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Articular cartilage metabolism in patients with Kashin–Beck Disease: an endemic osteoarthropathy in China

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Summary

Objective: The objective of this study was to investigate CD44 and proteoglycan metabolism in patients suffering from Kashin–Beck Disease (KBD), an endemic osteoarthropathy that affects 2.5 million of 30 million people living in the KBD regions of China.

Methods: Immunohistochemical analyses of cluster of differentiation-44 (CD44), BC-13 and 3-B-3(–) expression were performed in cartilage sections harvested from KBD and normal patients. In addition, the serum levels of soluble CD44 (sCD44), interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and matrix metalloproteinase-1 were determined using a sandwich enzyme linked immunosorbent assay.

Results: Hematoxylin & eosin and toluidine blue staining indicated that there was cell necrosis and proteoglycan loss in cartilage from both KBD children and adult cartilage. Strong immunohistochemical staining for CD44, BC-13 and 3-B-3(–) occurred in the majority of adult KBD patients and most KBD children. Furthermore, statistically significant elevated levels of sCD44, IL-1β and TNF-α were found in the sera of both adult and child KBD patients when compared to the levels of normal adult and child controls. Interestingly, IL-1β and TNF-α serum levels were all high in normal children from KBD regions when compared to normal children from non-KBD regions suggesting that unidentified factors (e.g., a genetic predisposition) may protect some people from KBD pathology.

Conclusion: Our results demonstrate that altered CD44, IL-1β and TNF-α metabolism occurs in the pathogenesis of KBD and there is an increased aggrecanase-generated proteoglycan loss from KBD adult and child cartilage. These primary metabolic changes are likely to be significant contributing factor causing pathological joint formation and instability that leads to secondary osteoarthritis in KBD patients.

Key words: Kashin–Beck Disease, CD44, Aggrecanase, Aggrecan metabolism, IL-1β, TNF-α, Osteoarthritis, Proteoglycan, Osteoarthropathy.

Introduction

Kashin–Beck Disease (KBD) is an endemic, chronic, and degenerative osteoarthropathy, which is manifests primarily in agricultural regions of Asia especially in the central regions of the PR China. KBD often occurs in children and is age-related. The symptoms include joint pain, morning stiffness in the joints, disturbances of flexion and extension in the elbows, enlarged inter-phalangeal joints and limited motion in the middle-sized and large joints of the body (Fig. 1). Pathologically, the epiphyseal plate of cartilage from young and adolescent patients shows necrosis in the hypertrophic layer near the adjacent subchondral bone thus explaining why these young KBD patients often have severe joints' deformities during development[1]. At present the etiology of KBD is unclear. One of the most popular hypotheses is that KBD is caused by fungal mycotoxins on stored food, especially T-2 toxin[2]. Other etiologies occur including selenium deficiency in soil and water in the KBD areas; nutrition deficiency and virus infections. Nonetheless, all of these hypotheses still lack adequate experimental evidence to support their proposal.

Osteoarthritis (OA) is a degenerative joint disorder that predominantly occurs in older people worldwide[3]. It has been suggested several years ago that a high prevalence of primary OA occurs in the KBD areas[4]. Thus, one might hypothesize that, in individuals moving into an endemic KBD area during adult life after growth plate closure, their articular cartilage would be susceptible to damage from the KBD causing agents resulting in the onset of a generalized secondary

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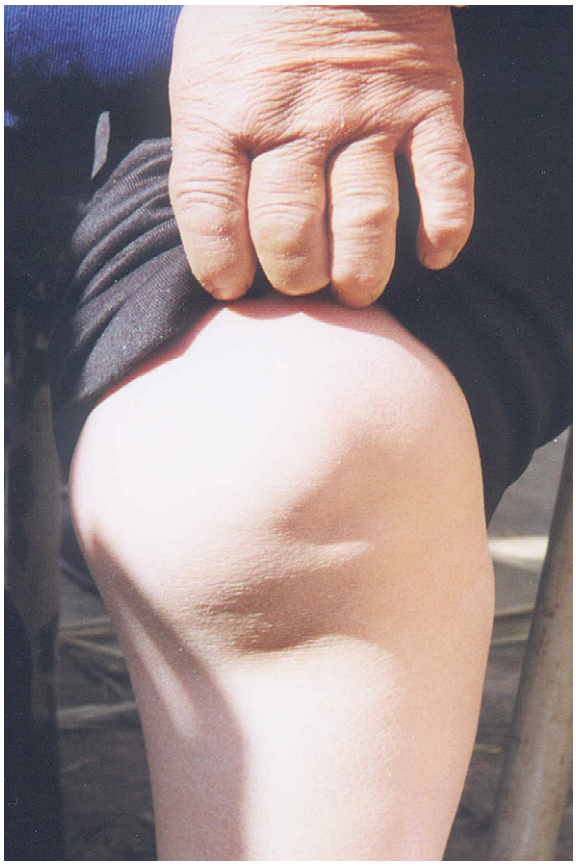

Fig. 1. Enlarged hand and knee joints of KBD patient. The patient was female, 56 years old, and was diagnosed as KBD stage III by X-ray.

OA. Although there have been very few studies investigating this hypothesis, there is a possible relationship between KBD and OA in their pathogenic pathways in spite of their different etiologies.

One of the central pathophysiological features contributing to cartilage erosion during OA is the catabolism and loss of the aggregating cartilage proteoglycan aggrecan[5]. Aggrecan exists as large aggregates in cartilage matrix *via* the interaction of its G1 domain with hyaluronan (HA) and link protein[6–8]. It has been confirmed that the accelerated loss of the aggrecan, and the consequent loss of glycosaminoglycan-bearing aggrecan fragments from articular cartilage, is an early event in the destruction of the articular cartilage seen in the pathogenesis of OA and rheumatoid arthritis (RA)[9]. The loss of aggrecan results in a decrease in functional and structural integrity of the cartilage matrix, which consequently makes the tissue lose its capacity to resist compression under load, eventually leading to irreversible mechanical and further enzymatic destruction of the cartilage[10]. Many proteolytic enzymes are involved in the process including a disintegrin-like and metalloprotease with thrombospondin motifs-4 and -5 (ADAMTS-4 and -5) and matrix metalloproteinases (MMPs). Several studies have identified the aggrecanases as the major proteases responsible for aggrecan degradation and loss from the cartilage at the early stage of degenerative joint disease[5,9,10]. The major proteolytic cleavage site responsible for cartilage pathology occurs in the interglobular domain (IGD) between Glu^{373} and ^{374}Ala[5,10]. Monoclonal and polyclonal antibodies are available to detect the neoepitopes generated at the aggrecanases and MMP cleavage sites in aggrecan[5,10].

Cluster of differentiation-44 (CD44) is a membrane glycoprotein and the primary HA receptor on chondrocytes in articular cartilage. It contains a hydrophobic transmembrane domain followed by a 70-amino acid intracellular domain[11]. The intracellular domain of CD44 contains motifs with the potential for transducing signals and controlling the spatial organization of the receptor in the plasma membrane, which is very important for cell–matrix interactions and proteoglycan retention around the chondrocyte. There is an accumulating evidence suggesting that perturbation of CD44 metabolism occurs during OA pathogenesis, although it is not clear whether this abnormal metabolism is a primary factor or just a consequence of the overall altered cartilage metabolism[12–14].

KBD is a unique endemic arthropathy that appears to have a different etiology when compared with OA, but it does have similar pathological outcome involving cartilage matrix degradation, leading to joint destruction. To date, there have been no studies investigating the metabolism of aggrecan and CD44 in KBD patient articular cartilage. In this study, we found that there was significant aggrecanase-mediated proteoglycan degradation in both adult and child KBD patients and altered CD44 metabolism was also involved in KBD pathogenesis.

Patients and methods

MATERIAL SOURCES

Child KBD cartilage samples were obtained from finger joints from four KBD patients aged from 3 through 7 years old with documented X-ray diagnosis of KBD. These children had died from accidents or other diseases such as bacillary dysentery. The adult cartilage samples were obtained from 16 KBD patients undergoing joint replacement surgery and were aged from 35 through 63 years old. The cartilage samples from the normal children and adults were obtained from patients who had died from clinical problems not involving joint pathology. Ethical approval for acquisition of these patient samples was approved by The Human and Ethical Committee for Medical Research at Xi'an Jiaotong University, School of Medicine (Dr Yong Liu, Director); documents have been provided to the Osteoarthritis Research Society International (OARSI). Ethics Committee with this paper submission. Both pathological (KBD) and normal cartilage samples were obtained within 2–4 h of death and fixed in 4% paraformaldehyde. KBD patient serum samples came from a separate KBD epidemiological survey where participants were subjected to X-ray diagnosis and blood collection. There were 18 KBD children (4–12 years old) samples, 18 normal children from a KBD area (4–12 years old) samples and 20 KBD adult samples (28–55 years old) from KBD areas of Shaanxi Province, PR China. Normal serum samples were obtained from people undergoing routine health examination in a non-KBD region. All of the patients and normal samples were obtained after getting the patient or guardian's consent as authorized by the Ethics Committee of Ministry of Health, Shaanxi Province.

IMMUNOHISTOCHEMICAL STAINING FOR CD44 AND NEOEPITOPES OF AGGRECAN METABOLISM

Cartilage was dissected from the subchondral bone, paraformaldehyde-fixed and then the paraffin-embedded cartilage samples were cut into 6-μm sections and placed on poly-L-lysine-coated glass slides. Immunohistochemical staining was performed with primary antibodies, biotin-conjugated secondary antibodies followed by detection using the strept–avidin–biotin–peroxidase complex (SABC) method (SABC kits: Boster Co, Wuhan, China). Briefly, after deparaffinization, endogenous peroxidase was blocked with 3% H_2O_2 for 15 min and the slides were washed with 0.01 M phosphate-buffered saline (PBS). The slides were then predigested using prewarmed (37°C) 0.1% trypsin (Maixin-Bio Co, Fuzhou, China) or 10 U/ml chondroitinase ABC (Sigma, USA), followed by rinsing with distilled water. After blocked using 10% normal goat serum, the sections were incubated with monoclonal antibodies recognizing either CD44 (Boster Co, Wuhan, China), BC-13 (Abcam, Cambridge, UK) or 3-B-3 (Seikagaku, Japan) or PBS as negative control for 18 h at 4°C followed by incubation with 1:200 biotinylated goat anti-mouse IgG (Boster Co, Wuhan, China). After incubation with the SABC complex, the sections were stained with 3-amino-9-ethylcarbozole. Counterstaining was performed with hematoxylin.

QUANTIFICATION OF CD44 POSITIVE CELLS

The stained slides were examined microscopically. Quantification of CD44 positive cells was performed over several different areas of each section. The percentage of positive cells was evaluated by counting 10 high magnification power fields (40×) in three consecutive tissue sections, using the following semi-quantitative criteria: 0 = negative; 1+ = less than 25% positive staining; 2+ = 25–50% positive staining; and 3+ = greater than 50% positive staining.

INVESTIGATION OF THE SERUM LEVELS OF SOLUBLE CD44 (sCD44) AND CYTOKINES IN KBD PATIENTS

sCD44 analysis was performed using enzyme linked immunosorbent assay (ELISA) kits (R&D Systems, UK) and the serum levels of interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α) and MMP-1 in patients were determined using ELISA kits from Jingmei Biotech Co, Ltd, PR China. These sandwich ELISAs were carried out according to the supplier's protocols and optical densities were determined using an automated reader (Biorad Co, USA). The concentration of sCD44 was determined using Microman2-reader software (Biorad Co, USA).

STATISTICAL ANALYSIS

Data are presented as mean ± S.E.M. Data were checked for normal distribution and equal variance. Student's *t* test for independent samples or one-way analysis of variance was carried out by SPSS 12.0 software. Differences were considered significant at *P* values of less than 0.05.

Results

HISTOCHEMICAL ANALYSES

Hematoxylin and eosin (H&E) staining of cartilage from normal and KBD patients is shown in Fig. 2. In the normal child cartilage, extensive matrix and cellular lacunae can be clearly seen [Fig. 2(A)]. However, in the KBD child articular cartilage, chondrocyte necrosis is seen in the lower hypertrophic layer, where few chondrocytes are found. Interestingly, there are also many cell clusters in areas above the sites of necrosis [Fig. 2(B) and (C)]. The cartilage surface of the normal adult was smooth, and chondrocytes were prevalent with territorial matrix staining around the chondrocyte in the hypertrophic layer and the tide-mark can be clearly seen [Fig. 2(D)]. In contrast, the cartilage from the KBD adult was fibrillated at the surface, and chondrocytes were absent in many areas especially in the deep layer where territorial matrix staining was absent. In addition, cell clusters were present above this area and no tide-mark was evident [Fig. 2(E) and (F)].

An example of toluidine blue staining of KBD child and adult articular cartilage is shown in Fig. 3. Strong toluidine blue staining was present throughout the depth of the normal child and adult articular cartilage [Fig. 3(A) and (D)]. In the cartilage from the KBD child [Fig. 3(B) and (C)], the staining in superficial and middle zones is similar to that seen in the normal child cartilage [Fig. 3(A)]. However, in the deeper hypertrophic layer, there are areas of weak staining, which coincide with the regions of chondrocyte necrosis are shown in Fig. 2(B) and (C). In the KBD adult cartilage, the staining was weak in all zones of the cartilage section compared with that from normal adult cartilage, especially in the superficial zone. In addition, territorial matrix staining is decreased in the areas surrounding the cell clusters above the necrotic zones [Fig. 3(E) and (F)].

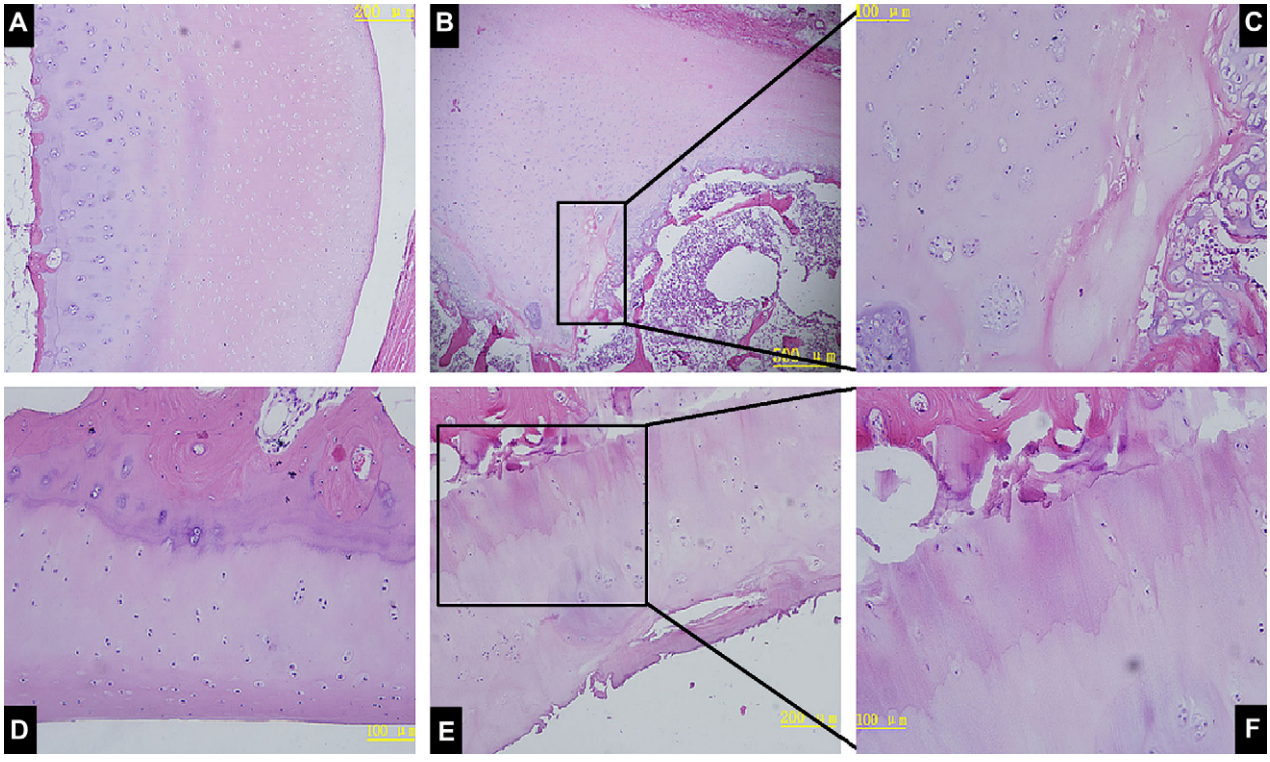

Fig. 2. H&E staining of cartilage from a normal child (A) and normal adult (D), as well as a KBD child (B and C) and a KBD adult (E and F). (A) 12-year-old child with no known history of joint disease. (B and C) 7-year-old KBD patient. There is a band of overt cell necrosis in the hypertrophic layer (B and C), with some cellular cluster formation above it (C). (D) Normal adult without history of joint disease. (E and F) KBD adult cartilage. There is a region with chondrocytes absent above the subchondral bone, and no obvious tide-mark.

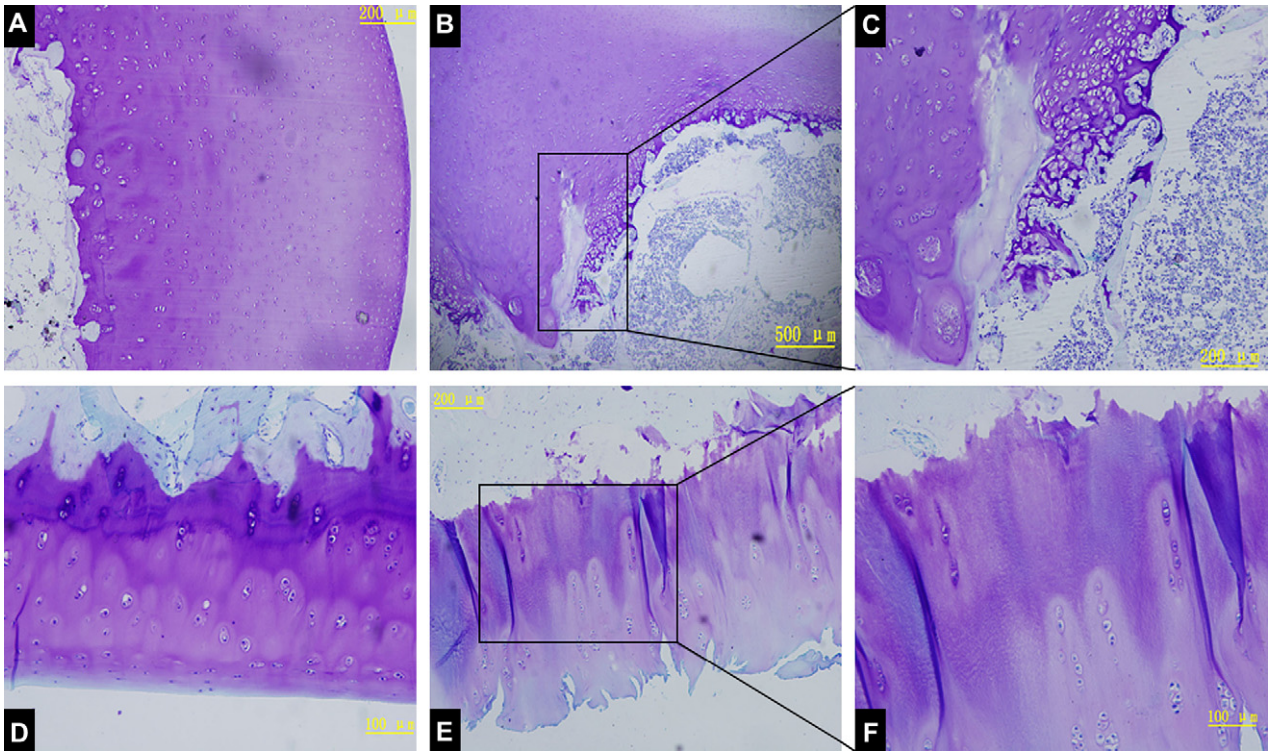

Fig. 3. Toluidine blue staining of the cartilage from a normal child (A) and a normal adult (D), as well as a KBD child (B and C) and a KBD adult (E and F). These sections were generated from the same samples as in Fig. 2. (A) 12-year-old child with no known history of joint disease. (B and C) 7-year-old KBD patient. The presence of areas with cell necrosis and cell clusters is similar to those seen with H&E staining in Fig. 2. In addition, reduced toluidine blue staining is evident in the KBD patient cartilage around the cell clusters above the necrotic regions of both child and adult cartilage.

CD44 IMMUNOHISTOCHEMISTRY

Examples of CD44 immunohistochemical staining in KBD patients and normal cartilage from both adults and children are shown in Figs. 4 and 5, respectively. Strong positive immunostaining for CD44 was only found in KBD patient cartilage from both adults and children. This was predominantly present in the lacunae of the KBD patients with some weak matrix staining. A summary of the analyses for CD44 immunostaining in all of the groups of normal and KBD cartilage (adult and child) was semi-quantified and is presented in Table I. Strong (+++) CD44 positive staining was found in 14 of the 16 adult patients, staining in two samples was moderate and no KBD patients had weak or negative staining (Table I). In contrast to the adult KBD samples, negative (two samples) or weak (two samples) CD44 immunostaining was found in normal adult cartilage sample (Table I). In the cartilage samples from the four KBD children, strong CD44 staining was found in three of them and weak staining in only one sample. Negative staining for CD44 was found in all three normal children cartilage samples that were analyzed (Table I).

sCD44 LEVELS IN NORMAL AND KBD PATIENTS

We also determined the sCD44 levels in KBD patients and normal people. The results of ELISA analyses of serum from patients are provided in Table II. Statistical analysis of these samples showed that the serum sCD44 level in KBD children was significantly higher than that of normal children from a non-KBD area ($P < 0.01$). Serum sCD44 levels from KBD adult patients were increased almost two-fold when compared to that of the normal adults from a non-KBD area ($P < 0.01$). In addition, the serum sCD44 levels from both the normal and KBD children were much higher than that found in the normal and KBD adults, i.e., 1062.01 and 1389 ng/ml compared with 362.72 and 660.88 ng/ml, respectively (Table II).

IMMUNOSTAINING FOR ANABOLIC AND CATABOLIC NEOEPITOPES IN KBD PATIENT ARTICULAR CARTILAGE

Immunostaining for the aggrecanase-generated …NITEGE^{373} neoepitope with monoclonal antibody BC-13 was performed on cartilage obtained from KBD and normal children (Fig. 6). There was no BC-13 positive staining in articular cartilage from a 12-year-old normal child [Fig. 6(A)]. In contrast, intense BC-13 staining occurred in cartilage obtained from a 7-year-old KBD child [Fig. 6(B)]. This BC-13 positive staining was seen in superficial, middle and deep zones, but mainly in the chondrocyte lacunae as well as a weaker staining in the territorial matrix [Fig. 6(B)]. No significant aggrecanase-generated BC-13 positive staining was seen in normal adult cartilage [Fig. 6(C)], but in contrast, there was significant positive BC-13 staining in the matrix of the adult KBD cartilage in particular in the surface zone [Fig. 6(D)].

Immunolocation with monoclonal antibody (mAb) 3-B-3 on normal and KBD children articular cartilage was used without prior chondroitinase predigestion, i.e., 3-B-3(−), see Fig. 7. Under this condition, 3-B-3(−) identifies a "native" chondroitin sulfate (CS) glycosaminoglycan epitope that occurs at the

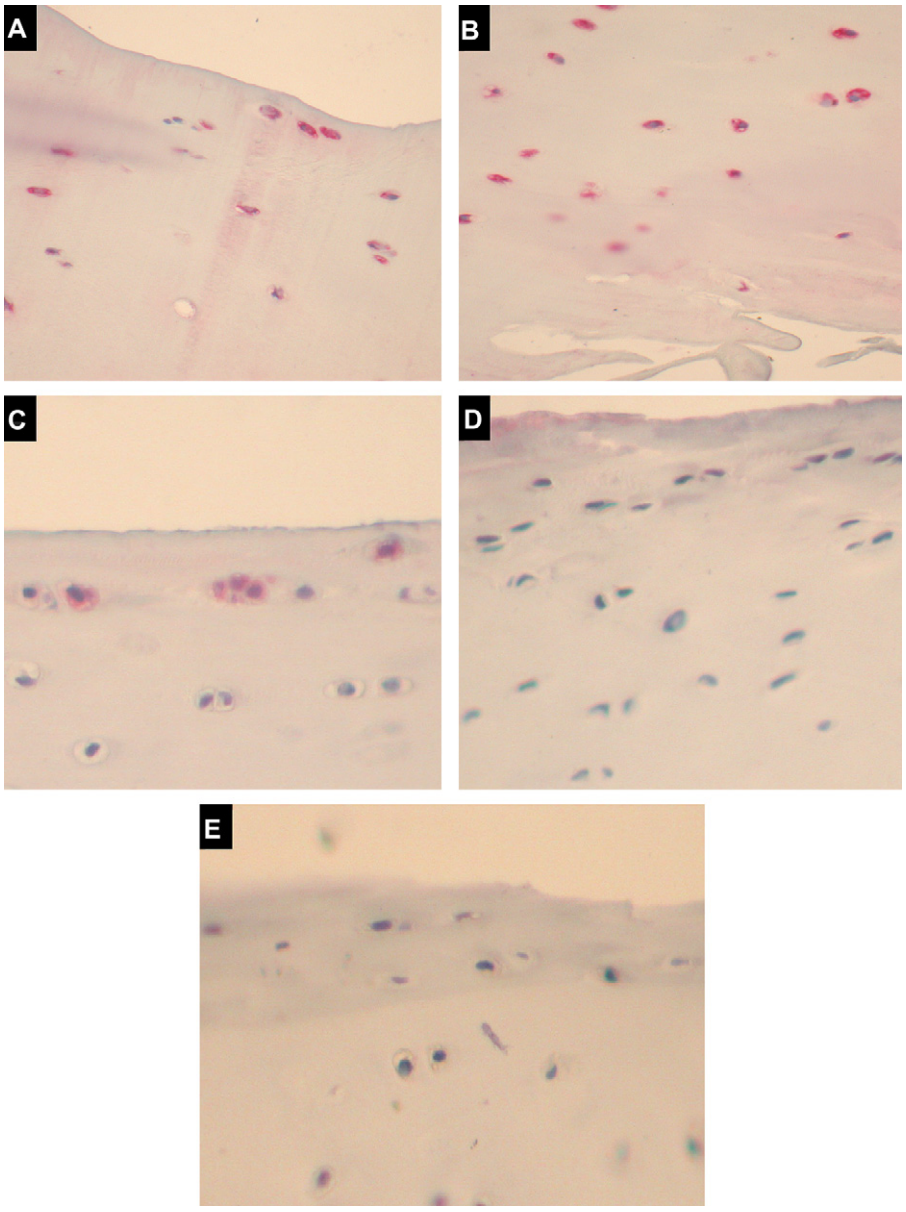

Fig. 4. CD44 immunostaining in normal and KBD adult cartilage (positive staining is red). (A and B) Adult KBD cartilage: A – staining of superficial and middle zones and B – staining of deep zone of KBD articular cartilage. (C and D) Normal adult cartilage: C – superficial zone and D – superficial and middle zones. (E) Negative control with PBS used instead of anti-CD44 primary antibody.

non-reducing terminal of some of the CS chains in newly synthesized aggrecan which is often present in OA cartilage[15,16]. There was no 3-B-3(–) positive staining in cartilage of normal children [Fig. 7(B)]. However, there was intense 3-B-3(–) staining in the surface and middle zones of the cartilage from the KBD child [Fig. 7(A)] with slightly weaker staining in the deep zone.

ANALYSIS OF LEVELS OF IL-1β, TNF-α AND MMP-1 IN SERUM FROM NORMAL AND KBD PATIENTS

ELISA analyses were used to determine the serum levels of IL-1β in normal and KBD patients (Table III). The serum levels of IL-1β in KBD children were significantly ($P < 0.05$) higher than that of the normal children in a non-KBD region. Interestingly, statistical analyses indicated that the serum IL-1β levels in both KBD children and normal children from a KBD region were higher than that found in the normal children from a non-KBD region ($P < 0.05$). However, there was no significant difference in the serum IL-1β levels between the KBD children and the normal children from a KBD region. Similarly, ELISA quantification indicated that the serum IL-1β levels of KBD adult patients were significantly higher than that of normal adults from the non-KBD region ($P < 0.05$).

ELISA quantification for the serum levels of TNF-α in children and adult KBD patients is also shown in Table III. Here, serum TNF-α levels in normal children from a KBD region were the highest and those of control children from a non-KBD region were the lowest. Statistical analyses showed that serum TNF-α levels of both KBD and normal children from a KBD region were higher than that of the

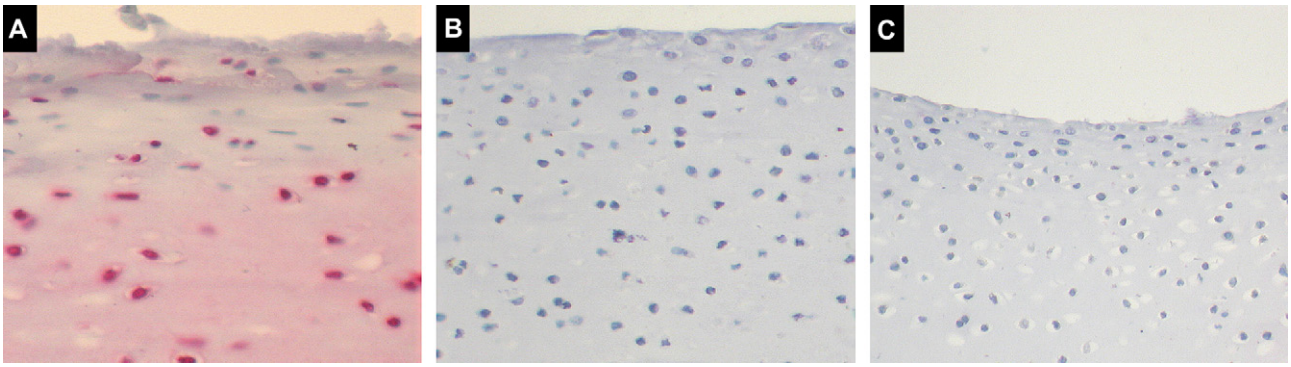

Fig. 5. CD44 immunostaining in KBD and normal child cartilage (positive staining is red). (A) KBD child cartilage where CD44 immunostaining is strong in the chondrocyte lacuna. There is also some weaker staining in the territorial matrix. (B) Normal child cartilage where there is no positive CD44 staining. (C) Negative control with PBS used instead of anti-CD44 primary antibody.

normal children from a non-KBD area ($P < 0.05$). However, there was no significant difference in serum TNF-α level between KBD children and normal children from a KBD region. In addition, the serum TNF-α levels in KBD adult patients were significantly higher than that of normal adults from a non-KBD region ($P < 0.05$).

Finally, serum MMP-1 levels in normal children from the non-KBD region, normal children from KBD region and KBD child patients' sera were also determined by ELISA and the results are shown in Table III. Here, the serum MMP-1 levels in KBD children were the highest and that of the normal children from non-KBD area were the lowest. However, statistical analysis showed that there was no significant difference in serum MMP-1 levels of all three groups ($P > 0.05$).

Discussion

The histochemical, immunohistochemical and biochemical analyses of tissue and sera obtained from KBD patients (children and adult) show several similarities and differences to those found in similar analyses of patients with degenerative joint disease. Histochemical analyses (H&E and toluidine blue) of articular cartilage sections obtained from children and adults KBD patients showed evidence for cellular necrosis and reduced proteoglycan content and surface fibrillation similar to that observed in different stages of the progression of degenerative joint diseases. In general, the presence of cellular necrosis occurred in patches above the subchondral bone, whilst diminished matrix staining occurred throughout all morphological zones of KBD patients' cartilage, which contrasts that generally found in the analyses of osteoarthritic cartilage where a progressive degeneration from the surface to deeper cartilage zones is observed[16]. This decrease in matrix staining was also present around chondrocyte clusters. At present, the mechanisms involved in chondrocyte necrosis in KBD patients are unclear. However, previous studies have shown that the cell apoptosis pathway may be involved in its pathogenesis[17].

Immunohistochemical analyses for CD44 expression in KBD patient and healthy articular cartilage indicated that CD44 was readily detectable around the periphery of chondrocytes in sections from both child and adult KBD patients but this expression was weak or absent in analyses of normal or child cartilage, respectively. This finding suggests that CD44 expression is up-regulated in KBD pathology and may be indicative of increased cartilage matrix metabolism in this disease. This result is similar to that found in the other degenerative joint diseases such as OA and RA where there was an increased expression of CD44 in the cartilage and synovial fluid[18,19]. These immunohistochemical findings are paralleled with the quantification of an increase in the levels of serum sCD44 in KBD patients when compared to that found in normal patients. sCD44 is found in serum and lymph. Its functions are not clear, but many studies have observed increased circulating levels of sCD44 in many diseases[20–22]. Our results show that significant increase in CD44 expression occurred in KBD patient cartilage, in both children and adults. Moreover, sCD44 levels in both KBD children and adult sera were significantly higher than that of normal samples. These results are similar to that seen in analyses of other osteoarthritic conditions[18,19]. The increasing levels of sCD44 in serum may result from two different phases of the disease process. The first occurs when KBD etiological factors directly disturb chondrocyte and/or CD44 metabolism, leading to CD44 shedding from cell surface. The second is that the increased CD44 shedding results from a damage and natural repair

Table I
Immunohistochemical expression of CD44 in normal and KBD cartilage tissues

Level of expression	Adult		Children	
	KBD	Normal	KBD	Normal
Negative	0	2	0	3
+	0	2	1	0
++	2	0	0	0
+++	14	0	3	0

Table II
Serum sCD44 levels (ng/ml) in normal and KBD patients

Group	*N*	sCD44 level ($\overline{X} \pm s$)
Normal children in non-KBD region	18	1062.01 ± 72.84
KBD children	18	1389.86 ± 55.62*
Normal adult in non-KBD region	20	362.72 ± 85.16
KBD adult	20	660.88 ± 200.50†

*$P < 0.01$, comparison with normal children in non-KBD region.
†$P < 0.01$, comparison with normal adult in non-KBD region.

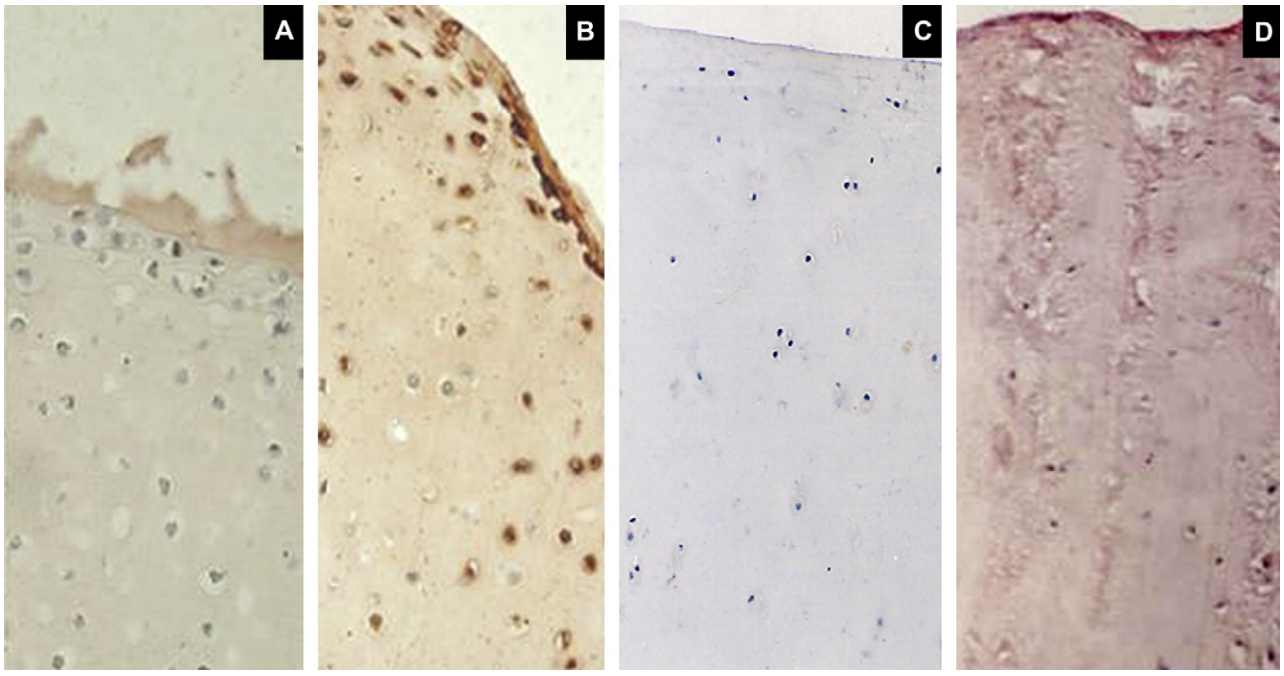

Fig. 6. Immunostaining for the aggrecanase-generated BC-13 neoepitope in cartilage obtained from KBD patients and normal people. (A) Finger joint cartilage from a 12-year-old child with no known history of joint disease. (B) Finger joint cartilage from a 7-year-old KBD patient. (C) Cartilage obtained from normal adult. (D) Cartilage obtained from an adult KBD patient. There is a significant BC-13 positive staining in the chondrocyte lacuna of the KBD child but no positive staining is evident in normal child cartilage. Similarly, there was no positive staining in the normal adult cartilage but significant BC-13 staining in the matrix of the KBD adult cartilage section.

mechanisms. Whether the increased sCD44 levels in KBD patients came from the first or the second phase or both of them is not clear at this point in time but our results indicated that CD44 metabolism is altered in KBD patients which may profoundly affect HA and proteoglycan metabolism. Interestingly, sCD44 levels in serum of normal children from KBD region were similar with that of KBD children from same region, and both of these were significantly higher than that of normal children from a non-KBD region. This result indicates that perturbed CD44 metabolism occurs in normal children from the KBD region although KBD pathology is not evident in all of those affected.

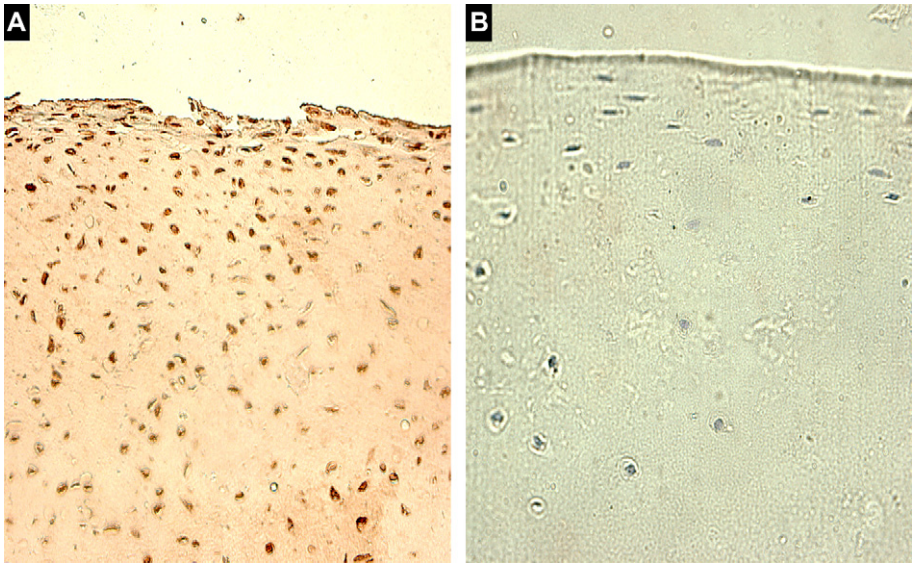

Fig. 7. Immunostaining for 3-B-3(−) CS glycosaminoglycan mimotope in cartilage obtained from KBD and normal children. (A) Finger joint cartilage from a 3-year-old KBD patient and (B) Finger joint cartilage from a 12-year-old child with no known history of joint disease. There is no positive staining in the normal child cartilage, but significant positive 3-B-3(−) staining is seen in the chondrocyte lacuna and weaker matrix staining in the KBD child cartilage.

Table III
Serum IL-1β, TNF-α and MMP-1 levels in normal and KBD patients

Group	*N*	IL-1β level (pg/ml) ($\overline{X} \pm s$)	TNF-α level (pg/ml) ($\overline{X} \pm s$)	MMP-1 level (ng/ml) ($\overline{X} \pm s$)
Normal children in non-KBD region	27	5.74 ± 2.79*,†	18.76 ± 5.35*,†	0.89 ± 0.26**
Normal children in KBD region	18	31.06 ± 6.94*	171.43 ± 32.40*	1.09 ± 0.64††
KBD children	18	68.96 ± 16.72†	88.09 ± 18.08†	1.18 ± 0.68
Normal adult in non-KBD region	20	2.49 ± 0.41‡	7.06 ± 0.83‡	N/A
KBD adult	20	4.82 ± 0.49‡	12.10 ± 1.10‡	N/A

*$P < 0.05$, normal children in a KBD region compared to normal children in a non-KBD region.
†$P < 0.05$, KBD children in a KBD region compared to normal children in a non-KBD region.
‡$P < 0.05$, KBD adult in a KBD region compared to normal adult in a non-KBD region.
**Only 18 normal children samples in non-KBD region were analyzed.
††Only 11 normal children samples in KBD region were analyzed.

We also investigated the occurrence of evidence for the presence of the aggrecanase-generated catabolic neoepitopes on aggrecan catabolites in KBD patient cartilage using mAb BC-13[5]. During the matrix damage–repair process, many proteolytic enzymes such as aggrecanases and MMPs are involved in cartilage matrix degradation. BC-13 is a monoclonal antibody used to identify the "catabolic neoepitope" …NITEGE^{373} generated after aggrecanase catabolism within the IGD of aggrecan. In this study, immunolocation results indicated that …NITEGE^{373} was present in both KBD child and adult cartilage but absent or only weakly expressed in normal child and adult cartilage. This finding indicates that there was active aggrecanases-mediated aggrecan degeneration in KBD cartilage, which is similar to that seen with OA. This study is the first to demonstrate that proteolytic cleavage within the IGD of aggrecan by aggrecanases is involved in the pathogenesis of KBD.

The occurrence of anabolic neoepitopes generated from aggrecan metabolism in tissue sections from normal and KBD patients was also investigated. Expression of the 3-B-3(−) non-reducing terminal disaccharide neoepitope indicates the presence of CS glycosaminoglycan chains on newly synthesized proteoglycan, which is a marker of altered repair or remodeling of the cartilage extracellular matrix in degenerative joint disease[6,15,16]. In our analysis, we found intense positive 3-B-3(−) staining in KBD child cartilage, which suggests that the chondrocytes are attempting to replace and repair proteoglycan loss caused by KBD pathogenesis and/or its unknown etiologies.

In addition to serum levels of sCD44 we also investigated the serum levels of the inflammatory cytokines (IL-1β and TNF-α) and the MMP-1 in KBD patients, normal patients from a KBD region and normal 'control' patients from an area not affected by KBD etiologies. Both IL-1β and TNF-α are cytokines which induce cartilage degradation in OA and RA[23,24]. As expected, IL-1β and TNF-α levels in both KBD children and adults were higher than that of the normal group from a non-KBD area. This result is similar to that reported in previous studies on adult KBD patients[25], where higher serum TNF-α levels were also observed in the adult KBD group when compared to the normal group from a non-KBD area; however, there was no difference in serum TNF-α levels between the adult KBD patients and the normal adults from a KBD area. Collectively, these results indicate that these two cytokines are likely to be involved in the pathogenesis of KBD. Interestingly, the IL-1β level in normal children from the KBD region was significantly higher than that of normal children from the non-KBD region (almost six times higher); however, the levels were lower than that of the KBD children from same area (i.e., 31.06 pg/ml vs 68.96 pg/ml). These results are also similar to the sCD44 results presented earlier and suggest that normal children from KBD regions are also affected by factors present in their KBD area environment, although for some reason overt KBD pathology does not occur in these children. Thus, there must be some unknown factors (e.g., a genetic predisposition) that protect these 'normal' children from the manifestation of KBD pathology.

In summary, for the first time, we have demonstrated that CD44 expression and metabolism increase in the cartilage isolated from both KBD children and adult patients. Furthermore, serum sCD44 levels from both KBD children and adult patients are higher than that found in normal people. These two results indicate that disturbed CD44 metabolism occurs in KBD pathogenesis. Also, for the first time, we have found that there is an increased level of proteoglycan degradation in both children and adult KBD cartilage, and that aggrecanases are implicated in this process. In addition, both IL-1β and TNF-α are also involved in the pathogenesis of KBD. Interestingly, several of our results suggest that some of the inhabitants of KBD areas are protected from the manifestation of KBD pathology.

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