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ORIGINAL ARTICLE

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Abnormal expression of Col X, PTHrP, TGF-β, bFGF, and VEGF in cartilage with Kashin–Beck disease

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Abstract The purpose of the current study was to investigate the abnormal expression of Col X, PTHrP, TGF-β, bFGF, and VEGF in cartilage from patients with Kashin–Beck disease (KBD) to understand the pathogenesis of chondronecrosis in KBD. Articular cartilage and growth plate cartilage collected were divided into four groups: control children (8 samples, 5 cases), KBD children (19 samples, 9 cases), control adults (8 samples, 6 cases), and KBD adults (16 samples, 15 cases). The presence of PTHrP, TGF-β_1, bFGF, VEGF, and collagen X in articular cartilage and in growth plate cartilage was analyzed by immunohistochemistry. Articular cartilage and growth plate were each divided in three zones, and the rate of positive cells was counted by light microscope for cytoplasmic and pericellular staining. Results showed that (1) in KBD children, Col X expression was lower in the deep zone of growth plate cartilage than in normal children; in articular cartilage of KBD adults, however, collagen X expression was higher in the middle zone compared to the controls; (2) staining for bFGF, PTHrP, TGF-β_1, and VEGF in KBD adult patients was prominent in the chondrocyte clusters and the eroded surface of articular cartilage, and the percentage of chondrocyte staining was significantly higher than in control samples (t = 3.64–10.34, df = 12 for children and 19 for adults, P = 0.002–0.0001); and (3) the enhanced PTHrP, TGF-β_1, and VEGF staining in the deep and middle zone of KBD articular cartilage correlated with the high incidence of chondronecrosis in the middle zone (48.5% ± 10.2%) and deep zone (70.6% ± 27.0%) of adult KBD cartilage. In conclusion, Col X expression was reduced in areas of chondrocyte necrosis in the deep zone of KBD articular cartilage, indicating changes in terminal chondrocyte differentiation. PTHrP, TGF-β_1, and VEGF expression was significantly altered and indicated degenerative changes in KBD cartilage, which initially resemble those occurring in osteoarthritis, but lead eventually to chondronecrosis, an event not observed in osteoarthritis.

Key words Kashin–Beck disease · Collagen type X · PTHrP · TGF-β · bFGF · VEGF · Cartilage

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Introduction

Kashin–Beck disease (KBD) is a chronic, endemic osteochondropathy affecting more than 2.5 million patients with KBD and 30 million people at risk in China [1]. The etiology of the disease is still under debate, although three 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 [2–4]. The basic pathological features of the disease are degeneration and necrosis, mainly in growth plate cartilage and articular cartilage, which can result in growth retardation, secondary osteoarthrosis, and disability in advanced stages. Because the growth plate cartilage is the growth center of bone, the developmental deformities are most likely a result of impaired chondrocyte differentiation and endochondral ossification. The younger the patient and the more serious the condition, the more serious will be the resulting deformities. Clinically, the disease manifests in enlarged interphalangeal joints, shortened fingers, and deformed and enlarged joints, as well as limited motion of joints, in the extremities (Fig. 1), which develops in four stages: the early stage, and the first, second, and third degree. In all stages, joints from limbs to spine are progressively damaged [5]. Seriously affected children suffer from shortened stature or dwarfism and disability in daily living.

Degenerative changes in KBD cartilage are characterized by multiple foci of chondronecrosis in zones of

Fig. 1. Patient with Kashin–Beck disease (KBD) (male, 45 years old) manifested as enlarged phalangeal joints, shortened fingers, and deformed and enlarged elbow joints as well as limited motion of joints in the extremities

cartilage that are secluded from the blood supply. In fetal and juvenile cartilage, most of those changes are located in the maturing and in the hypertrophic chondrocyte zone; some necrotic fields extend to the transition region between proliferative and hypertrophic zones in growth plate cartilage, in some serious cases even to all zones of cartilage. Before overt degenerative changes in the extracellular matrix, chondrocyte necrosis can be visualized earlier under the electron microscope [6]. Besides chondrocyte necrosis, chondrocyte modulation and formation of chondrocyte clusters and osteophytes have been reported as secondary pathological changes in KBD.

The histological and morphological changes seen in the articular cartilage of KBD [7,8] include significant alterations in chondrocyte phenotype based on major changes in collagen distribution. The collagen types synthesized by chondrocytes can be used as specific markers to define various differentiation states of this cell type [9]: types II, IX, and XI collagen assemble into the collagen heterofibril of hyaline cartilage and thus are characteristic for the hyaline

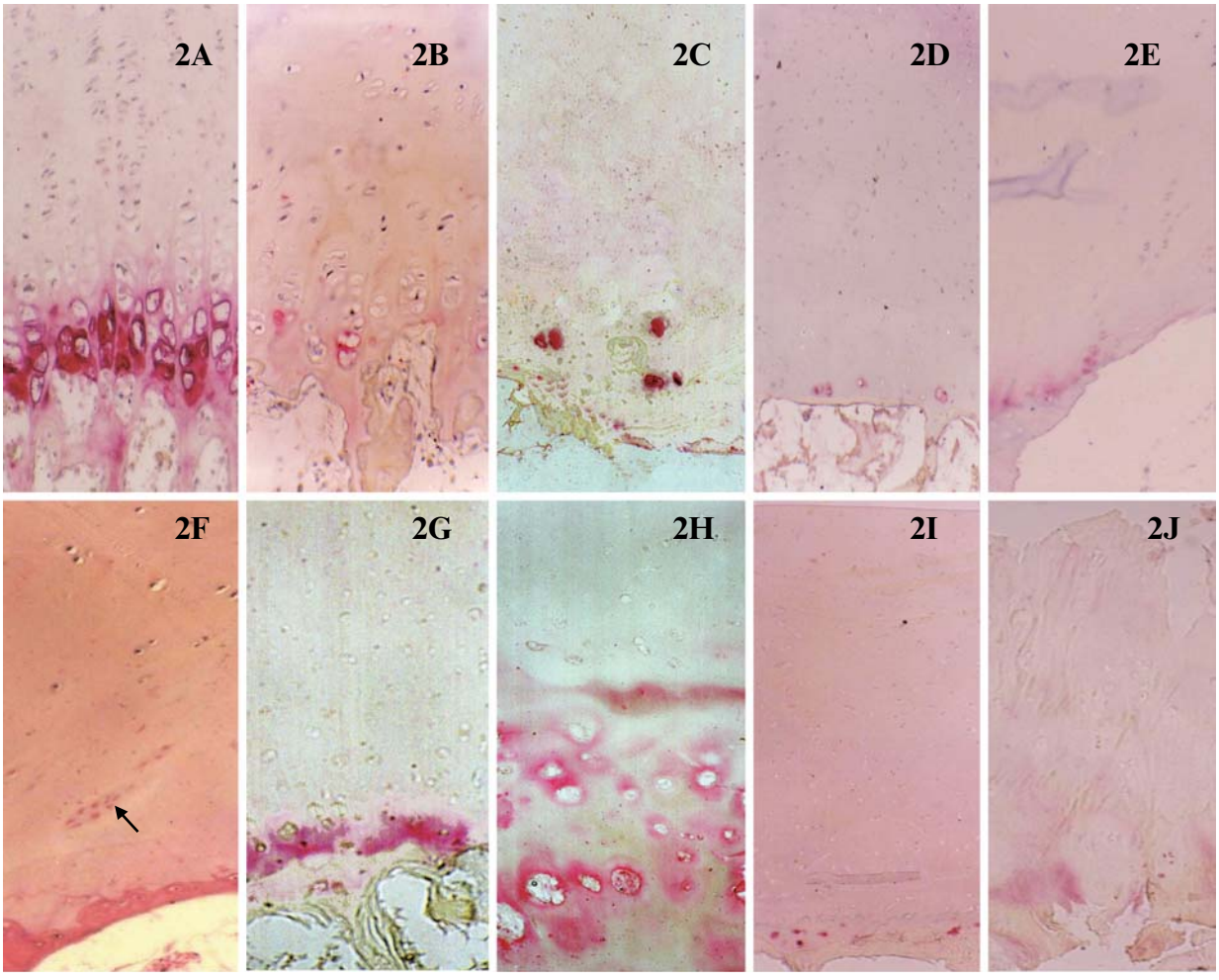


Fig. 2. Immunohistochemical staining (IHS) of collagen X (COL X) staining in growth plate cartilage (GPC) fingers of control child (6-year-old girl) from non-KBD area (**A**) and KBD child (7-year old girl) from KBD area (**B**). IHS of COL X in the calcified zone in articular cartilage (AC) in control child (8-year-old boy; **C**), deep zone in AC (**D**), chondrocyte clusters (**E**) and H&E of chondronecrosis (*arrows* in **F**), compared with fingers (**E**) from KBD child (6-year-old boy). HIS of COL X in the deep zone of AC in knee of a 31-year-old man from non-KBD area (**G**, **I**) and a 48-year-old KBD man with second-degree KBD (**H**,**J**) from KBD area. **A–H** ×100; **I**,**J** ×40

functional chondrocyte. Expression of types I and/or III and V collagen indicates phenotypic alterations to fibroblastic cells, and type X collagen (Col X) is characteristic for hypertrophic chondrocytes [9,10]. Previously, we observed an abnormal collagen pattern in KBD articular cartilage with a more pronounced type I collagen expression in the superficial zone but lack of any collagen staining in local chondrocyte necrosis areas [11]. The reasons for these changes are still unclear. Here we investigate the possibility that alterations in cytokines, growth factors, and other factors regulating chondrocyte differentiation and metabolism are responsible for these changes in collagen expression and associated modulation in KBD cartilage.

Numerous growth factors have been shown to be involved in the control of cartilage growth and chondrocyte differentiation at various stages of chondrocyte development [12]. One of them, the parathyroid hormone-related peptide (PTHrP), is expressed in periarticular, perichondrium, and prehypertrophic chondrocytes [13,14], but PTHrP mRNA was recently detected with reverse transcription-polymerase chain reaction (RT-PCR) in both proliferative and mature chondrocytes [15]. PTHrP stimulates proliferation and hypertrophic of chondrocytes in those zones and delays terminal differentiation of chondrocytes during endochondral bone development [16]. Transforming growth factor-β_1 (TGF-β_1) can induce cartilage formation in perichondrium [17] but inhibits terminal chondrocyte differentiation. It has been shown to act upstream of PTHrP to regulate the rate of hypertrophic differentiation, suggesting that TGF-β_1 has both PTHrP-dependent and PTHrP-independent effects on endochondral bone formation [14,18]. Another factor involved in enchondral ossification is vascular endothelial growth factor (VEGF), a potent and specific endothelial cell mitogen responsible for the induction of angiogenesis and the growth of new blood vessels [19], but also for autocrine regulation of chondrocyte metabolism [20]. It exists at high levels in the synovium of patients with rheumatoid arthritis and causes the formation of synovial pannus and destruction of articular cartilage [21]. Basic fibroblast growth factor (bFGF) inhibits the terminal phase of chondrocyte differentiation by decreasing growth plate chondrocyte proliferation and cellular hypertrophy and, at high concentrations, cartilage matrix production [22,23]. Therefore, investigation of the expression of PTHrP, TGF-β, bFGF, and VEGF as well as collagen type X as markers of chondrocyte differentiation may provide clues to the role of alterations in chondrocyte differentiation and chondronecrosis in the pathogenesis of KBD.

Materials and methods

Tissue preparation and groups

Cartilage samples collected were divided into four groups. (1) Control group of children: 8 samples of articular cartilage and 5 samples of growth plate cartilage in phalanges of hands were collected from 5 children unaffected with KBD from non-KBD areas, 3 boys and 2 girls between 6 and 12 years old who had died of nonrelated causes such as falling or a road traffic accident (3 cases) or acute diarrhea or acute pneumonia (2 cases). (2) KBD group of children (KBD children): 19 samples of articular cartilage and 6 samples of growth plate cartilage in phalanges of hands were collected from 9 cases of KBD children from the diseased areas, including 5 boys and 4 girls aged from 6 to 12 years old who had died of diseases such as acute pneumonia, or acute diarrhea (3 cases), drowning (2 cases), or falling or a road traffic accident (4 cases). (3) The adult control group: 8 samples of articular cartilage from knee joints were collected from 6 adult cases in hospitals of non-KBD areas: 5 men and 1 woman between 24 and 48 years old who had died in road traffic accidents without KBD. (4) KBD group of adults (KBD adults): 16 samples of articular cartilage from knee joints were directly collected from 15 adult cases of KBD from diseased areas by joint debridement in hospitals including 10 men and 5 women aged from 25 to 50 years old. The KBD patients were diagnosed as first degree (9 cases, 6 males and 3 females) and second degree (6 cases, 4 males and 2 females). The defected and eroded surface and osteophytes were seen in articular cartilage of knee joint, and some cartilage pieces without or with subchondral tissues were taken from KBD patients during joint debridement under anesthesia.

KBD children and KBD adults were diagnosed as the early stage or the first and second degree based on the national diagnosing criteria of Kashin–Beck disease in China [24] by X-ray films of right hand and cartilage sections after hematoxylin and eosin (H&E) staining. The health status of control children and adults was diagnosed by histological examination of cartilage sections after H&E staining.

Cartilage slices (0.5–1 cm) were fixed with 4% paraformaldehyde for 24 h after removal of the tissue and decalcified in 3% ethylenediaminetetraocetic acid (EDTA). The samples were dehydrated in a series of alcohol, cleared in xylene, and embedded in paraffin wax. Paraffin sections (6–8 μm) were cut, mounted on slides, pretreated with 2% with 10% poly-L-lysine, and stored at room temperature until used.

Antibodies and reagents

Mouse monoclonal antibodies against human recombinant type X collagen (X-53) were from the Institute of Experimental Medicine I, University of Erlangen-Nurmberg (Germany), and the specificity was tested by enzyme-linked immunosorbent assay (ELISA), Western blotting, and by immunostaining on test tissues [25,26]. The polyclonal immunohistochemistry kits for bFGF, PTHrP (1–34), VEGF, and TGF-β_1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and their specificity was tested by ELISA, Western blotting, and immunostaining on test tissues [27,28]. Biotin-labeled IgG antibodies of sheep against mouse and rabbit against human were purchased from Sigma (St. Louis, MO, USA).

Immunohistochemistry

Collagen type X staining

Deparaffinized cartilage sections were incubated with testicular hyaluronidase [2 mg/ml, phosphate-buffered saline (PBS), pH 5, 30 min at room temperature] and protease type XXIV (Sigma P 8038, 0.02 mg/ml, PBS, pH 7.3, 30 min at room temperature) according to Aigner et al. [25]. Primary monoclonal antibodies to Col X were incubated overnight at 4°C and visualized using alkaline phosphatase-labeled secondary antibodies. Color development was continued for 30 min at room temperature using 3-hydroxy-2-naphthoic acid 2,4-dimethylanilide as a substrate. Finally, nuclei were counterstained with hematoxylin.

Staining for PTHrP, bFGF, TGF-β1 and VEGF

Deparaffinized cartilage sections were briefly pretreated in a microwave oven to expose antigens and incubated in 3% H_2O_2 for 10 min according to the protocol recommended by the manufacturer of the immunohistochemical staining kits for bFGF, PTHrP, TGF-β, and VEGF (Santa Cruz Biotechnology). For the detection of bFGF, PTHrP, and TGF-β_1, sections were incubated overnight at 4°C with primary antibodies and visualized using alkaline phosphatase-labeled secondary antibodies. Color development was continued for 30 min at room temperature using 3-hydroxy-2-naphthoic acid 2,4-dimethylanilide as a substrate. Finally, nuclei were counterstained with hematoxylin. For the detection of VEGF, primary antibodies were incubated 1 h at 37°C and Envision–polyenzyme complexes were incubated 30 min. Color development was continued for 30 min at room temperature using 0.5% diaminobenzedine (DAB) as a substrate. Finally, nuclei were counterstained with hematoxylin.

Classification of cartilage zones

Chondrocytes of articular cartilage were divided similar to the fetal growth plate in five zones to define three cell morphologies by light microscopicy criteria, namely, (1) the superficial (surface) zone, (2) the upper zone (corresponding to the reserve or resting zone of the fetal growth plate), (3) the middle (transitional or intermediate) zone), (4) the deep zone, and (5) the calcified cartilage zone below the tide mark (corresponding to the hypertrophic zone of the growth plate). Chondrocytes in the superficial zone are relatively small and flat, oriented with the long axis parallel to the surface. Chondrocytes of the upper and middle zone are larger and show the typical rounded cellular profile of hyaline cartilage; they are randomly distributed in a matrix with fibers running in oblique directions. Cells in the deep zone were of increasing size and arranged in columnar manner perpendicular to the surface, similar to the proliferating zone of the fetal growth plate [29].

Statistical analysis

Tissue sections were examined and counted by light microscope for cytoplasmic and pericellular staining by mono- or polyclonal antibodies of Col X, bFGF, PTHrP, TGF-β_1, and VEGF as cellular signals of chondronecrosis. Four to six randomly selected fields in each zone were counted at 400× magnification. The average rate in different zones was calculated for each case and then for the different groups. The values were analyzed as statistically significant if $P < 0.05$ by independent sample tests (t test).

Results

Alterations in collagen X localization in growth plate and articular cartilage of KBD children and adults

In the growth plate of the fingers of control children, Col X staining was mainly localized to the deep zones of growth plate cartilage, as expected (Fig. 2A). In KBD children, Col X staining was remarkably reduced in the same zone of growth plate cartilage (Fig. 2B). Similarly, in the deep zone of articular cartilage of KBD children (Fig. 2D), Col X was significantly reduced as compared to normal children (Fig. 2C), but some Col X staining was also seen in chondrocyte clusters (Fig. 2E) in the upper, middle, and deep zones in the articular cartilage of KBD children.

In the articular cartilage of control adults, Col X is expressed only in the calcified zone below the tide mark (Fig. 2G,I). In contrast, in the deep and middle zones of articular cartilage of KBD adults (Fig. 2H,J), Col X staining was seen in restricted chondrocyte clusters in cell-free areas, indicating chondronecrosis (Fig. 2E; compare with Fig. 2F).

Localization of bFGF, PTHrP, TGF-β_1 and VEGF in articular cartilage of KBD patients

In control articular cartilage, chondrocytes existed as single cells or in pairs, separated by an abundant extracellular matrix in the upper zone and as flattened cells in the middle zone (Fig. 3A). No significant staining for bFGF (Fig. 3B), PTHrP (Fig. 3C), VEGF (Fig. 3E), or TGF-β_1 (Fig. 3F) was seen in the upper and middle zones of articular cartilage from control adults. In adult KBD patients, strong staining for bFGF (Figs. 3H and 4B), PTHrP (Figs. 3I and 4C), VEGF (Figs. 3L and 4D), and TGF-β_1 (Figs. 3M and 4E) was prominent in chondrocyte clusters (Figs. 3G and 4A) appearing in the eroded surface of articular cartilage. Compared with the adult articular cartilage, PTHrP was mainly localized in the proliferative and hypertrophic zones of growth plate cartilage in KBD children (Fig. 3J), but significantly increased as compared to normal children (Fig. 2D). These factors were, however, not all located in the same chondrocytes or chondrocyte clusters in parallel sections. In contrast to the control (Fig. 4J), toluidine blue staining for proteoglycans was absent in KBD cartilage (Fig. 4F).

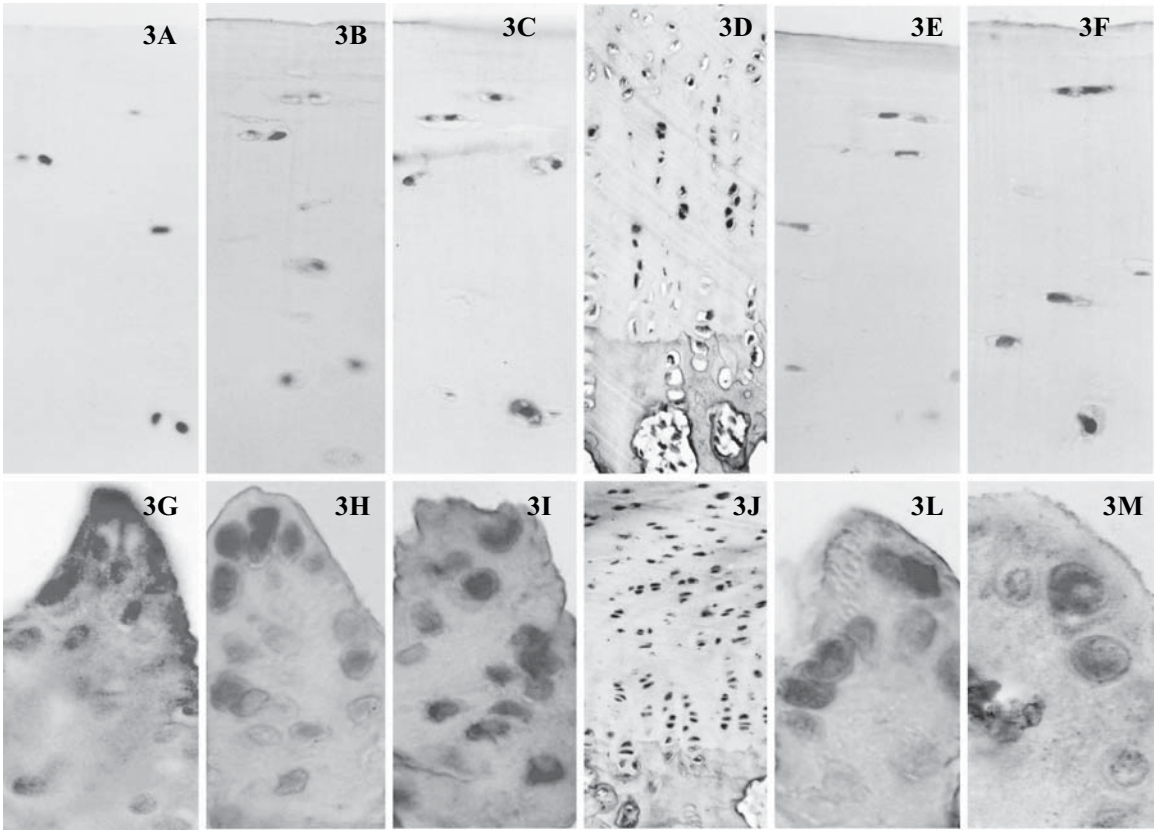
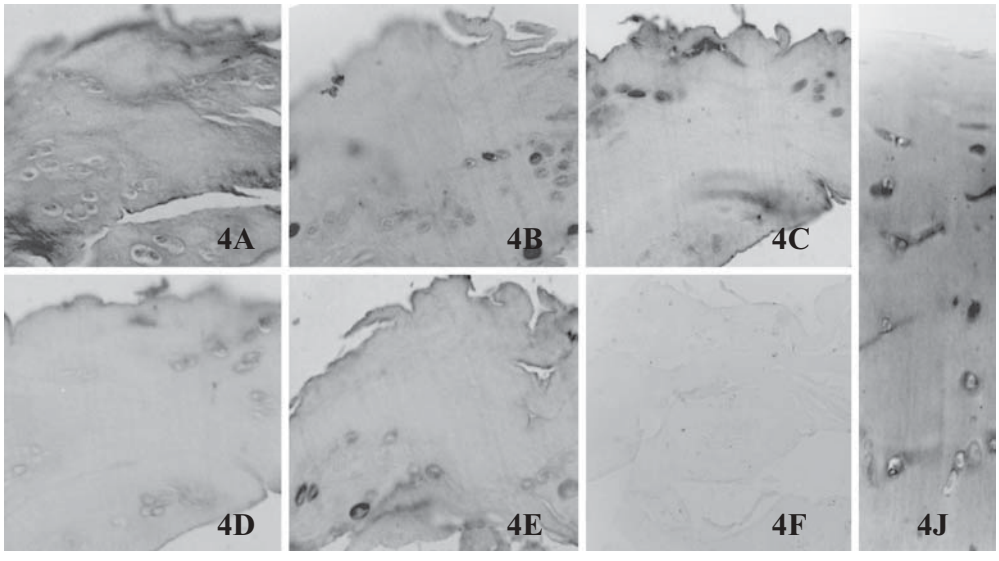


Fig. 3. Immunohistochemical localization of basic fibroblast growth factor (bFGF) (**B**,**H**), parathyroid hormone-related peptide (PTHrP) (**C**,**I**), vascular endothelial growth factor (VEGF) (**E**,**L**), and transforming growth factor-β (TGF-β) (**F**,**M**) in articular cartilage of a healthy donor (40-year-old normal man from non-KBD area) and a KBD patient (43-years old with clinical manifestations of a second-degree KBD, and PTHrP (**D**,**J**) in growth plate cartilage of middle phalanx in the second finger of a healthy donor (6-year-old normal boy from non-KBD area) and a KBD child (7 years old with clinical manifestations of a first-degree KBD). **A**,**G** Hematoxylin and eosin (H&E) staining. **A–C**, **E–I**, **L**,**M** ×400; **D**,**J** ×200

Fig. 4. Pathological changes of articular cartilage in the knee joint of a KBD patient (male, 43 years old) with second-degree KBD. H&E staining (**A**) and immunohistochemical localization of bFGF (**B**), PTHrP (**C**), TGF-β (**D**), and VEGF (**E**). Toluidine blue staining shows the loss of proteoglycans from large areas on KBD cartilage (**F**) and control (**J**; 40-year-old man from a non-KBD area). ×200



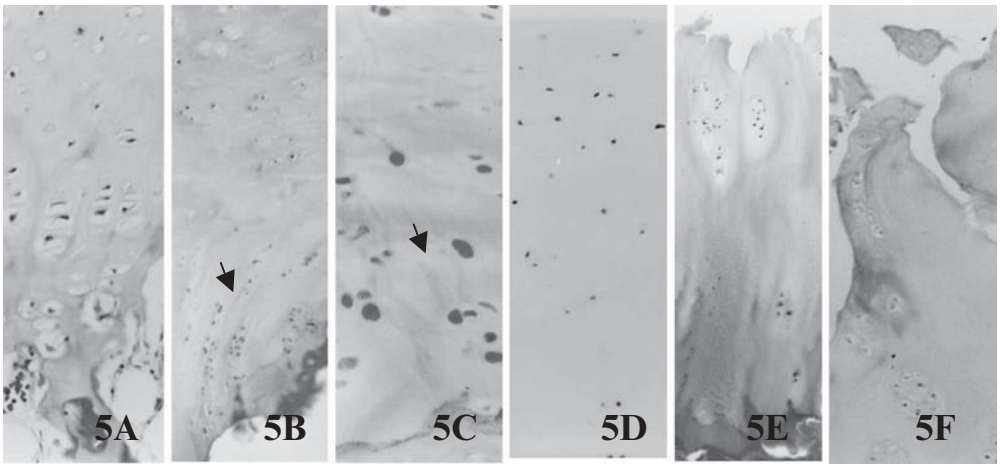


Fig. 5. A Hematoxylin and eosin staining of control growth plate cartilage of finger from a 7-year-old girl. **B**,**C** Chondronecrotic areas (*arrows*) in the growth plate cartilage of fingers from a 7-year-old KBD girl. **D** Hematoxylin and eosin staining of adult control articular cartilage (40-year-old man). **E**,**F** Chondrocyte clusters in the eroded surface of articular cartilage of knee joint from a 45-year-old KBD man. **A**,**B** ×200; **C** ×400; **D–F** ×100

Chondronecrosis and osteoarthritic degenerative changes in growth plate cartilage and articular cartilage of KBD patients

The pathological changes of hyaline cartilage in KBD patients have been described extensively by Mo and Sokoloff [7,8]. Typically, in the growth plate cartilage of KBD children large chondronecrotic areas without cells were observed in the deep zone (arrows in Fig. 5B,C), which was not seen in control cartilage (Fig. 5A). The chondrocyte size was significantly reduced (Fig. 5B), but cell membranes were intact. The nuclei were dissolved, broken, and compressed, associated with red staining in the chondrocyte cytoplasm (Fig. 5C), and acellular fibrillated areas appeared. In contrast to control cartilage (Fig. 5D), chondrocyte clusters (Fig. 5E) and foci of chondronecrosis with red staining plasma, a compressed nucleus, and incomplete cell membrane appeared in the upper and middle zone in the articular cartilage of KBD adults (Fig. 5F).

Histomorphological analysis of Col X, bFGF, PTHrP, TGF-β_1, and VEGF in chondrocytes from KBD cartilage

The percentage of chondrocytes staining intra- or extracellularly for Col X in the deep zone of the growth plate decreased from 63.0% ± 24.2% in the control children to 31.2% ± 15.4% in KBD children ($t = 3.03$, $df = 12$, $P = 0.01$; see Table 1), whereas there was no staining for collagen X in the middle and upper zone in juvenile articular cartilage. In adults, however, the average rates of Col X staining in the middle zone increased from 15.0% ± 11.9% of the control group to 30.9% ± 21.6% in the KBD group ($t = 2.15$, $df = 19$, $P = 0.046$). These findings demonstrate that Col X expression in KBD cartilage is significantly reduced in the deep zone of growth plate cartilage as well as articular cartilage of KBD children.

The histomorphometric analysis of growth factor distribution in KBD articular cartilage revealed striking differences between normal and KBD cartilage, as well as between different zones. In the chondrocyte clusters and the eroded surface in articular cartilage, but also in the deep zone of KBD articular cartilage, the percentage of chondrocytes staining for bFGF, PTHrP, TGF-β_1, and VEGF was significantly higher than in the control ($t = 3.64$–10.34, $df = 12$ for children and 19 for adults, $P = 0.002$–0.0001; see Table 1). The same factors were also enhanced in the middle zone ($t = 4.37$–8.97, $df = 12$ for children and 19 for adults, $P = 0.0001$), except for bFGF. Most striking was the upregulation of VEGF, but also of TGF-β_1 and PTHrP in the deep and middle zone of KBD articular cartilage; this correlated with a high incidence of chondronecrosis in the middle zone (48.5% ± 10.2%) and deep zone (70.6% ± 27.0%) of adult KBD cartilage as compared to the controls (see Table 1). In both child and adult cartilage there were also differences in growth factor staining in the upper zone of articular cartilage, but this was not as significant as in the middle or deep zone. Interestingly, growth factor staining for bFGF, PTHrP, and TGF-β_1 in the upper zone was relatively high in normal children, but lower in adult cartilage, with the exception of bFGF.

Discussion

The basic pathological features of growth plate cartilage and articular cartilage from KBD patients in children and adults are multiple focal chondronecrotic areas in the deep and middle zone and the formation of chondrocyte clusters such as seen in osteoarthritic cartilage. The degenerative changes in KBD cartilage show a number of similarities with osteoarthritis (OA) such as surface fibrillation, chondrocyte clusters, collagen II degradation, and loss of proteoglycans from the matrix. There is, however, usually no indication of chondronecrosis in OA cartilage. Here we have investigated the abnormal differential expression of collagen type X as a marker for hypertrophic differentiation of chondrocytes in relation to the expression of PTHrP, TGF-β_1, bFGF, and VEGF, which are involved in the regulation of chondrocyte differentiation, and report a

Table 1. Percentage of chondrocyte staining for collagen X (Col X), parathyroid hormone-related peptide (PTHrP), transforming growth factor-beta (TGF-β), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF) in articular cartilage and growth plate cartilage of Kashin–Beck disease (KBD) children and adults

Groups	*n*	Upper zone: positive (%)	Middle zone: positive (%)	Deep zone: positive (%)
Col X				
Control children*	5	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	$63.0 \pm 24.2^{a\#\#}$
KBD children*	9	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	$31.2 \pm 15.4^{b\#\#}$
Control adults	6	0.0 ± 0.0^{a}	$15.0 \pm 11.9^{b\#\#}$	$43.0 \pm 8.7^{b\#\#}$
KBD adults	15	0.0 ± 0.0^{a}	$30.9 \pm 21.6^{c\#\#}$	$43.1 \pm 7.2^{b\#\#}$
bFGF				
Control children	5	$21.0 \pm 11.6^{a\#\#}$	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
KBD children	9	6.0 ± 4.9^{b}	12.0 ± 3.8^{b}	$37.0 \pm 21.1^{b\#\#}$
Control adults	6	$19.0 \pm 8.2^{a\#\#}$	15.0 ± 3.3^{b}	1.2 ± 0.9^{a}
KBD adults	15	48.6 ± 11.3^{c}	38.3 ± 8.9^{c}	$10.0 \pm 4.2^{c\#\#}$
PTHrP				
Control children	5	1.4 ± 0.5^{a}	1.2 ± 0.4^{a}	3.0 ± 1.5^{a}
KBD children	9	23.8 ± 17.2^{b}	25.0 ± 4.5^{b}	29.8 ± 9.8^{b}
Control adults	6	5.2 ± 2.5^{a}	0.0 ± 0.0^{a}	3.3 ± 0.8^{a}
KBD adults	15	55.1 ± 7.7^{c}	46.2 ± 11.6^{c}	$16.3 \pm 5.7^{c\#\#}$
TGF-β_1				
Control children	5	$35.2 \pm 7.0^{a\#\#}$	1.6 ± 0.9^{a}	0.0 ± 0.0^{a}
KBD children	9	$52.7 \pm 6.1^{b\#\#}$	39.2 ± 5.0^{b}	33.6 ± 12.6^{b}
Control adults	6	$17.3 \pm 6.4^{c\#\#}$	2.3 ± 0.8^{a}	1.2 ± 0.8^{a}
KBD adults	15	45.5 ± 9.7^{b}	$73.7 \pm 7.2^{c\#\#}$	44.1 ± 10.5^{b}
VEGF				
Control children	5	7.2 ± 1.9^{a}	5.4 ± 1.8^{a}	10.0 ± 1.6^{a}
KBD children	9	66.3 ± 14.1^{b}	77.0 ± 12.0^{b}	$82.4 \pm 12.2^{b\#}$
Chondronecrosis				
Control children	5	0.0 ± 0.0^{a}	3.4 ± 2.0^{a}	5.4 ± 2.5^{a}
KBD children*	9	2.4 ± 1.4^{a}	$28.8 \pm 6.1^{b\#\#}$	$76.6 \pm 12.8^{b\#\#}$
Control adults	6	2.7 ± 1.4^{a}	6.3 ± 2.2^{a}	$9.5 \pm 2.3^{a\#}$
KBD adults	15	9.5 ± 3.7^{b}	$48.5 \pm 10.2^{b\#\#}$	$70.6 \pm 27.0^{b\#\#}$

* Growth plate cartilage

[a,b,c] $P < 0.05$–0.001 if letters are different and $P > 0.05$ if letters are same among groups by independent samples test (*t* test)

[#] Mean difference is significant at 0.05 level by *t* test; [##] Mean difference is significant at 0.01 level by *t* tests

correlation to chondronecrosis in cartilage samples from patients affected with Kashin–Beck disease.

Collagen type X (Col X)

Two representative alterations in Col X expression were observed in KBD cartilage. One was the lower level of Col X expression in the deep zone of growth plate cartilage in KBD children as compared to age-matched normal cartilage. There was an inverse relation between Col X expression and chondronecrosis in the deep zone of cartilage in KBD children, i.e., the lower expression the Col X, the higher the rate of chondronecrosis. The data indicate that the synthesis of Col X in the deep zone of KBD cartilage was impaired, perhaps resulting from chondrocyte necrosis. Another remarkable feature of KBD articular cartilage was that Col X expression extended from the deep zone to the middle zone, indicating that chondrocytes became hypertrophic, similar to osteoarthritic cartilage [9].

Basic fibroblast growth factor (bFGF)

Growth factors of the FGF family and their receptors are involved in almost all steps of chondrocyte differentiation, cartilage matrix synthesis, and skeletal development starting from mesenchymal precursor cells to hyaline and hypertrophic chondrocytes, to endochondral bone formation, in both a stimulatory and inhibitory manner [30]. Basic FGF (FGF-2) can accelerate chondrocyte proliferation, differentiation and matrix synthesis, restrain alkaline leukocyte phosphatase (ALP) excretion, decrease the chondrocyte maturation, and maintain the chondrocyte phenotype in cartilage. In early chondrogenesis, bFGF stimulates chondrogenesis and proteoglycan synthesis [31], whereas it inhibits terminal differentiation of chondrocytes [32] and longitudinal bone growth [33]. On the same line, mutations in the FGF receptor FGFR3 leading to constitutive activity are a major cause of achondroplasia, the most common form of dwarfism [34]. Short stature is also a common feature among KBD patients. Here we show that bFGF expression was strongly enhanced in the middle and deep zones of articular cartilage in KBD children in comparison to normal cartilage and further expanded to the upper zone in KBD adults.

Generally, enhanced levels of extracellular bFGF protein, which does not have a signal sequence to be secreted from the cell through the normal pathway, may be explained either by release from dead cells or by shedding with particles of the broken cell membranes. Thus, bFGF expression in KBD cartilage in zones of high chondronecrosis rates in the deep and middle zones may be, among other factors, responsible for the development of pathological changes in advanced stages of KBD. There are several possibilities how released bFGF could affect cartilage development and cartilage matrix synthesis. First, FGF2 has been shown to activate phosphorylation of Sox9 [35], a major transcription factor required for chondrogenic differentiation and for expression of cartilage-specific collagens II and XI and aggrecan [36]. Second, the low levels of Col X in KBD cartilage may result from enhanced secretion of MMP13 or other collagenases that are unregulated by bFGF. The exact role of bFGF in cartilage maintenance and necrotic degeneration in KBD remains, however, to be investigated.

Parathyroid hormone-related peptide (PTHrP) and transforming growth factor-β_1 (TGF-β_1)

In KBD children and adults, PTHrP and TGF-β_1 were expressed in enhanced amounts in all zones of growth plate cartilage and articular cartilage, with a predominance in the middle and deep zone. In comparison to normal cartilage from non-KBD areas, PTHrP and TGF-β_1 were pronounced, especially in chondrocyte clusters around foci of chondronecrosis. PTHrP has been shown to stimulate chondrocyte proliferation and to delay hypertrophic differentiation and Col X synthesis of chondrocytes in the fetal growth plate [14,37–40].

The role of TGF-β_1 in chondrocyte differentiation and matrix synthesis is more complex; on the one hand, it is able to induce chondrogenic differentiation of chondroprogenitor cells in vitro [41] and in vivo, e.g., in periosteum [17], and to stimulate collagen and integrin synthesis in connective tissue cells [42,43]. On the other hand, it inhibits terminal chondrocyte differentiation [44,45]. Inactivation of the TGF type II receptor in transgenic mice promotes terminal chondrocyte differentiation [46].

In KBD cartilage, TGF-β_1 expression was higher in all zones of articular and growth plate cartilage of KBD adults and children than in healthy controls, but it was absent from areas of chondronecrosis. Most chondrocyte clusters resulting from reactive hyperplasia in the upper and middle zones of KBD adults were positive for TGF-β_1 expression, indicating that TGF-β_1 expression in KBD cartilage is associated with the formation of chondrocyte clusters and repair of degenerating cartilage areas.

Some of the effects of TGF-β may be explained by its stimulatory activity on PTHrP expression, but other effects are PTHrP independent [18]. To what extent the degenerative changes seen in KBD cartilage are related to the upregulation of PTHrP and TGF-β_1 expression shown here remains to be investigated. PTHrP expression was higher in KBD adults with increasing secondary pathological changes than in KBD children with primary cartilage changes. The enhanced PTHrP expression seen in KBD cartilage is similar to OA cartilage, which also has been shown to contain increasing amounts of PTHrP [47].

TGF-β has been shown to upregulate expression of PTHrP in hypertrophic chondrocytes [48] and thus affects chondrocyte differentiation, which is blocked by PTHrP [49]. In comparison to the control, in KBD articular cartilage the expression of PTHrP and bFGF was higher in the middle and deep zones of coinciding with areas of high chondronecrosis; this would be consistent with the ability of TGF-β_1 to upregulate PTHrP and the high PTHrP expression levels in areas of high (TGF-β_1 expression in the upper and middle zones of cartilage where chondrocytes remained at a low maturity level. TGF-β_1 expression in cartilage of KBD children was, however, more widespread than that of PTHrP, especially in the upper zone, with a decreasing tendency from the upper to the deep zone, which was not the case with PTHrP. In addition, TGF-β_1 was expressed in chondrocyte clusters around the chondronecrosis areas, but PTHrP was not, which indicated the action of TGF-β_1 and PTHrP expression may be partially independent and relate to different functions in KBD cartilage.

Vascular endothelial growth factor (VEGF)

Low levels of VEGF expression in articular cartilage of control children are consistent with other studies, as normal hyaline cartilage is avascular. In KBD children, the VEGF expression was strongly enhanced, especially in the deep zone. Similar findings were reported in osteoarthrosis (OA) [50]. In OA, VEGF has been shown to be expressed in the upper and middle zones but not in the deep zone [48]. In contrast to OA, in KBD children VEGF expression was seen in the entire articular cartilage, especially in the deep zones, which is consistent with the distribution of chondronecrosis in the deep to middle zones. VEGF is not only essential for the induction of vascularization of bone but also plays an important role in the differentiation of hypertrophic chondrocytes, osteoclasts, osteoblasts, and endothelial cells [50,51]. Thus, high VEGF levels may induce degenerating and modulating changes following chondrocyte necrosis in KBD cartilage.

In contrast, the lower expression of Col X with chondronecrosis in the deep zone, the higher expression of bFGF in middle and deep zones, and of PTHrP, TGF-β_1, and VEGF in all zones of growth plate cartilage in KBD children were not reported in human OA. With development of the disease and growth with age, the higher expression of Col X in the middle zone, the prominent staining for PTHrP, TGF-β_1, and VEGF in the chondrocyte clusters, and the eroded surface of articular cartilage in KBD adult patients were similar to human OA cartilage.

In conclusion, Col X and bFGF expression was reduced in areas of chondrocyte necrosis in the deep zone of KBD articular cartilage and affected terminal chondrocyte differentiation. PTHrP, TGF-β_1, and VEGF expression were

significantly altered, which indicated degenerative and perhaps regenerative changes in KBD cartilage, similar to OA.

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