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Expression and localization of connective tissue growth factor (CTGF/Hcs24/CCN2) in osteoarthritic cartilage

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Summary

Objective: The investigation of the expression and localization of connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24/CCN family member 2 (CTGF/Hcs24/CCN2) in normal and osteoarthritic (OA) cartilage, and quantification of CTGF/Hcs24-positive cells.

Methods: Cartilage samples of patients ($n = 20$) with late stage OA were obtained at total joint replacement surgery. Morphologically normal cartilage was harvested for comparison purposes from the femoral heads of 6 other patients with femoral neck fracture. Paraffin-embedded sections were stained by Safranin O. The severity of the OA lesions was divided into four stages (normal, early, moderate, and severe). The localization of protein and mRNA for CTGF/Hcs24 was investigated by immunohistochemistry and *in situ* hybridization, respectively. The population of CTGF/Hcs24-positive chondrocytes in OA cartilage and chondro-osteophyte was quantified by counting the number of the cells under light microscopy.

Results: Signals for CTGF/Hcs24 were detected in a small percentage of chondrocytes throughout the layers of normal cartilage. In early stage OA cartilage, the CTGF/Hcs24-positive chondrocytes were localized mainly in the superficial layer. In moderate to severe OA cartilage, intense staining for CTGF/Hcs24 was observed in proliferating chondrocytes forming cell clusters next to the cartilage surface. In chondro-osteophyte, strong signals were found in the chondrocytes of the proliferative and hypertrophic zones.

Conclusion: CTGF/Hcs24 expression was detected in both normal and OA chondrocytes of human samples. The results of the current study suggested that expression of CTGF/Hcs24 was concomitant with development of OA lesions and chondrocyte differentiation in chondro-osteophyte.

Key words: CTGF/Hcs24/CCN2, Cartilage, Osteoarthritis, Chondro-osteophyte.

Introduction

Osteoarthritis (OA) is a proliferative joint disease characterized by articular cartilage degeneration, osteophyte formation, subchondral bone sclerosis, and secondary induced synovitis[1]. In the course of development of OA, there is an imbalance between cartilage degeneration and attempts to repair as seen in chondrocyte cloning, covering of fibrous tissue over the cartilage, and marginal osteophyte formation[2]. Numerous studies have suggested that the relative concentration of growth factors and cytokines, or altered response of chondrocytes might determine the rate of progression of this disease[3]. Mature chondrocytes and their precursors produce and respond to a wide range of growth factors[4], such as fibroblast growth factors (FGF)[5,6], epidermal growth factors (EGF)[7,8], insulin-like growth factors (IGF)[8,9], transforming growth factors (TGF)[6,10], and bone morphogenic proteins (BMP)[11–13]. We previously cloned by the differential display PCR method[14] an mRNA predominantly expressed in hypertrophic chondrocytes from a newly established immortal, clonal chondrocytic cell line (HCS-2/8)[15–19]. Its gene product, named hypertrophic chondrocyte-specific gene product 24(Hcs24), was identical with connective tissue growth factor (CTGF)[20–23], which is a member of the so called "connective tissue growth

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factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family"[20]. CTGF/Hcs24 is induced by growth factors such as TGF-β, and regulates cell proliferation, migration, angiogenesis, and wound healing[20,24]. We also reported that CTGF/Hcs24 specific receptors were present on chondrocytic cells[25] and recombinant CTGF/Hcs24 promoted proliferation and differentiation of chondrocytes *in vitro*[26]. Moreover, CTGF/Hcs24 has a role in the proliferation, migration and tube formation of vascular endothelial cells *in vitro* and angiogenesis *in vivo*[27,28]. Although recent reports suggested that CTGF/Hcs24 is expressed in normal and OA chondrocytes[11,29], the precise localization and relationship with cartilage degeneration has not been fully understood.

In the current study, we examined the expression and localization of CTGF/Hcs24 in normal and different stages of OA cartilage lesions and in chondro-osteophyte at the mRNA and protein levels by *in situ* hybridization and immunohistochemistry, respectively.

Materials and methods

PATIENT SELECTION AND TISSUE PREPARATION

Human OA cartilage was obtained at the time of total joint replacement surgery (10 hips and 10 knees) from 18 OA patients (2 males and 16 females, aged 43–78 years, mean 66.8 years) who lacked indication of other inflammatory diseases. All the cases satisfied the American College of Rheumatology criteria for OA of the hip and knee. Comparison samples of macroscopically normal cartilage were obtained from the resected femoral heads of 6 patients with femoral neck fracture. Informed consent was obtained from all the patients. The chondro-osteophyte samples were also obtained from the 15 samples from the different series of OA patients previously described[30]. The samples were immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), decalcified in 0.3 M EDTA (pH 7.5) for 2–3 weeks, and embedded in paraffin. Three sections from each sample were used for the histological and immunohistochemical studies, and 5 sections from 5 OA samples were used for *in situ* hybridization analysis.

HISTOLOGICAL EVALUATION OF OA SEVERITY

All sections were stained with Safranin O and observed by light microscopy. Histological classification of the severity of OA lesions was graded on a scale of 0–13, using the modified Mankin score reported by van der Sluijs et al.[31], a combined score assessing structure; (0–6 points), cellular abnormalities; (0–3 points), and matrix-staining (0–4 points). As a result, 0 points represent normal and 13 points represent the most severe cartilage lesions. The modified Mankin score point-range of 14 points was divided into 4 ranges in the current study: normal (0 point), early OA (1–3 points), moderate OA (4–8 points), and severe OA (9–13 points).

IMMUNOHISTOCHEMICAL STAINING

The paraffin sections were soaked in xylene to remove paraffin, then dehydrated in a graded alcohol series (50–100%). Antigen retrieval was performed by autoclaving for 15 min at 121°C. Additional enzymic digestion by testicular hyaluronidase (1.0 mg/ml, 37°C for 3 h) for epitope unmasking did not affect the stainability of CTGF/Hcs24 (data not shown). After cooling, endogenous peroxidase activity was quenched with 0.3% H_2O_2 in PBS. Anti-CTGF/Hcs24 antibodies were raised in the rabbit by immunization with a synthetic peptide of CTGF/Hcs24 composed of 20 amino acids (240–259: RPCEADLEENIKKGKKCIRT), the sequence of which was the same as that of Fisp12 (the mouse homolog of CTGF), but not the same as that of Cyr61, Nov, WISP-1/Elm-1, WISP-2/rCop-1/CTGF-3, or WISP-3. Using Western blotting techniques, we previously confirmed that the synthetic peptide of CTGF/Hcs24, but not the synthetic peptide of the corresponding region of Cyr61, inhibited the binding of the anti-CTGF/Hcs24 antibodies to the 38 kDa CTGF/Hcs24 protein[27]. After blocking with normal goat serum, polyclonal antibodies against CTGF/Hcs24 was applied as primary antibody for 2 h, and then incubated for 30 min with biotinylated anti-rabbit antibody. After rinsing in PBS, specimens were incubated with ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA), and then washed with distilled water. The reaction was visualized by diaminobenzidine (DAB) resulting in a brown color, and counterstained with hematoxylin. Stained sections incubated with normal non-immune rabbit immunoglobulin (IgG) or without primary antibodies were used as negative controls.

SEMI-QUANTITATIVE ANALYSIS FOR CTGF/HCS24-POSITIVE CELLS

Immunoreactivity was evaluated by light microscopy. The chondrocytes with definite, diffusely stained cytoplasm or nuclei were regarded as positively stained. The populations of CTGF/Hcs24-positive cells in the superficial, middle and deep layers of OA cartilage, and in the fibrocartilage, proliferative, hypertrophic zones of chondro-osteophyte[30] were quantified by counting the number of the cells within the three layers and the three zones, respectively. Cell number counting was done in at least 3 fields, at ×100 magnification, and averaged. Judgment was based on the consensus of at least two of the authors (SO and MS) without reference to the patients' clinical information. The number of positive chondrocytes was divided by the total number of chondrocytes within all 3 layers and 3 zones to calculate the positive chondrocyte ratio. The marginal areas between each layers and each zones were avoided for cell counting to reduce the inter-observer errors. The frequency levels of CTGF/Hcs24-positive cells were divided into six groups: −: 0%; ±: 0–10%; +: 11–30%; ++: 31–50%; +++: 51–70%; ++++: 71–100%.

IN SITU HYBRIDIZATION FOR CTGF/HCS24 MRNA

The paraffin sections were washed with PBS, refixed with 1% paraformaldehyde, and treated with 2 mg/ml glycine–PBS. Then they were immersed in 0.1 M triethanolamine–HCl (pH 8.0), after which acetic acid-anhydride was added and the slides were rinsed twice with 4× standard saline–citrate (SSC) for 10 min, and immersed in prehybridization buffer (2× SSC, 50% formamide and 1 mM levamisole) at 70°C for 15 min to decrease the intrinsic alkaline phosphatase activity. *In situ* hybridization was performed at 52°C using DIG-labeled sense and antisense CTGF/Hcs24 riboprobes (0.35 μg/ml) as previously described[32]. Sense and antisense RNA probes were synthesized with T3 and T7 RNA polymerases from cDNA templates of CTGF/Hcs24 cDNA (1.1 kb whole coding

region) by using a digoxigenin labeling kit (Roche Diagnostics Corp., Indianapolis, IN). After hybridization, the color reaction was performed with 1:2000 dilution of 150 U/200 μL anti-DIG alkaline phosphatase-conjugated antibody (Roche), and nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyphosphate (BCIP) in an alkaline buffer solution containing 1 mM levamisole in a dark room at room temperature[32].

Results

EXPRESSION OF CTGF/HCS24 PROTEIN IN OA CARTILAGE

CTGF/Hcs24-positive chondrocytes were expressed in a small population of chondrocytes throughout the layers of cartilage with morphologically normal structure [Fig. 1(a–d)]. In early stage OA cartilage (1–3 points), the population of immuno-positive chondrocytes in the superficial layer (51–70%) was higher than those in middle (11–30%) and deep (0–10%) layers [Fig. 1(e–h)]. In moderate OA cartilage (4–8 points), the superficial layer was much reduced or destroyed, and fibrillated. Immuno-localization of CTGF/Hcs24 was observed in individual and clustering chondrocytes in the middle (51–70%) and deep (31–50%) layers [Fig. 1(i–k)]. In severe OA cartilage (9–13 points), the superficial layer and a part of the middle layer had disappeared, and chondrocytes in the middle (71–100%) and deep (51–70%) layers showed significant reaction for CTGF/Hcs24 [Fig. 1(l–n)]. The vascular endothelial cells and periosteal osteoblastic cells also showed a weak to moderate signal for CTGF/Hcs24, which was similar to that previously reported by Nakata *et al.*[33]. No positive immuno-reaction was seen in the control specimens. Results of semi-quantitative analysis for CTGF/Hcs24-positive cells in OA cartilage are summarized in Table I .The *in situ* hybridization analysis confirmed the expression of CTGF/Hcs24 by OA chondrocytes at the mRNA level, and the distribution of positive chondrocytes was similar to that of CTGF/Hcs24 immuno-positive chondrocytes (Fig. 2).

EXPRESSION OF CTGF/HCS24 PROTEIN IN CHONDRO-OSTEOPHYTE

Chondrocytes forming chondro-osteophyte also showed positive reaction for CTGF/Hcs24. A weak staining for CTGF/Hcs24 was observed in the chondrocytes of the fibrocartilage zone (0–10%), and a strong signal was detected in the chondrocytes of the proliferative (11–30%) and hypertrophic (31–50%) zones. The endothelial cells and osteoblastic cells in the calcified zone also showed a weak to moderate signal for CTGF/Hcs24 [Fig. 3(A, B)]. Results of semi-quantitative analysis for CTGF/Hcs24-positive cells in chondro-osteophyte are summarized in Table I. *In situ* hybridization showed significant expression of CTGF/Hcs24 mRNA by proliferating and hypertrophic chondrocytes within chondro-osteophyte [Fig. 3(D, E)]. No positive signal was detected in specimens used for specificity of immunohistochemistry and *in situ* hybridization [Fig. 3(C, F)].

Discussion

Kumar *et al.*[29] first demonstrated that CTGF/Hcs24 was one of the top 20 most abundant genes expressed in both normal (seventeenth) and OA (sixth) cartilage libraries. The result of expressed sequenced tags (ESTs) was further confirmed by Northern blot analysis showing that CTGF/Hcs24 was detected in two of four control samples and three of four OA cartilage samples. In the present study, we demonstrated the expression and localization of CTGF/Hcs24 in the normal and OA joint at protein and mRNA levels. The expression of CTGF/Hcs24 was seen in chondrocytes in all layers of normal cartilage, and up-regulated in chondrocytes in OA cartilage, as well as in the proliferative and hypertrophic zones of chondro-osteophyte.

The early structural changes of OA are characterized by a conspicuous increase in chondrocyte numbers. A remarkable process of proliferation of the cells remaining between the fibrillary clefts begins, and this alteration parallels the increased synthesis of macromolecules characteristic of the early disease. Although the mechanism of chondrocyte proliferation is poorly understood, a potential role for growth factors, such as TGF-β, IGF-1, BMP, FGFs has been assumed[34]. It was reported that recombinant CTGF/Hcs24 protein (20–50 ng/ml) promotes the proliferation of chondrosarcoma cells (HCS-2/8) and rabbit growth cartilage cells in sparse and growing cultures[35]. In addition, CTGF/Hcs24 increases the proteoglycan synthesis and gene expressions of aggrecan and collagen type II, typical markers of chondrocyte maturation in both types of cells in confluent cultures where they were maturing[20]. Interestingly, the phenotype of CTGF/Hcs24 mutant showed that CTGF/Hcs24 is required for chondrocyte proliferation and enchondral ossification[36]. These results suggest that chondrocyte differentiation and hypertrophy in the course of cartilage alteration may stimulate the production of CTGF/Hcs24, which can act as an autocrine and paracrine growth factor to further promote the proliferation and differentiation of the cells. CTGF/Hcs24 may also play a role in production of extracellular matrix molecules such as type I and type II collagen, or production of proteoglycans by chondrocytes, as a part of the attempt to repair.

Previous studies indicated that the gene expression of CTGF/Hcs24 is up-regulated in various fibrous disorders. CTGF/Hcs24 was shown to promote the proliferation and migration of fibroblasts *in vitro*, and induce expression of extracellular molecules, including type I collagen, fibronectin, and α5 integrin[37]. Recently, we have shown that the expression of CTGF/Hcs24, especially in proliferative and hypertrophic chondrocytes, is increased at mRNA and protein levels at sites of cartilage regeneration during fracture healing in mouse ribs[33]. These facts suggest that the expression of CTGF/H24 by cloning chondrocytes in OA cartilage exposed to the synovial fluid might be linked with the development of the fibrous tissue over the damaged cartilage.

Pfander *et al.*[38] reported vascular endothelial growth factor (VEGF) mRNA and protein expression in OA cartilage. They found a significant increase of VEGF immuno-positive chondrocytes in severe OA cartilage compared with normal articular cartilage. Enlarged chondrocytes clustered next to the cartilage surface showed strong immuno-reaction for VEGF, which is consistent with the localization of CTGF/Hcs24 in the current study (Fig. 1). As VEGF and CTGF/Hcs24 are down-stream effectors of TGF-β, an interaction between VEGF and CTGF/Hcs24 in OA cartilage can be speculated. Inoki *et al.*[39] reported that CTGF/Hcs24 inhibits $VEGF_{165}$-induced angiogenesis by forming a complex of the two growth factors. It is also reasonable to speculate that increased expression of CTGF/Hcs24 by OA chondrocytes might have roles in the promotion of fibrosis in damaged cartilage, and in the regulation of VEGF function.

In the current study, we also showed that CTGF/Hcs24 is expressed by hypertrophic chondrocytes of chondro-osteophyte. The histological appearance of chondro-osteophyte

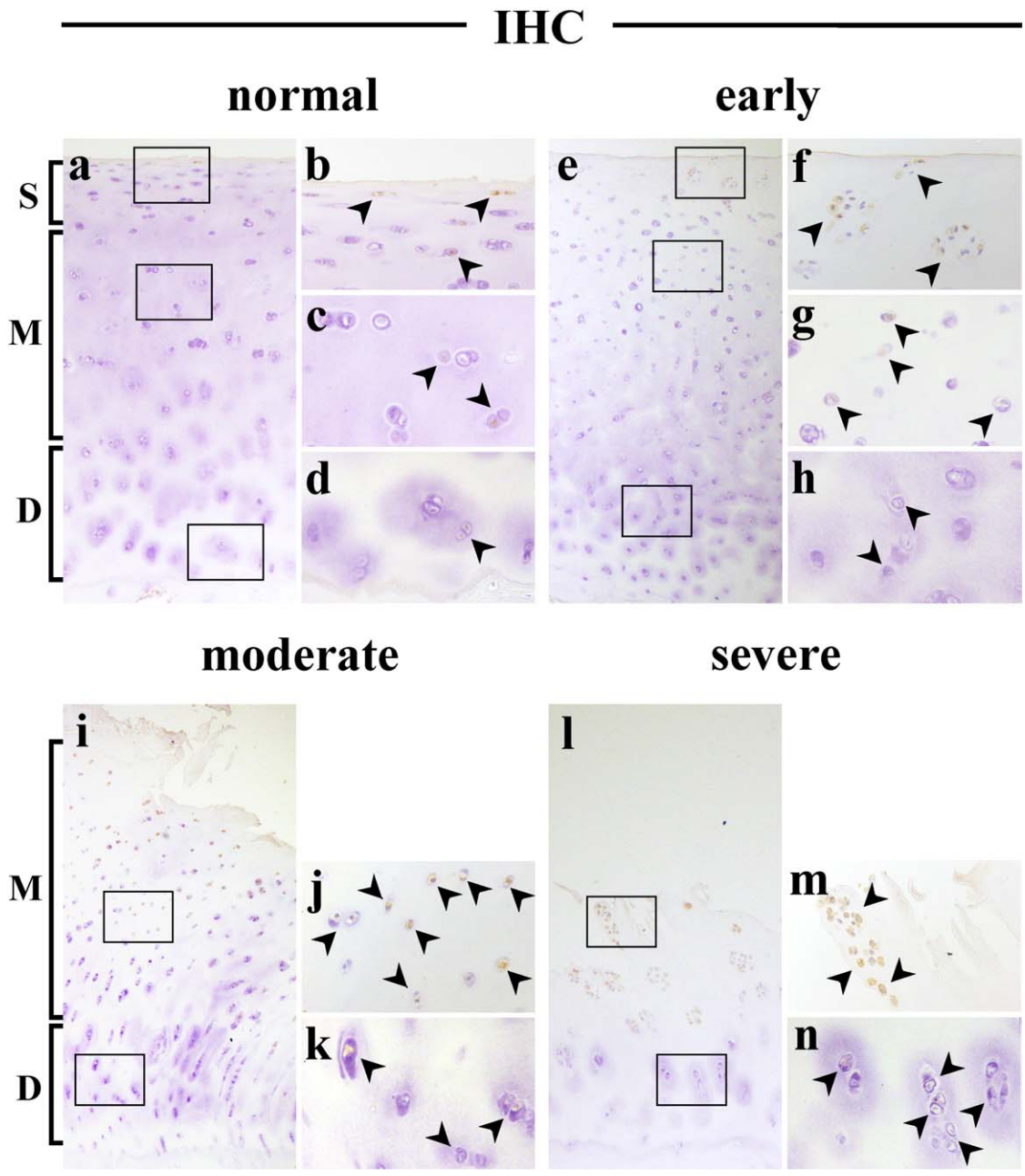

Fig. 1. Expression and localization of CTGF/Hcs24 in morphologically normal cartilage and early, moderate and severe stage OA cartilage detected by immunohistochemistry (IHC). Signals for CTGF/Hcs24 protein were distributed in chondrocytes throughout the layers of normal cartilage (a–d). In early stage OA, the expression of CTGF/Hcs24 was up-regulated mainly at the superficial layer of early stage OA cartilage (e–h; arrows). In moderate and severe OA cartilage, superficial zone cartilage was much reduced, and immuno-localization of CTGF/Hcs24 was observed in individual and clustering chondrocytes in the middle and deep cartilage layers (i–n; arrows). (Original magnification; a, e, i, l: ×50, b–d, f–h, j, k, m, n: ×100.) S, superficial layer (b, f); M, middle layer (c, g, j, m); D, deep layer (d, h, k, n).

resembles normal enchondral ossification at the epiphyseal plate[30]. Ivkovic *et al.*[36] reported that CTGF/Hcs24 deficiency leads to skeletal dysmorphisms as a result of impaired chondrocyte proliferation and extracellular matrix composition within the hypertrophic zone of the growth plate cartilage. Interestingly, a mutation in WISP3, a member of CCN family, is associated with the autosomal recessive skeletal disorder progressive pseudorheumatoid dysplasia, which clinically shows continued cartilage loss and bone destruction[40]. The data of the current study support the idea that expression of CTGF/Hcs24 chondrocytes in proliferative and hypertrophic zones of chondro-osteophyte contributes to chondrocyte proliferation, differentiation, and cell death as well as the penetration of blood cells into calcifying cartilage from bone marrow. Moreover, TGF-β has been shown to be highly expressed in the hypertrophic chondrocytes of chondro-osteophyte[41], the predominant expression of CTGF/Hcs24 in the proliferative and hypertrophic chondrocytes in chondro-osteophyte may be related to the high level of TGF-β.

Table I
Summary of semi-quantitative analysis for CTGF/Hcs24-positive chondrocytes in OA cartilage and chondro-osteophyte

OA stage	Modified Mankin score	Cartilage layer		
		Superficial	Middle	Deep
Normal	(0)	+	±	±
Early	(1–3)	+++	+	±
Moderate	(4–8)	*	+++	++
Severe	(9–13)	*	++++	+++
Chondro-osteophyte			Zone	
		Fibrocartilage	Proliferative	Hypertrophic
		±	+	++

Positive cell ratios labeled by anti-CTGF/Hcs24 antibody were expressed: −: 0%, ±: 0–10%, +: 11–30%, ++: 31–50%, +++: 51–70%, ++++: 71–100% of cells positive.
*: Superficial zone was already destroyed.

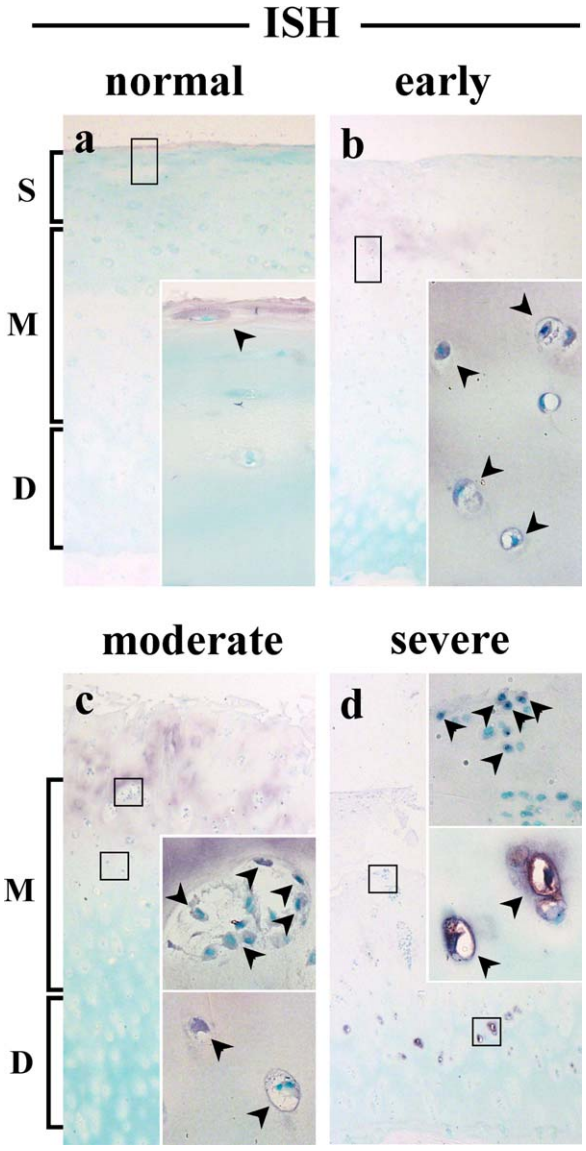

Fig. 2. Expression and localization of CTGF/Hcs24 detected by *in situ* hybridization (ISH). Expression of mRNA of CTGF/Hcs24 was identified throughout the layer of normal cartilage (a), especially in the superficial layer (a; arrows), and observed in the superficial and middle layer of early stage OA cartilage (b; arrows). In moderate and severe stage, CTGF/Hcs24 mRNA was expressed in the middle and deep layer chondrocytes, especially in the cloned chondrocytes (c, d; arrows). (Original magnification; a–d: × 50, in insets: × 200.) S, superficial layer; M, middle layer; D, deep layer.

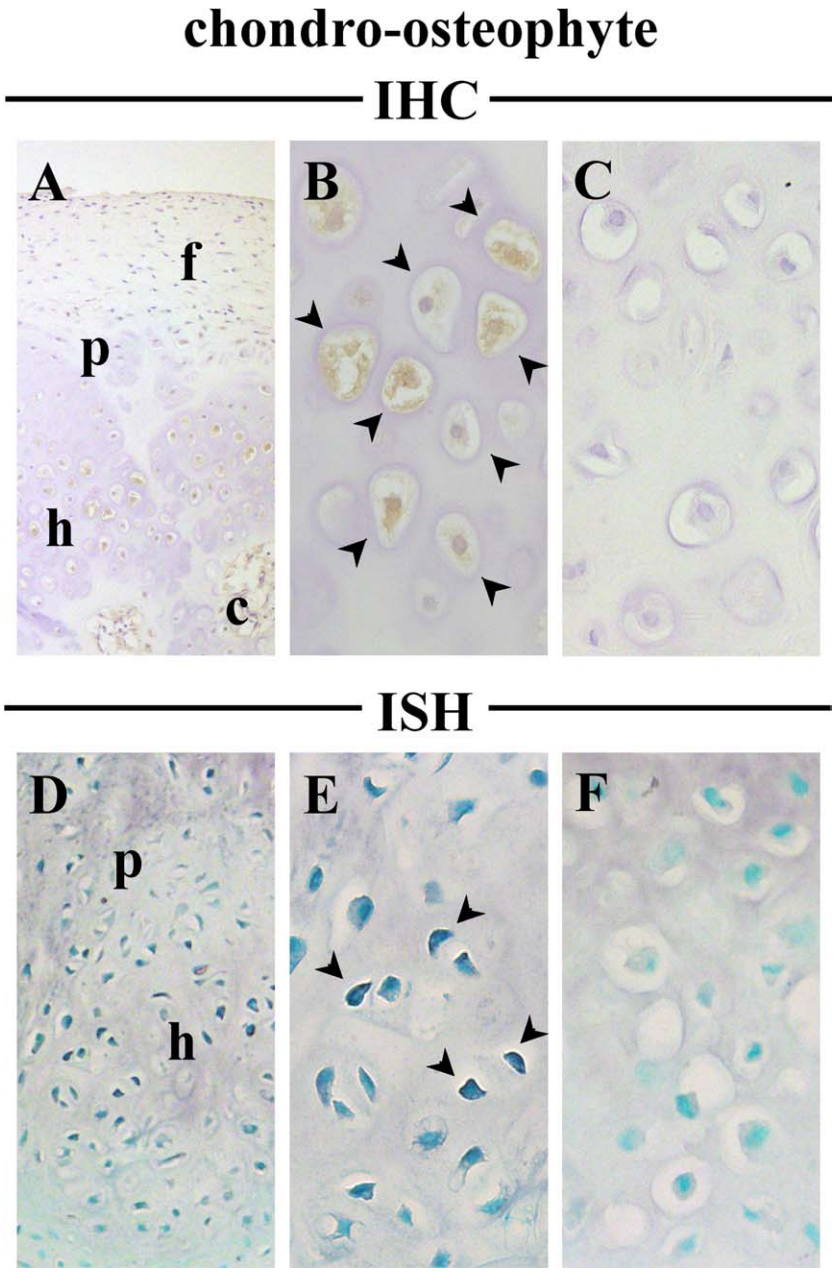

Fig. 3. Expression and localization of CTGF/Hcs24 at protein and mRNA levels were detected in proliferating and hypertrophic chondrocytes which form the chondro-osteophyte by immunohistochemistry (IHC) (A, B; arrows) and *in situ* hybridization (ISH) (D, E; arrows), respectively. Negative controls for IHC obtained by excluding the primary antibody, and negative controls for ISH using the sense human CTGF/Hcs24 RNA probe are shown in C and F, respectively. (Original magnification; A, D: ×50, B, C, E, F: ×200.) f, fibrocartilage zone; p, proliferative zone; h, hypertrophic zone; c, calcified zone.

In conclusion, CTGF/Hcs24 is expressed by normal and OA chondrocytes at the mRNA and protein levels. A strong positive immuno-reaction for CTGF/Hcs24 was seen in the clustering chondrocytes next to the damaged cartilage surface, as well as in proliferating and hypertrophic chondrocytes in chondro-osteophyte, suggesting the expression of CTGF/Hcs24 appeared to be related to cartilage degeneration and chondrocyte differentiation. The results of the current study may contribute to understanding of the anabolic activity of chondrocytes in the course of cartilage destruction of OA.

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