Morphology and Phenotype Expression of Types I, II, III, and X Collagen and MMP-13 of Chondrocytes Cultured from Articular Cartilage of Kashin-Beck Disease

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ABSTRACT. ***Objective.*** We investigated the characteristics of cell morphology and expression of types I, II, III, and X collagen and matrix metalloproteinase-13 (MMP-13) of chondrocytes from articular cartilage of adult patients with Kashin-Beck Disease (KBD) *in vitro* to understand the pathogenesis in chondrocytes.

Methods. Samples of articular cartilage were divided into 2 groups: KBD group (8 samples, 8 cases) and the control (8 samples, 8 cases). KBD patients were diagnosed according to "Pathological Criteria to Diagnose KBD in China." Hyaline cartilage was digested with collagenase into cell suspensions and cultured in monolayers. Chondrocyte ultrastructure was observed by electron microscope at 10th day *in vitro*. Primary articular chondrocytes were seeded on microscope slides and immunostained on 12th day of cultivation for types I, II, III, and X collagens and MMP-13. Positive findings were counted by light microscopy and confirmed by flow cytometric analyses.

Results. Considerable amounts of vacuoles and distorted nuclei, as well as thickening and irregular arrangement of collagen fibrils, were seen in the KBD samples by electron microscopy. Types I, III, and X collagen were stained in the KBD, but not in the control cultures. The percentages of positive staining for type II collagen were significantly lower in KBD than those in controls ($t_{col\ II} = -5.54$, $p < 0.001$), and for MMP-13 in the KBD group were significantly higher ($t_{MMP\text{-}13} = 3.70$, $p < 0.01$).

Conclusion. Phenotype expressions of types I, II, III, and X collagen and MMP-13 in chondrocytes cultured *in vitro* were significantly different between the KBD and control cultures, indicating degenerative and hypertrophic changes in chondrocytes of KBD articular cartilage. (First Release Mar 1 2008; J Rheumatol 2008;35:696–702)

Key Indexing Terms:
KASHIN-BECK DISEASE COLLAGEN MATRIX METALLOPROTEINASES
CARTILAGE CHONDROCYTES

Kashin-Beck disease (KBD) is a chronic, endemic osteochondropathy affecting over 0.74 million patients with 10.342 million people at risk in China[1]. The disease is mainly distributed in the area ranging from the northeastern to the southwestern parts of China, as well as some areas in Russia and North Korea[2,3]. The etiology of the disease is still under debate, although 3 major environmental hypotheses have been proposed in the past 50 years: endemic selenium deficiency, serious cereal contamination by mycotoxin-producing fungi, and high humic acid levels in drinking water[4-8]. Clinical symptoms include enlarged interphalangeal joints, shortened fingers, deformed and enlarged joints, and limited motion of joints[9]. Pathologically, the disease is manifested as degeneration and necrosis mainly in the growth plate and the articular cartilages, resulting in growth retardation, secondary osteoarthrosis, and disability in advanced stages[10,11]. Such an elusive etiology and intolerable agony of patients have caught the attention of physicians and researchers to investigate the etiopathogenesis and treatments of KBD in recent decades. *In vitro* cultivation of chondrocytes is an important way to investigate the etiopathogenesis of the disease. However, chondrocyte cultures have not been utilized for the investigation of cartilage with KBD until now.

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As in other degenerative joint diseases, the large weight-bearing joints are the most degenerated ones in patients with KBD, especially hyaline cartilage of the knee joint. Hyaline cartilage is an avascular deformable tissue, whose physical properties depend mainly on extracellular matrix (ECM) produced by chondrocytes. It covers articulating surfaces and protects them against the damaging effect of repetitive load and friction associated with joint movement. Collagen is one of the major components of cartilage. The collagen types synthesized by chondrocytes can be used as specific markers to define various differentiation states of chondrocytes[12]. Types II, IX, and XI collagen assemble into the collagen heterofibril of hyaline cartilage, and thus are characteristic for the functional hyaline chondrocyte. Expression of types I and/or III and V collagen indicates phenotypic alteration to fibroblastic cells, and type X collagen is characteristic for hypertrophic chondrocytes[12,13]. Destruction of collagen within cartilage depends in part on collagen-degrading matrix metalloproteinases (MMP). The MMP, including MMP-13, are responsible for the degradation of ECM components in physiological processes such as morphogenesis, wound healing, and cell migration[14,15].

Previously, abnormal expression of collagens was observed in KBD articular cartilage with a more pronounced type I collagen expression in the superficial zone, but lack of any collagen staining in the local chondrocyte necrosis areas[16,17]. Type X collagen expression was reduced in areas of chondrocyte necrosis in the deep zone of KBD articular cartilage, indicating changes in terminal chondrocyte differentiation[11]. However, the phenotype expression in KBD chondrocytes has not been examined using an *in vitro* human cartilage system, and little is known about the changes of the phenotype and MMP in adult KBD articular chondrocytes.

Several culture models have been developed to investigate cartilage biology. Culture models including monolayer and perfusion cultures[18], 3-dimensional culture systems like alginate cultures[19], high density pellet cultures, and explanted cultures[20,21] have been reported to revert and prevent the dedifferentiation of chondrocytes, and the chondrocytic phenotype of human chondrocytes can be maintained in many culture systems for at least 14 days[20,21].

We determined differences in the expression levels of types I, II, III, and X collagen and MMP-13 in KBD chondrocytes *in vitro*, compared with controls. We also present the prospect for utilizing a cell culture model to investigate KBD cartilage chondrocytes.

MATERIALS AND METHODS

Cartilage samples. The articular cartilage samples collected were divided into the KBD and healthy control groups. In the KBD group, articular cartilages were collected from the femoral condyles of adult patients undergoing total knee replacement surgery at Xi'an Red Cross Hospital (5 women and 3 men, ages 31–42 yrs). The selected KBD patients were diagnosed as the first and second degrees based on the national diagnosis criteria of KBD in China[22] using the analysis of radiographic films of the right hand, knee, and hip joints; cartilage sections underwent hematoxylin and eosin (H&E) staining. The control group consisted of 8 samples of articular cartilage collected from the same region of fresh cadaver knees (from 2 women and 6 men, ages 27–39 yrs). The cadaver donors, who died in traffic accidents, were from non-KBD areas and excluded KBD, and status of control cartilage samples was diagnosed by histological examination with H&E staining, and excluding the genetic bone and cartilage diseases, osteoarthritis, and rheumatoid arthritis. This investigation was approved by the Human Ethics Committee of Xi'an Jiaotong University. All patients and relatives of donors provided an informed consent.

Cultivation of human articular chondrocytes. Within 4 h after surgery or 12 h of death, cartilage tissues were transported immediately in Dulbecco's modified Eagle's medium (DMEM, Gibco) from the surgical operating room. After rinsing with phosphate buffered saline (PBS) 3 times, chondrocytes were prepared from cartilage by overnight digestion using 0.25% collagenase (type II, Gibco; 1 ml solution per 100 mg tissue)[23]. Chondrocytes were filtered through gauze to remove undigested cartilage fragments. Chondrocytes from individual donors were kept strictly separated in all experiments. The released cells were plated in cell culture flasks in DMEM supplemented with 12% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 mg/ml ascorbic acid, and maintained in a humidified atmosphere at 5% CO_2 and 37°C. The medium was replaced 2 or 3 times a week. Primary cells, without passage, were used in all experiments.

Electron microscopy. Primary chondrocytes, seeded in 25 cm^2 cell culture flasks in numbers of 4×10^5, were harvested by trypsin/EDTA after incubation at 37°C under 5% CO_2 for 10 days, centrifuged to pellets, and fixed, dehydrated, embedded and sectioned. The sections were stained with lead citrate and uranyl acetate, and observed by electron microscopy (Hitachi, Japan). In order to confirm the phenomenon, we observed 3 samples cultured separately in each group. The characteristic morphologic changes of chondrocytes from the KBD and the control groups were recorded.

Antibodies and reagents. Mouse monoclonal antibodies against human recombinant types I, II, and III collagen, and MMP-13 were purchased from Sigma (St. Louis, MO, USA). Mouse monoclonal antibody against human recombinant type X collagen (X-53) was kindly donated by Prof. Klaus von der Mark from the Institute of Experimental Medicine I, University of Erlangen-Nurnberg, Germany, and the specificity was tested by ELISA, Western blotting, and immunostaining on test tissues[24]. The horseradish peroxidase-diaminobenzidine (DAB) immunostaining kit (including biotin-labeled IgG antibodies of sheep against mouse; R&D Systems) was purchased from Zymed (San Francisco, CA, USA).

Immunocytochemical staining. For immunocytochemical staining, primary chondrocytes were seeded in 24-well plates, in which 7×7 mm^2 microscope slides coated with P-L-polylysine were placed, at a density of 1×10^4 cells/well, expanded in human articular chondrocyte culture medium. Cells were fixed on the 12th day with 4% paraformaldehyde in PBS for 20 min, and rinsed with PBS. Then, immunocytochemistry investigation was performed with the avidin-biotin-peroxidase method according to the manufacturer's recommendation. In brief, when staining intracellular antigens, the cells were permeabilized with 0.2% Triton X-100 for 15 min. Before incubation with primary antibodies, the cells were incubated in 10% normal serum in PBS (1 h, 37°C) and washed with PBS. Primary antibodies were incubated overnight at 4°C, and secondary antibodies were incubated at room temperature (RT) for 45 min. After washing with PBS, the peroxidase reaction was visualized with 0.3% (v/v) DAB buffer containing 0.1% (v/v) hydrogen peroxide. When needed, the cells were stained with hematoxylin. Finally, the cells were washed with distilled water, dehydrated through an ethanol series (70%, 95%, and 99.9%) and xylene, mounted, and photographed using a Nikon light microscope. The negative controls for secondary antibody were performed by treating slides according to the protocol, but omitting the primary antibody.

Flow cytometric analysis. Primary cells were seeded at density of 1×10^5

cells per well in 6-well plates and every sample was seeded in 6 parallel wells. Cells were harvested with trypsin/EDTA after incubation at 37°C under 5% CO_2 for 12 days and the cell suspensions were washed twice with PBS and fixed in 2% paraformaldehyde (15 min RT), and then washed with PBS prior to permeabilization with PBS containing 0.2% Triton X-100 (15 min at RT). After incubation in 10% normal serum in PBS (1 h, 37°C), the cells were incubated with primary antibodies overnight at 4°C. Next, cells were rinsed with PBS and then incubated with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (Sigma; 25 min RT). All monoclonal antibodies were titrated in preliminary experiments, and a negative control with an isotype-matched antibody was included. After 2 washing steps with SAP-buffer (0.1% saponin in PBS), the cells were resuspended in 1 ml PBS and analyzed in a FACS Calibur flow cytometer using Cell Quest software (Becton Dickinson, Mountain View, CA, USA). Between 10,000 and 30,000 events per test were acquired. To analyze the expression of types I, II, III, and X collagen and MMP-13, a gated area was selectively determined. Results were expressed as frequency of positive cells and mean fluorescence intensity referred to staining with isotype control monoclonal antibody.

Statistical analysis. Chondrocyte numbers in slides were counted by light microscopy for cytoplasmic staining. Six randomly chosen fields in each slide were counted at 400× magnification. The average rate was calculated in each slide in each case, and then for the different groups. All flow cytometric analysis experiments were performed for 8 individuals. Mean and SD was used for descriptive statistics. Differences between means were assessed by unpaired Student's t test. P values less than 0.05 were considered statistically significant.

RESULTS

Morphology and ultramicrostructure. In electron microscopy, the chondrocytes in the KBD group appeared oval, and the nuclei were clearly distorted and the cells had a high nucleus to cytoplasm ratio. The cytoplasm contained abundant rough endoplasmic reticulum and a large number of vacuoles, and smooth plasma membranes with few filopodia were observed (Figure 1A, 1C). ECM showed collagen fibers of larger diameter with disordered orientation (Figure 1E, 1G). Compared with KBD samples, the chondrocytes of the control group showed normal cytoplasmic and nuclear morphology, with a large amount of endoplasmic reticulum and mitochondria in the cytoplasm (Figure 1B, 1D). In addition, filopodia were seen on the plasma membrane, and normal ECM with well arranged and dense collagen fibers could be observed (Figure 1B, 1D, 1F, 1H).

Immunocytochemistry. On the 12th day of the experiment, positive immunostaining for types I, III, and X collagen could be observed in chondrocytes of the KBD group, but not in the cells of the control cultures, and the percentage of the KBD chondrocytes positive for type II collagen was significantly lower than that in controls ($t_{col\ II} = -5.54$, $df_{col\ II} = 14$, $p < 0.001$; Table 1, Figure 2). The percentage of positive staining for MMP-13 in the KBD group was significantly higher than that in the controls ($t_{MMP\text{-}13} = 3.70$, $df_{MMP\text{-}13} = 14$, $p < 0.01$).

Flow cytometric analysis. In the parallel experiments, the expression of types I, II, III, and X collagen and MMP-13 in monolayer cultivation at the 12th day were analyzed by flow cytometry. In accord with the differences indicated by immunocytochemistry, a lower percentage of positive cells for type II collagen ($t_{col\ II} = -5.27$, $df_{col\ II} = 14$, $p < 0.001$; Figure 3) and a higher percentage for MMP-13 ($t_{MMP\text{-}13} = 3.66$, $df_{MMP\text{-}13} = 14$; $p < 0.01$) were seen in the KBD group compared with those in the controls. Expression of types I, III, and X collagen was observed in the KBD groups, but was hardly detectable in the control group. In the KBD group, mean fluorescence intensities for type II collagen and MMP-13 were also significantly different ($t_{col\ II} = -4.98$,

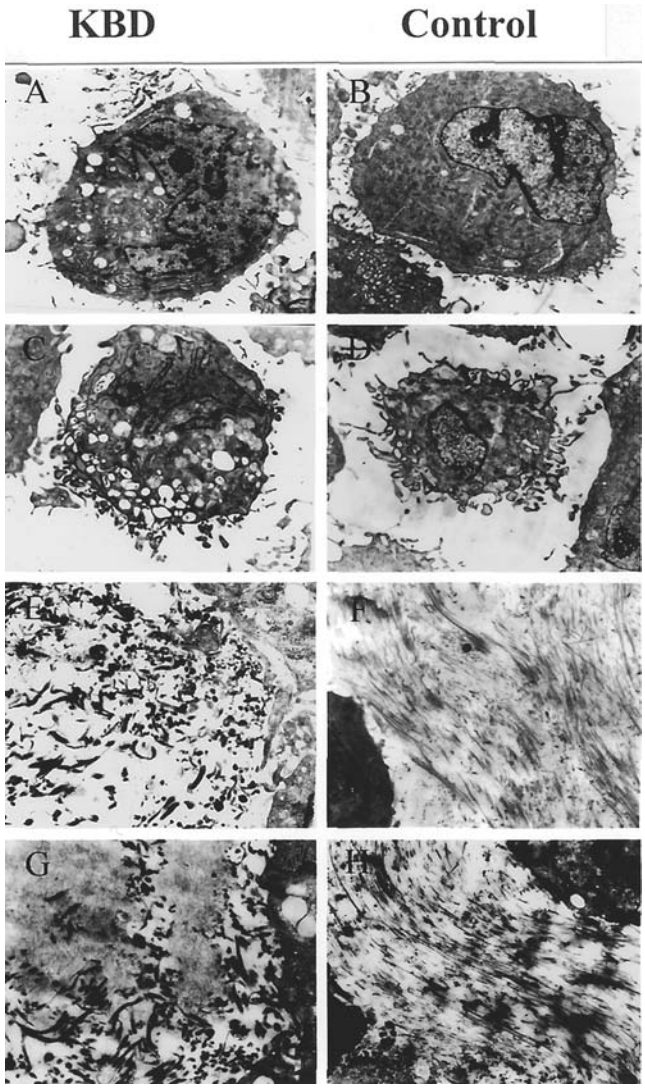

Figure 1. Morphology of the cultured chondrocytes under electron microscopy. A, C: KBD chondrocytes were oval and had distorted nuclei, a high nucleus to cytoplasm ratio, and the cytoplasm contained abundant rough endoplasmic reticulum. Many vacuoles and smooth cell membrane appeared in cytoplasm (TEM ×5000). B, D: The control chondrocytes showed normal cytoplasmic and nuclear morphology. Large amounts of endoplasmic reticulum and mitochondria were present in the cytoplasm. Filopodia were seen on the plasma membrane (TEM ×5000). E, G: ECM in KBD chondrocytes appeared to have a larger diameter of collagen fibers with disordered orientation (TEM ×10,000). F, H: ECM in chondrocytes cultured from the control group was well arranged, and had dense collagen fibers (TEM ×10,000).

Table 1. Percentage of positive staining in chondrocytes for types I, II, III, and X collagen and MMP-13 between the KBD and control groups at 12th day, analyzed by immunocytochemistry. Data are mean ± SD.

Groups		n	Positive Stain, %
Col I	Control	8	0.0 ± 0.0
	KBD	8	32.0 ± 4.4**
Col II	Control	8	84.7 ± 6.6
	KBD	8	62.1 ± 9.7**
Col III	Control	8	0.0 ± 0.0
	KBD	8	35.6 ± 7.8**
Col X	Control	8	0.0 ± 0.0
	KBD	8	21.6 ± 3.2**
MMP-13	Control	8	22.3 ± 6.9
	KBD	8	37.0 ± 9.0*

* Mean difference is significant at 0.05 level between 2 groups by t-test. ** Mean difference is significant at 0.001 level between 2 groups by t-test. MMP: matrix metalloproteinase; KBD: Kashin-Beck disease.

$df_{col\ II} = 14$, $p < 0.001$, $t_{MMP\text{-}13} = 3.91$, $df_{MMP\text{-}13} = 14$, $p < 0.01$; Figure 3, Table 2).

DISCUSSION

KBD is a chronic, endemic osteochondropathy with pathological features of multiple focal chondronecrotic lesions in the deep zone. The degenerative changes in the KBD articular cartilage appear as surface fibrillations, chondrocyte clustering in adult patients, type II collagen degradation, and loss of proteoglycans from the ECM[25]. We investigated the cellular ultrastructure and the expression of types I, II, III, and X collagen and MMP-13 in chondrocytes isolated from KBD articular cartilage and cultured *in vitro*.

Since the numbers of primary chondrocytes available from human cartilage are limited, in-depth and longterm quantifications of chondrocyte protein expression are technically difficult. Spontaneous dedifferentiation of chondrocytes during *in vitro* expansion with longterm and multiple passages further limits the population doublings useful for the experiments. In order to observe KBD chondrocyte characteristics, we focused on monolayer culture and limited our experiments to short-term culture of primary chondrocytes. In our experiments, we analyzed the ultrastructure on the 10th day and protein expression on the 12th day to maintain the differentiated phenotype and proliferation under monolayer culture conditions[20]. The selected incubation time was considered necessary to allow sufficient metabolic activity to take place, and to enable us to detect the possible differences between the KBD and the control cells.

The ECM plays an active and complex role in regulating the behavior of the chondrocytes by influencing their development, migration, proliferation, and metabolic function. Specialized matrix surrounding the articular chondrocytes contributes to their unique structure and function. Interactions among matrix protein molecules and the interactions of those molecules with the chondrocytes have been considered to be essential for the maintenance of tissue properties[26-28]. Expression of collagen type II is a marker of proliferating chondrocytes[29], and the synthesis and the presence of type II collagen indicates the differentiated state of chondrocytes[30,31]. Moreover, type II collagen is the principal component of the ECM of articular cartilage, comprising 15%–25% of wet weight, about half the dry weight, and 90%–95% of the total collagen content of the cartilage[32]. Type II collagen fibrils are composed of a triple helix of 3 identical α-chains, which can form fibrils stabilized by intermolecular crosslinks. These fibrils provide tensile strength[33] and maintain the integrity of cartilage. This fibrillar network resists the swelling pressure that results from the hydration of polyanionic proteoglycan aggregates in the ECM. It has been reported that increased cleavage[34-36] and denaturation[37,38] of the triple helix of type II collagen were found in KBD articular cartilage samples[39]. Type I and type III collagen are synthesized as procollagens containing propeptide extensions at both ends of the molecule. After secretion into the ECM, the propeptides are removed and the collagen molecules aggregate to form the fibril. Where metabolic activity is low, as in normal human adult articular cartilage, expression of types I and III collagen is not detectable[40]. In our study, low expression of type II collagen and the differentiated characteristics in the KBD chondrocytes were evident, and confirmed by increased expression of types I and III collagen in monolayer cultivation. A larger diameter of disorderly-oriented collagen fibers and a significant amount of distorted nuclei and vacuoles were detected at the ultrastructural level. It is interesting that there were fewer filopodia in the KBD cells. Hyaluronan synthase overexpression has been shown to induce the formation of filopodia, while treatment of cells with hyaluronidase destroyed them[41]. Since hyaluronan is a major organizer of cartilage matrix, it may play a role in the pathogenesis of KBD, perhaps also affecting the filopodia. These phenomena indicate that dystrophy and decreased ability of chondrocytes to produce type II collagen likely influence the pathology of the aging process of KBD articular cartilage.

Hypertrophic chondrocytes are marked by the expression of type X collagen. According to our results, the percentages of chondrocytes positive for type X collagen were significantly higher in the KBD patients than in the controls *in vitro*. Although type X collagen is absent from most normal adult articular cartilages, it has been detected in deep and even heavily fibrillated superficial regions of osteoarthritic cartilage[42]. In macroscopically normal canine cartilage, it was detectable after extended removal of ECM[43]. Calcium ions and a variety of other agents are known to induce the synthesis of type X collagen[44]. Type X collagen has been shown to bind to annexin V and alter calcium ion uptake of matrix vesicle-mediated mineralization[45]. The differential expression of type X collagen between the 2 groups suggests a premature hypertrophy phenomenon in the KBD

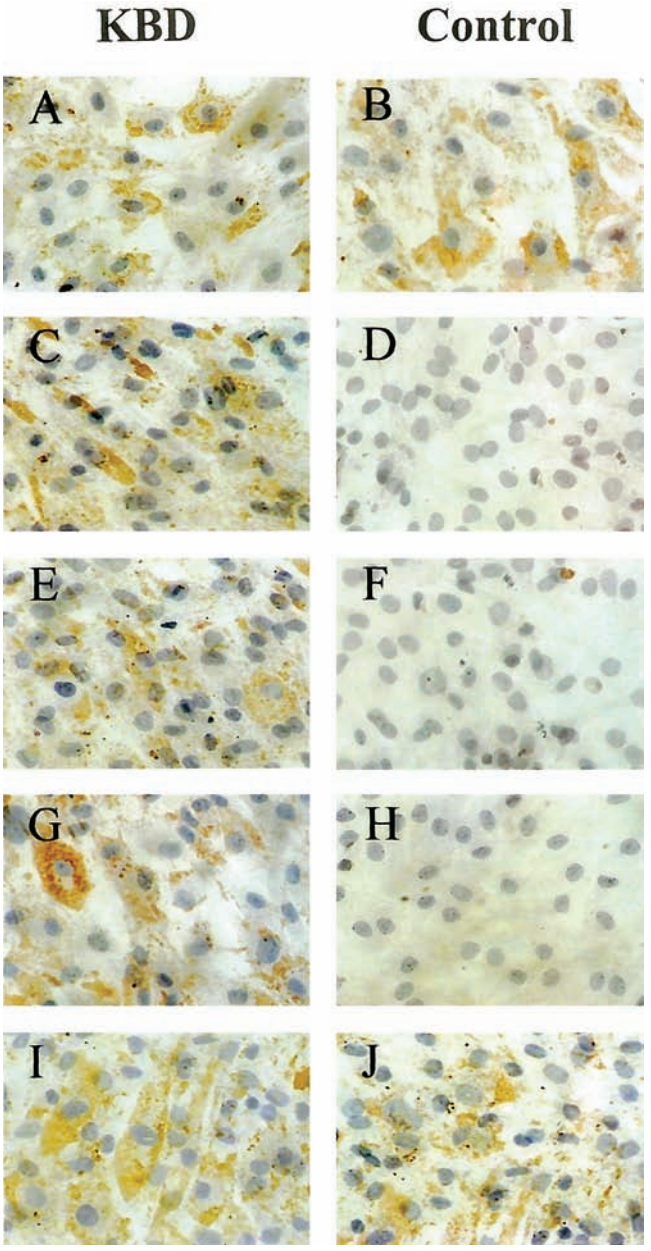

Figure 2. Immunocytochemical staining for types I, II, III, and X collagen and MMP-13 in monolayer chondrocytes of patients with KBD and controls. Immunostaining for types II (A), I (C), III (E), X (G) collagen and MMP-13 (I) in KBD chondrocytes at the 12th day. Immunostaining for types II (B), I (D), III (F), X (H) collagen and MMP-13 (J) in control chondrocytes at 12th day. Magnification 400×.

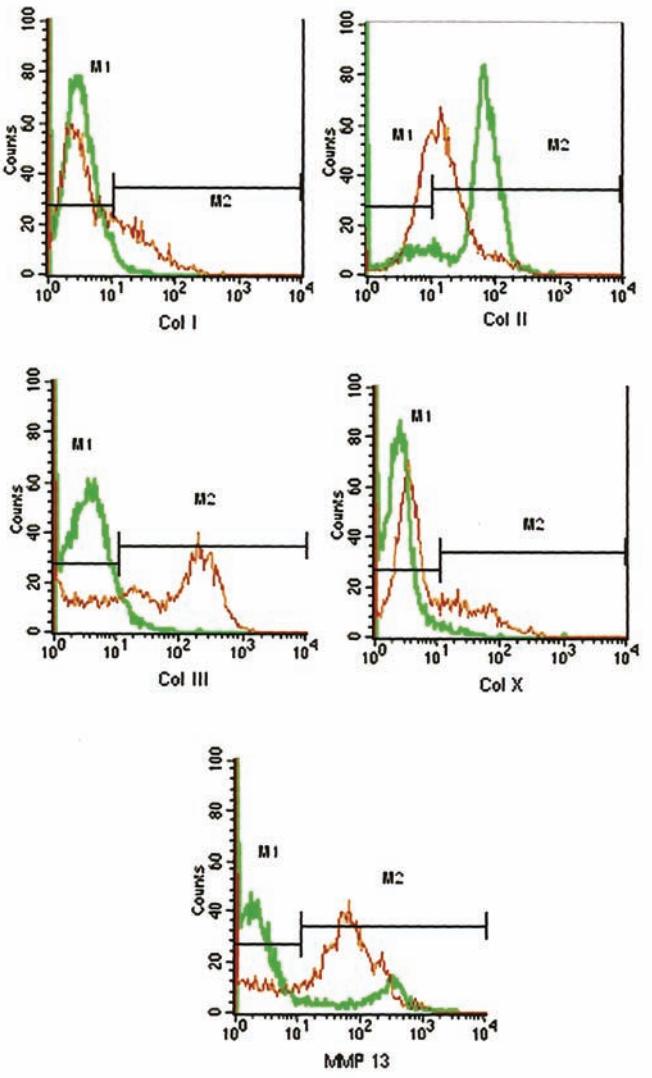

Figure 3. Expression of type I, II, III, X collagen (Col I, Col II, Col III, Col X) and MMP-13 in KBD and control chondrocytes. Flow cytometry profiles of a representative experiment are shown with overlay histogram of staining in gated cells (1×10^4). Fluorescence intensity of FITC-labeled secondary antibodies in the overlay histogram is shown on the X axis for the KBD (orange) and control (green) groups. M1: negative expression; M2: positive expression.

chondrocytes, which suggests that the aging process of chondrocytes in the KBD articular cartilage is accelerated, and the terminal cells are not able to differentiate normally.

Changes in ECM components and organization can modulate cellular behavior. Thus, the turnover of ECM is an integral part of development, morphogenesis, and tissue remodeling. While various types of proteinases participate in matrix turnover, MMP are the principal matrix-degrading proteinases[44]. The MMP are a family of calcium-dependent zinc-containing endopeptidases, which are capable of degrading a wide variety of ECM components[46]. Our data indicated that collagenase-3 (MMP-13) might play important roles in this degradation. It has been found that inhibition of matrix resorption by an inhibitor of collagenase MMP-13 can suppress type II collagen cleavage and also arrest the expression of MMP-13[47], suggesting that degradation products of type II collagen may themselves induce collagenase expression. The induction of collagenase activity was clearly accompanied by an increase in MMP-13 in chondrocyte cultures[48]. Those findings showed the subtle differences in the ECM, which was synthesized by cultured

Table 2. Expression for types I, II, III and X collagen and MMP-13 between the KBD and control groups at 12th day, analyzed by flow cytometry. Data are percentage of positive cells and mean fluorescence intensity (MFI).

Groups		n	Positive Cells, %	MFI, ± SD
Col I	Control	8	2.4 ± 0.7	13.7 ± 3.5
	KBD	8	39.6 ± 6.7**	208.4 ± 36.5**
Col II	Control	8	95.2 ± 2.6	547.1 ± 77.3
	KBD	8	82.5 ± 6.3**	346.9 ± 83.6**
Col III	Control	8	3.0 ± 0.8	29.3 ± 10.8
	KBD	8	41.0 ± 6.4**	155.2 ± 20.3**
Col X	Control	8	2.8 ± 0.7	28.0 ± 10.2
	KBD	8	33.8 ± 8.9**	143.7 ± 22.3**
MMP-13	Control	8	33.6 ± 6.8	79.3 ± 9.7
	KBD	8	48.8 ± 9.6*	112.0 ± 21.6*

* $p < 0.05$, ** $p < 0.001$ between 2 groups by t-test. MMP: matrix metalloproteinase; KBD: Kashin-Beck disease.

chondrocytes, and underline the complexity of the cell-ECM interaction. A disturbed matrix results in a cellular response to correct the underlying abnormality. These changes indicated the degenerative process taking place in the hyaline articular cartilage of patients with KBD.

There are cases where the intracellular composition is not representative of the extracellular character. For instance, in pseudoachondroplasia, mutations in cartilage oligomeric protein (COMP) disturb the secretion of COMP, causing intracellular accumulation of the protein[49]. In our study, we noted certain changes in the secreted collagens, and we used flow cytometric analyses of permeabilized cells to determine whether intracellular staining of nascent proteins would reflect expression of the composition of ECM. Our finding was that both pools were rather similar in their composition.

Our objective was to analyze the extracellular collagens synthesized by cultured chondrocytes; further studies comparing matrix extracellular and intracellular properties are needed in order to get more information.

In summary, the chondrocytes of KBD articular cartilage *in vitro* showed obvious differences compared with normal control samples, indicating pre-hypertrophy and accelerated aging in monolayer cultures from KBD articular cartilage.

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