellular activities and to maintain the structural and mechanical properties of cartilage through interactions with fibronectins, integrins and other matrix proteins [6,7]. Although a *COMP*-deficient mice study did not show a phenotype resembling the clinical manifestations of PSACH/MED in humans, the mechanism of these diseases might be caused by folding defects or extracellular assembly abnormalities due to dysfunctional mutated COMP [8].

As COMP seems to play a key role in the assembly and stabilization of cartilage matrix, it was hypothesized that anabolic growth factors might up-regulate its expression

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*Corresponding author: Tel, 358-3-31178109, 86-10-62017691; Fax, 86-10-62017700; E-mail, tianhua@medmail.com.cn

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and contribute to cartilage maintenance, remodeling and repair. Among these factors, transforming growth factor-β, bone morphogenetic protein-2, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and insulin-like growth factor (IGF) are the key regulators [9–13]. Although it has already been reported that the first two mentioned can stimulate *COMP* expression [13], there is no information about the role of the last three in chondrocyte-mediated synthesis of COMP. Although they seem to be good candidate molecules in positive regulation of *COMP*, a set of experiments need to be designed and executed to investigate if they indeed regulate *COMP*.

In this study, two different cellular models, primary human chondrocytes and a chondrogenic cell line ATDC5, were used. The eventual dose- and time-dependent manner of the effects of recombinant human FGF-1, PDGF-BB and IGF-I on *COMP* mRNA levels were studied using quantitative real time RT-PCR followed by confirmatory studies with transfection of a full-length mouse *COMP* promoter cloned upstream of a luciferase reporter gene.

Materials and Methods

Cell culture

Chondrocytes were released from normal human knee articular cartilage by enzyme digestions as previously described [14] and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) for no more than six passages (Invitrogen, Carlsbad, USA). 1.2 million of both the chondrocytes and ATDC5 cells were plated and grown in 6-well plates until 80% confluence and were changed to serum-free medium ITS-DMEM 24 h prior to each experiment. Cells were then treated with fresh ITS-DMEM with or without 100 ng/ml human recombinant IGF-I, 100 ng/ml human recombinant FGF-1 and 10 ng/ml human recombinant PDGF-BB respectively for chondrocytes, and 100 ng/ml human recombinant IGF-I only for ATDC5 cells for 36 h. All of these cytokines were obtained from GroPep (Adelaide, Australia). In the analysis of regulation kinetics or dose-dependence of COMP statement by IGF-I, the same amount of plated chondrocytes were treated with IGF-I as mentioned above for various time or at indicated concentrations, and cells were harvested for measuring the *COMP* mRNA level by real time PCR.

Total RNA extraction and real time polymerase chain reaction

Total RNA was extracted from either chondrocytes or ATDC5 cells using RNA easy kit (Qiagen, Hilden, Germany), and 0.5 μg of RNA was reverse-transcribed with Superscript II (Life Technology, Espoo, Finland). Real-time quantitative PCR amplification of *hCOMP* and *GAPDH* were performed on Cepheid Smart-Cycler. The primers and TaqMan probes for both genes were synthesized by Applied Biosystems Incorporation (Framingham, USA). Primers were designed and selected using Primer Express software (PE Applied Biosystems). Sequences of the forward and reverse primers are shown in **Table 1**.

An optimal real-time PCR condition was achieved through test runs by monitoring the growth curve and verified by the liner correlation between the logarithmic amount of either *hCOMP* or *GAPDH* cDNA of each sample and the cycle threshold (Ct, i.e., the cycle number at which the amount of amplified gene of interest reached a fixed threshold). The PCR mixture consisted of 1×TaqMan PCR Master Mix, which includes AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference (indicate the vendor) and PCR buffer, 200 nM forward and reverse primers, 300 nM probe and cDNA of samples in a total

Table 1 The sequences of primers used in this study

Gene	GenBank accession No.	Primer Sequence (5′→3′)	
hCOMP	NM_000095	Forward	GCTCTGTGGCATACAGGAGA
		Reverse	CATAGAATCGCACCCTGATG
hGAPDH	NM_002046	Forward	GAAGGTGAAGGTCGGAGTC
		Reverse	GAAGATGGTGATGGGATTTC
mCOMP	NM_016685	Forward	TGGGTGATGCCTGTGATAGT
		Reverse	CGTCATTGTCATCATCGTCA
mGAPDH	NM_199472	Forward	ACCACAGTCCATGCCATCAC
		Reverse	TCCACCACCCTGTTGCTGTA

volume of 50 μl. PCR was carried out using the following protocol: initial activation of AmpliTaq Gold DNA polymerase at 94 ºC for 5 min, 40 cycles of 94 ºC for 15 s and 60 ºC for 1 min. Each sample was performed in triplicate. Direct detection of PCR product was monitored in real time by measuring the increase in fluorescence caused by the released Fam dye from dsDNA, using the Cepheid Smart-Cycler Sequence Detection System. Subsequently, the Ct was determined and relative transcription levels were then calculated. Relative quantification of *COMP* mRNA statement was calculated by the comparative Ct method described by the manufacturer (PE Applied Biosystems). The relative quantification value of the target, normalized to an endogenous control and relative to a calibrator, is expressed as $2^{-\Delta\Delta Ct}$, where ΔCt=Ct(*COMP*)–Ct(*GAPDH*), ΔΔCt=ΔCt(sample)–ΔCt(calibrator).

Transfection

For luciferase reporter assay, an approximately 1.9 kb of 5′-flanking region (–1925 to +15) of mouse *COMP* gene, which has promoter activity was cloned upstream of luciferase gene in pGL-2 basic vector (Promega, Pittsburgh, USA) and the resultant vector was termed as pGL.mCOMPp. The construct was transfected into ATDC5 cells along with CMV-beta-galactosidase statement vector as an internal control as described previously [14]. Briefly, ATDC5 cells were plated out into 60-mm dishes with 1.2 million cells per dish just before the day of transfection. After incubation at 37 ºC overnight, cells should reach 70%–80% confluence. Five micrograms of either pGL.mCOMPp or pGL2.basic was then transfected into the cells with Lipofectamine according to the manufacturer's instructions (Life Technology). Eight hours after incubation with DNA-lipid complex, full growth medium with 10% FBS was added and kept for 24 h. The cells were then incubated with serum-free medium with ITS for another 24 h. Next, cells were treated with 100 ng/ml IGF-I. The transcriptional activities of mCOMPp were assayed in cell lysates for luciferase activity 36 h post-stimulation.

Statistical analysis

F test was used and the Student-Newman-Keuls (S-N-K) was applied to comparison between groups when screening factors and doing transfection. T test was used for analyzing the induction of *COMP* gene expression by IGF-I in ATDC5 cells. $P<0.01$ was indicated significant difference. Data are expressed throughout as mean and standard deviation of the mean.

Results

Insulin-like growth factor-I up-regulates cartilage oligomeric matrix protein mRNA levels in human chondrocytes in a dose- and time-dependent fashion

Human adult chondrocytes were treated with IGF-I, FGF-1, PDGF-BB or empty control for 24 h before samples were collected for the measurement of the *COMP* mRNA levels using real time RT-PCR technique. IGF-I significantly increased the *COMP* mRNA expression by almost 5-fold (**Table 2**), whereas FGF-1 and PDGF-BB had hardly any effect. Subsequent experiments therefore focus on the effects of IGF-I on *COMP* gene expression.

Table 2 Screening factors to identify which has effects on cartilage oligomeric matrix protein (COMP) expression

Group	*n*	COMP
Negative	3	18.8±0.94
IGF-I	3	88.0±4.80*
FGF-I	3	27.6±4.21
PDGF-BB	3	21.2±1.84

Human adult chondrocytes with insulin-like growth factor-I (IGF-I), fibroblast growth factor-I (FGF-1), platelet-derived growth factor-BB (PDGF-BB) and empty control cultured for 24 h and quantitatively measured the *COMP* mRNA level using real time reverse transcript-polymerase chain reaction (RT-PCR). Relative expression level of *COMP* was calculated. F test and the Student-Newman-Keuls (S-N-K) were applied to comparison between groups ($F=293.3$, $P<0.01$). *Compared with the control group, there is significant difference. *n*, number of experiment. Data were represented as mean±SD.

In the next set of experiments, it was found that this up-regulatory effect of IGF-I on *COMP* is in a dose-dependent manner, with the optimal concentration of 100 ng/ml. The *COMP* expression level reached a peak at 36 h post stimulation (**Fig. 1**).

Insulin-like growth factor-I induces cartilage oligomeric matrix protein expression in a chondrogenic ATDC5 cell line

To further examine the molecular basis of the effect of IGF-I, mouse chondrogenic ATDC5 cells were used. In a non-stimulated resting state, ATDC5 cells did not express *COMP*; but upon IGF-I stimulation, *COMP* expression was effectively induced (**Table 3**).

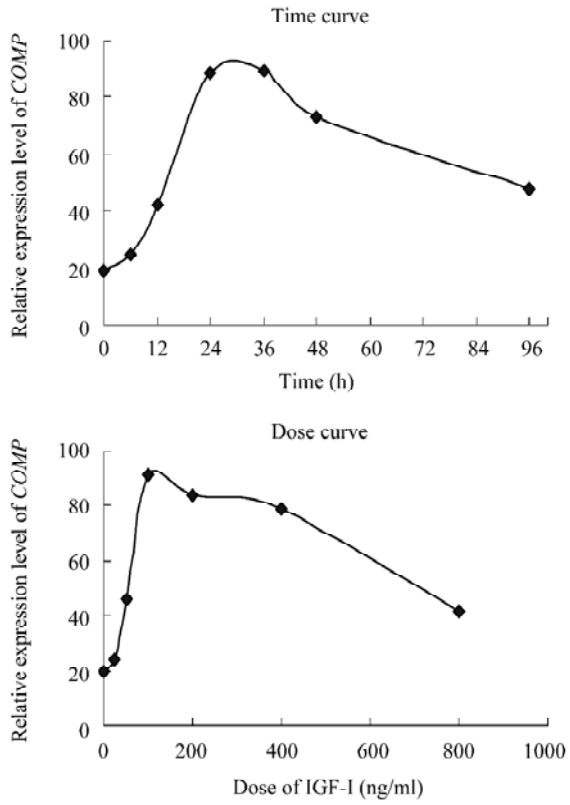

Fig. 1 Time- and dose-denpendent pattern of *COMP* expression stimulated by IGF-I.

The stimulation effects of IGF-I on the *COMP* expression in adult chondrocytes are dependent on the time and dose. Time point of 36 h and dose of 100 ng/ml produced the maximum effects.

Table 3 Induction of cartilage oligomeric matrix protein (*COMP*) gene expression by insulin-like growth factor-I (IGF-I) in ATDC5 cells

Group	*n*	COMP
Negative	3	0.2±0.12
IGF-I	3	22.3±2.71

When grown to 80%–90% confluence, ATDC5 cells were treated with or without 100 ng/ml of IGF-I for 36 h. Cells were harvested and the total RNA was extracted. Total RNA was used for real time reverse transcript-polymerase chain reaction (RT-PCR) to amplify *COMP* gene. T test was used (t=14.089, $P<0.01$). *n*, number of experiment; Data were represented as mean±SD.

The IGF-I mediated transcriptional responses of *COMP* gene could be further verified by a transient transfection of a full-length mouse *COMP* promoter-luciferase reporter construct into ATDC5 cells. As shown in **Table 4**, IGF-I up-regulated the expression of the reporter gene by 8-fold in such ATDC5 cells.

Table 4 Effect of insulin-like growth factor-I (IGF-I) on the *mCOMP* promoter activity in ATDC5 cells

Group	*n*	Activity
Negative (empty vector)	3	89.4±3.78
Negative (promoter)	3	83.1±3.55
IGF-I (empty vector)	3	90.7±3.64#
IGF-I (promoter)	3	713.0±17.43*

ATDC5 cells were transfected with *mCOMP* promoter reporter construct. After 24 h of transfection, cells were stimulated with or without 100 ng/ml of IGF-I. Cells were harvested 36 h post stimulation and luciferase activity was examined. The increase of *mCOMP* promoter activity after IGF-I stimulation was expressed as the relative activity to the unstimulated group. F test was used and the Student-Newman-Keuls (S-N-K) was applied to comparison between groups (F=3380, $P<0.01$). *Compared with the negative control with empty vector; # compared with the IGF-I (promoter) group, there is significant differences. Data were represented as mean±SD.

Discussion

Chondrocytes play an important role in maintaining cartilage matrix by modulating the synthesis and degradation of the various components in both normal and pathological conditions. As an important non-collagenous protein component of the extracellular matrix, COMP was first assumed to represent relatively specific to articular chondrocytes [1], although it has later been found in other cells as well [3]. To avoid other sources as confounding factors, a decision was made to assess the effect of FGF-1, PDGF-BB and IGF-I on pure chondrocyte cultures. Accumulating evidence has shown that COMP is one of the common target molecules regulated in various disease conditions [5,15]. This study identified quantitatively, for the first time, IGF-I as an important up-regulator of the *COMP* gene expression in both human adult chondrocytes and mouse chondrocytic ATDC5 cells. Perhaps as importantly but against our expectations, FGF-1 and PDGF-BB did not have such effect.

It has been proven that IGF-I is the main anabolic growth factor for articular cartilage. It plays a key role in cartilage homeostasis, balancing the extracellular matrix synthesis and breakdown. In animal models, IGF-I significantly improved the quantity and quality of defect repair through increasing the expression of collagen type II and proteoglycan [16–18]. The present study demonstrates that up-regulation of *COMP* also belongs to the positive effects of IGF-I, which is in agreement with the earlier observations concerning other properties of IGF-I when stimulating cartilage and chondrocytes. Our main conclusion is supported by the clear dose- and time-dependent

manner of the effect of IGF-I on *COMP* mRNA copy numbers. In this study, it was shown that IGF-I in the concentration of 100 ng/ml reached maximal effect on the expression of *COMP*, and then it was decreased slowly from 100 ng/ml to 400 ng/ml and sharply down thereafter. Hui *et al*. also found that 100 ng/ml of IGF-I blocks the collagen release and down-regulates the expression of metalloproteinases in cultured cartilage explant [19]. In addition, the time-dependent curve showed that the effect of IGF-I was kept at a high level between 24 and 36 h after stimulation, then went down gradually.

The ATDC5 cell line is a well characterized chondrogenic cell line derived from a mouse teratocarcinoma. It retains the properties of chondroprogenitor cells very well. Phornphutkul and co-workers found that IGF-I has a dual ability to promote ATDC5 cell proliferation and differentiation. The former effect was dependent on the ability of IGF-I to activate the mitogenic pathway involving Erk1 and Erk2 [20]. As these protein kinases are expressed in chondrocyte, it was thought that COMP would be synthesized in these cells and would also be eventually up-regulated by IGF-I. Actually there was no *COMP* expression in the unstimulated and resting ATDC5 cells as *COMP* mRNA was not found at all even though a sensitive real time PCR method was used. However, after stimulation with IGF-I, *COMP* expression was effectively induced. This could be due to the fact that the half-life of type II collagen in articular cartilage is very long (e.g., in rabbit over 100 years), and the mRNA level of type II collagen in mature cartilage tissue is very low [21]. It might be that there is no need for a resting chondrocyte to synthesize collagen type II or COMP, whereas it becomes necessary when the cells were stimulated.

In order to provide additional information about the regulation of IGF-I on the expression of *COMP*, a full-length mouse *COMP* promoter directed luciferase gene was transfected into ATDC5 cells. The result showed that IGF-I increased the luciferase activity by 8-fold, compared with the empty control. The ability of IGF-I to up-regulate *COMP* expression may relate to its anabolic effects on the extracellular matrix proteins, which had been demonstrated for proteoglycan and type II collagen, and is now at least to a limited extent also shown for COMP.

The current study has some limitations, including just investigating the effects of IGF-I on *COMP* in the mRNA level and just studying *in vitro* cultured cells. Nevertheless, this study has provided much valuable new information about the gene regulation of *COMP*. Our future study will focus on the effects of IGF-I on *COMP* in protein levels, signaling mechanisms and the effects of IGF-I on *COMP in vivo*.

In conclusion, IGF-I seems to be an important positive regulator of COMP, which might play an important role in an attempted repair of either traumatized or degenerated cartilage. Work in experimental arthritis indicates that it is possible to modulate cartilage proteoglycans and COMP by liposomally packaged clodronate [22]. The present work suggests that somewhat similar results could be obtained via local application and/or up-regulation of IGF-I.

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