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A molecular and histological characterization of cartilage from patients with Morquio syndrome

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Summary

Objective: To investigate the gene expression profile and the histological aspects of articular cartilage of patients affected by Morquio syndrome, a lysosomal storage disease characterized by the accumulation of glycosaminoglycans within the cells which result in abnormal formation and growth of the skeletal system.

Method: Articular cartilage samples were obtained from the femoral condyle of two siblings with Morquio syndrome during surgery performed to treat valgus knee. As controls, four biopsy samples of healthy cartilage were obtained from four different male multiorgan donors. A Real-Time Polymerase Chain reaction (RT-PCR) analysis was performed to evaluate the expression of type I and II collagens and aggrecan mRNAs. Histological and immunohistochemical analyses for some matrix proteins were carried out on paraffin embedded sections.

Results: Type I collagen mRNA mean level was higher in the samples of patients with Morquio syndrome compared to controls. Type II collagen and aggrecan mRNAs' mean expression was instead lower. The morphological appearance of the cartilage showed a poorly organized tissue structure with not homogeneously distributed cells that were larger compared to normal chondrocytes due to the presence inside the vacuoles of proteoglycans which were not metabolized. Chondrocytes were negative for collagen II immunostaining while the extracellular matrix was weakly positive. Collagen type I immunostaining was positive at cellular level. Keratan sulfate showed diffuse positivity and chondroitin-6-sulfate was present throughout the cartilaginous thickness.

Conclusion: In cartilage of patients with Morquio syndrome, a low expression of collagen type II and a high expression of collagen type I both at protein and molecular levels are evidentiated. This finding could give evidence of the reduction in ankle and knee joint movement observable in these patients.

Key words: Glycosaminoglycans, RT-PCR, Keratan sulfate, Chondroitin-6-sulfate.

Introduction

Morquio syndrome, also known as Morquio–Brailsford syndrome belongs to mucopolysaccharidoses. It was so called by the physician who first described this pathology in a Swedish family[1,2]. Also called mucopolysaccharidosis type IV A (MPS IV A), it is an autosomal recessive inborn error resulting from the deficient activity of the lysosomal enzyme *N*-acetylgalactosamine-6-sulfatase (GALNS)[3–5]. From a biochemical point of view MPS IV A is different from type B which is caused by the deficiency of the β-galactosidase enzyme[6,7]. GALNS is one of the sulfatases required to degrade glycosaminoglycans, such as keratan sulfate and chondroitin-6-sulfate[8]. To date, over 90 different mutations have been identified including around 70 missense mutations. Genotype/phenotype correlation exists for some of these mutations; most deletions lead to the severe form of MPS IV A, while point mutations produce a broad range of phenotypes[9–12]. These vary from the classical form with bone dysplasia, spinal deformity, cardiac abnormalities, corneal opacities, deafness and dental abnormalities to attenuated forms with fewer manifestations[13]. Compared to other types of mucopolysaccharidoses, visceral organs and mental development in Morquio disease are normal. There are very few data in the literature reporting histological and/or molecular evaluations in this pathology. Some arthroscopy findings suggest that pathologic alterations are at the cartilage–bone junction[14]. We had the opportunity to receive biopsies from two brothers with Morquio syndrome who had undergone surgery to correct valgus knee and we used immunohistochemical analyses to look at the morphological aspect of the cartilage tissue. We performed, for the first time in this pathology, a Real-Time PCR analysis to evaluate the gene expression profile

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of the main molecules of the extracellular cartilaginous tissue.

Materials and methods

PATIENTS

Cartilage biopsies were obtained from two siblings (one man and one woman; 35 and 38 years old, respectively) affected by the more severe form of Morquio syndrome as diagnosed at Istituto Giannina Gaslini (Genova, Italy)[15]. The patients were both homozygous for the same mutation of the gene which encodes for the GALNS enzyme. This mutation is responsible for the lack of enzyme activity through the exon-2 skipping which contains the catalytic site of the enzyme. It was shown that the proximity of exon-2 at the mutation site leads to this alternative splicing[15]. The biopsies were obtained from the femoral condyle of the knee during surgery performed to treat valgus knee. As controls, four biopsy samples of healthy cartilage were obtained from four different male multiorgan donors (aged 20, 47, 35, and 38) who did not have a known history of arthritis or other joint pathology. Informed consent was obtained from the patients and the work was approved by the Ethical Committee of Istituti Ortopedici Rizzoli.

CHONDROCYTE ISOLATION

The chondrocytes were isolated from the biopsies by enzymatic procedure as previously reported[16]. Briefly, fragments of the cartilaginous tissues were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Paisley Scotland) with 25 mM *N*-(2-Hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid); 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) (HEPES) (Sigma), penicillin–streptomycin 10,000 U/ml and 10,000 μg/ml, respectively (Gibco), and 50 mg/ml gentamycin (Biological Industries, Kibbutz Beth Haemek, Israel). The chondrocytes were isolated by sequential enzymatic digestions: 30 min with 0.1% hyaluronidase (Sigma, St Louis, MO, USA), 1 h with 0.5% pronase (Sigma) and 1 h with 0.2% collagenase (Sigma) at 37°C. The isolated chondrocytes were filtered by 100 μm and 70 μm sterile nylon mesh filters to remove cell raft and matrix debris. The filtrate was then centrifuged for 10′ at 1800 rpm and the pellet was washed twice with DMEM supplemented with 10% fetal calf serum (FCS, Biological Industries, Kibbutz Beth Haemek, Israel). The cell number and viability were assessed by vital staining with eosin.

ANALYSIS OF CARTILAGE-SPECIFIC MOLECULE EXPRESSION BY REAL-TIME RT-PCR

RNA extraction and reverse transcriptase

5×10^5 isolated cells were pelleted and lysed in 0.5 ml of RNAzol B reagent (Biotecx Laboratories, Houston, TX, USA); total RNA was subsequently isolated according to the manufacturer's instructions. Complementary DNA was synthesized from 1 μg of total RNA per sample with 45 min incubation at 42°C, using Moloney murine leukemia virus reverse transcriptase (Perkin Elmer, Norwalk, CT, USA) and oligo-(dT) priming.

Primer design

PCR primer for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as an internal control was obtained from published Refs.[17–19]. PCR primers for types I and II and aggrecan were designed using the PRIMER 3 software (Steve Rozen, Helen J, Skaletsky 1998 Primer 3) http://www.genome.wy.mit.edu/genome_software/other/primer3.html. Specific primer pairs, PCR products' length, annealing temperatures and references are reported in Table I.

LightCycler Real-Time PCR

Real-Time PCR was run in triplicate in a LightCycler Instrument (Roche, Applied Science) using the QuantiTect™ SYBR® Green PCR Kit (Qiagen, GmbH, Germany) with the following protocol: initial activation of HotStarTaq™ DNA Polymerase at 94°C for 15 min, 45 cycles of 94°C for 15 s, 56–60°C for 20 s and 72°C for 10 s.

To determine absolute mRNA copy numbers, standard curves were generated for collagens I and II and aggrecan using 10-fold dilution series of gel-purified PCR products. The increase in PCR product was monitored for each amplification cycle by measuring the increase in fluorescence caused by the binding of SYBR Green I dye to dsDNA. The crossing point values (i.e., the cycle number at which the detected fluorescence exceed the threshold value) were determined for each standard and sample and specificity of the amplicons was confirmed by melting curve analysis. Collagens I and II and aggrecan mRNA levels were normalized to the housekeeping gene GAPDH.

Table I
Real-Time RT-PCR primers' description

RNA template	Primer sequences	Amplicon size (base pairs)	Annealing temperature (°C)	References*
Aggrecan	5′-TCG AGG ACA GCG AGG CC 3′-TCG AGG GTG TAG CGT GTA GAG A	85	60	PRIMER 3
Type I collagen	5′-AGG TGC TGA TGG CTC TCC T 3′-GGA CCA CTT TCA CCC TTG T	105	58	PRIMER 3
Type II collagen	5′-GAC AAT CTG GCT CCC AAC 3′-ACA GTC TTG CCC CAC TTA C	257	58	PRIMER 3
GAPDH	5′-TGG TAT CGT GGA AGG ACT CAT GAC 3′-ATG CCA GTG AGC TTC CCG TTC AGC	190	60	17–19

*Primer sequences were obtained from published references where indicated or designed using PRIMER 3 or LightCycler Probe Design Software.

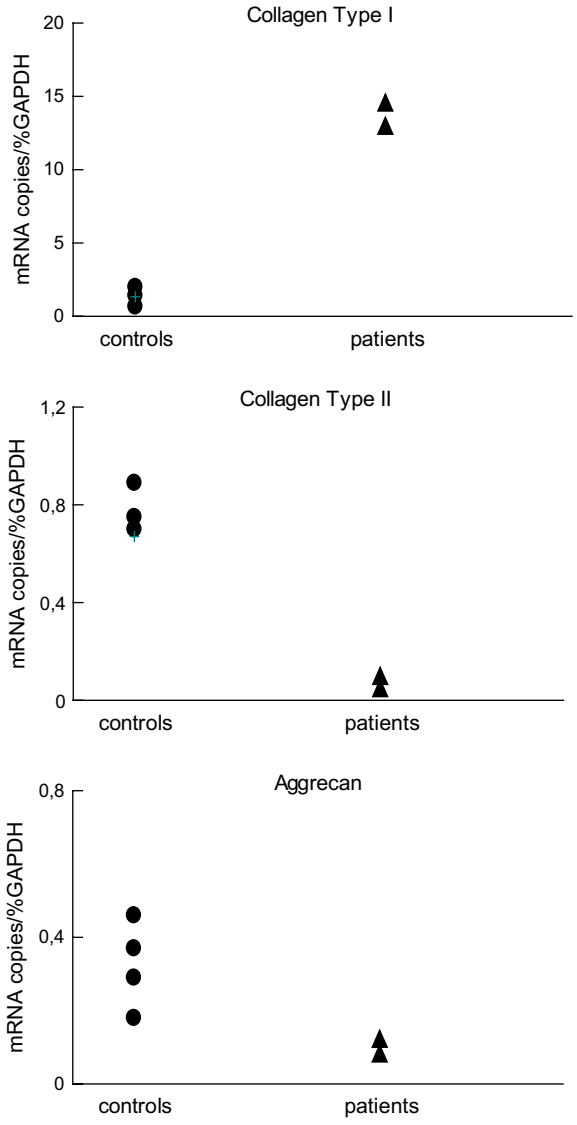

Fig. 1. Real-Time PCR analysis of collagens I and II and aggrecan mRNAs' expression in chondrocytes isolated from patients with Morquio syndrome and from controls. Data were normalized to GAPDH and expressed as mRNA copies/%GAPDH.

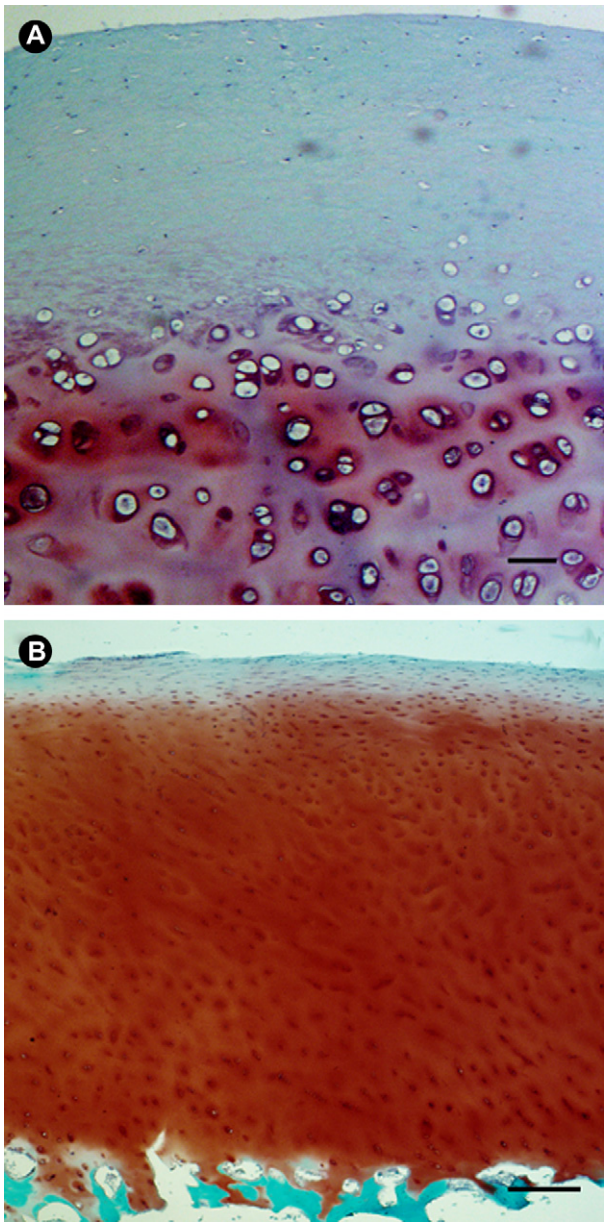

Fig. 2. Representative patient light microscopy evaluation after Safranin-O staining showing a poorly organized morphological structure with larger and not homogeneously distributed cells both in the superficial layer and in the deep zone (A) (bar = 200 μm) compared to normal hyaline cartilage (B) (bar = 320 μm).

HISTOCHEMISTRY

Biopsies for histological analysis were fixed in 10% buffered formalin, washed and decalcified with Formical-2000 (Decal Chemical, Congers, NY, USA) for 2 h at room temperature. The samples were then dehydrated through a graded series of alcohol and embedded in paraffin. Sections, 4 μm thick, were obtained from the cartilage specimens, and the slides were stored at room temperature. The slides were stained with 0.001% Fast Green and 0.1% Safranin-O (Sigma) and 1% Alcian Blue 8GX (Sigma).

IMMUNOHISTOCHEMISTRY: TYPE I AND II COLLAGENS

For these immunohistochemical analyses, the following primary antibodies were used: mouse monoclonal anti-human type I collagen (Chemicon International, Temecula, CA, USA); anti-human-collagen type II mouse monoclonal antibody (Chemicon International). Paraffin sections were deparaffinized and rehydrated. For epitope unmasking the samples were treated with 0.1% hyaluronidase (Sigma) in phosphate buffered saline (PBS) at 37°C for 5 min. After washes, the slides for the detection of type I and II collagens were incubated at room temperature for 30 min in 1X PBS containing 5% of normal goat serum (NGS) (Dako, Carpinteria, CA, USA) to prevent non-specific bindings. The slides were incubated with the anti-human type I and II collagen primary antibodies diluted 1:20 in 0.04 M

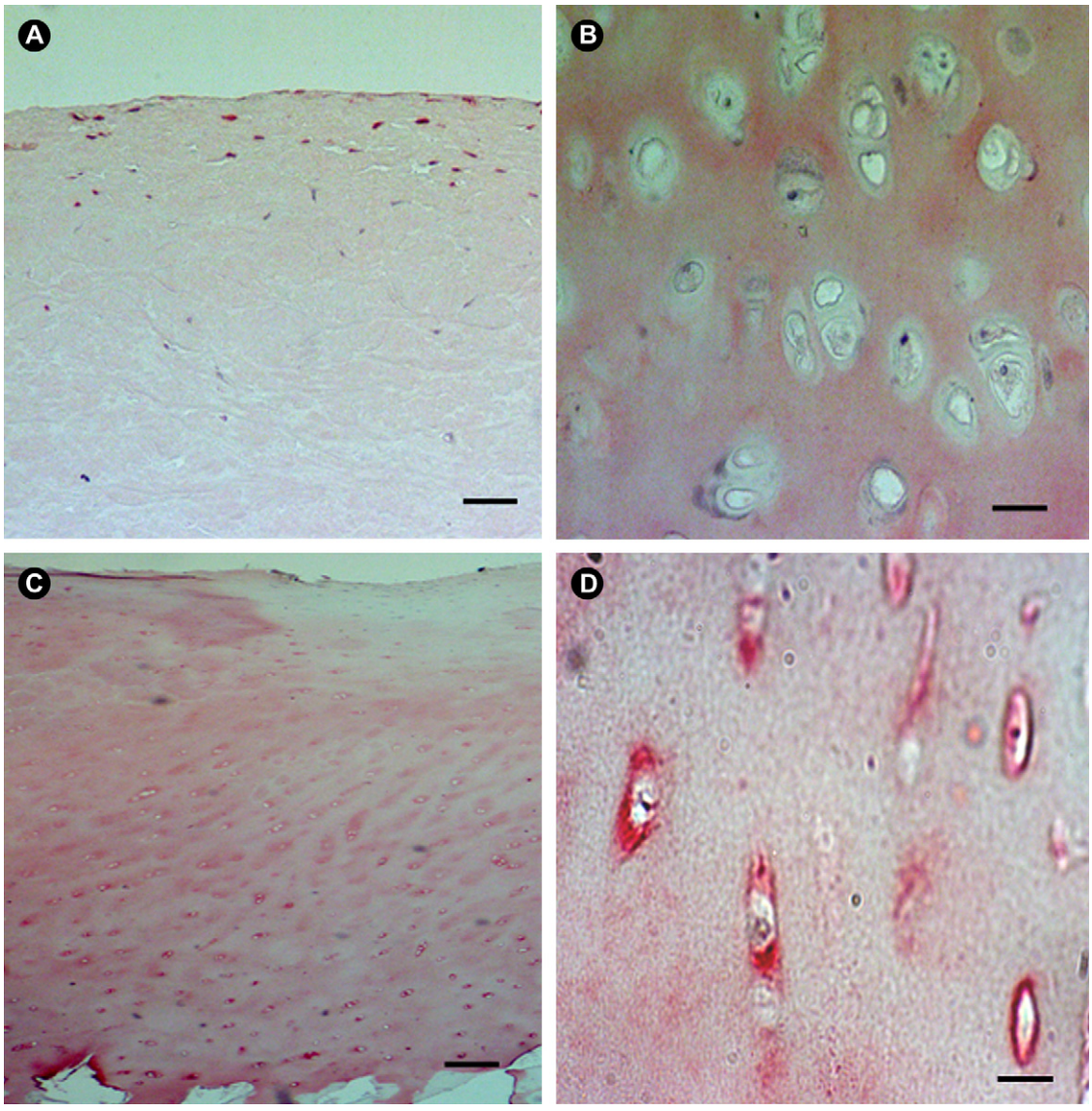

Fig. 3. Immunostaining for collagen type II in cartilage from a patient with Morquio disease shows a weak positivity which is confined to the extracellular matrix of both the superficial layer (A) (bar = 200 μm) and the deep zone while the cells are negative (B) (bar = 500×). The healthy tissue shows a positivity which is evident both for the extracellular matrix (C) (bar = 160 μm) and the cells (D) (bar = 500×). Collagen type II was developed using new fuchsine (red is positive stain).

trizma base saline (TBS) pH 7.6 containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 for 1 h at room temperature. The slides were washed three times with 0.04 M TBS pH 7.6 and incubated with goat anti-mouse and anti-rabbit immunoglobulins labeled with dextran molecules-alkaline phosphatase (Envision, Dako, Carpinteria, CA, USA) at room temperature for 30 min. After three washes with 0.04 M TBS pH 7.6 the reactions were developed using the new fuchsin kit (Kit New Fuchsin Substrate System, Dako, Carpinteria, CA, USA) in the presence of 5 mM Levamisole (Sigma) to block endogenous alkaline phosphatase. Negative staining controls were performed either by omitting the primary antibody or using a control isotype-matched antibody. Slides were counterstained with hematoxylin and mounted in glycerol gel. All the samples were analyzed using a Zeiss Axioscope Microscope (Carl Zeiss, Oberkochen, Germany).

IMMUNOHISTOCHEMISTRY: TOTAL PROTEOGLYCANS

For immunohistochemical staining of proteoglycans tissue samples were deparaffinized and rehydrated, then epitopes were unmasked by 20 min deep in 0.02 U/ml Chondroitinase ABC in Tris−HCl pH 8 at 37°C (Sigma). After washes in 0.04 M TBS, tissue sections were blocked with 5% NGS in 1X PBS, 30 min at room temperature. The slides were then exposed to monoclonal mouse anti-adult cartilage proteoglycans (Chemicon) antibody diluted 1:50 in 0.04 M TBS + 1% BSA for 1 h at room temperature. The antibody recognizes short peptides on the core protein of proteoglycans of human articular cartilage. After washes, incubation with goat anti-rabbit/mouse labeled with alkaline phosphatase (Envision, Dako) was performed and finally the reactions were developed using the new fuchsin kit in the presence of 5 mM Levamisole (Sigma) to block

endogenous alkaline phosphatase. Negative staining controls were performed either by omitting the primary antibody or using a control isotype-matched antibody. The slides were counterstained with hematoxylin and mounted in glycerol gel.

IMMUNOHISTOCHEMISTRY: CHONDROITIN-6-SULFATE AND KERATAN SULFATE

After the samples had been deparaffinized and rehydrated, the epitopes were unmasked by 20 min deep in 0.02 U/ml Chondroitinase ABC in Tris–HCl pH 8 at 37°C (Sigma). After the washes and the block of non-specific binding, as reported above, an incubation of 90 min at 4°C with anti-chondroitin-6-sulfate and anti-keratan sulfate antibodies (Chemicon) diluted 1:100 in 0.04 M TBS + 3% normal rabbit serum (NRS) was performed. After further washes, a Rabbit Anti-Mouse TRITC (Tetramethyl-rhodamine-isothiocyanate, Dako) conjugated secondary antibody was added to the slides for the detection of chondroitin-6-sulfate, while a Rabbit Anti-Mouse FITC (Fluoresceine isothiocyanate) conjugated secondary antibody was added to the slides for the detection of keratan sulfate. Both the antibodies were diluted 1:40 in 0.04 M TBS + 3% NRS for 30 min at room temperature. Negative staining controls were performed either by omitting the primary antibody or using a control isotype-matched antibody. After two washes the slides were mounted with an anti-fading solution (1,4 diazobicyclo 2.2.2. octane, Sigma) and visualized with a fluorescent microscope (Zeiss Axioscope microscope, Carl Zeiss).

Results

SPECIFIC GENES mRNA EXPRESSION

Real-Time PCR allowed rapid and sensitive detection of the extracellular matrix molecule mRNAs evaluated. Type I collagen mRNA levels were higher in the patient samples analyzed with respect to controls. Type II collagen mRNA expression was lower in the patient samples than in the controls. Aggrecan mRNA showed lower levels compared to controls (Fig. 1).

HISTOCHEMISTRY

Cartilage from patients with Morquio syndrome [Fig. 2(A)] showed a poorly organized morphological tissue structure, both in the superficial layer and in the deep zone. The cells were not homogeneously distributed and larger than normal chondrocytes [Fig. 2(B)], due to the presence of not metabolized proteoglycans inside the vacuoles. The collagen fibers were tangentially oriented in the superficial layer of the tissue but thicker. The tidemark was not well evident.

IMMUNOHISTOCHEMISTRY

In the cartilage from Morquio patients chondrocyte cells were positive for collagen II staining at superficial layers and the extracellular matrix was weakly positive [Fig. 3(A) and (B)], while control samples showed a strong positivity both at cellular [Fig. 3(C)] and extracellular levels [Fig. 3(D)]. Conversely, collagen type I immunostaining was positive at cellular level in both the superficial and deep zones (Fig. 4). Total proteoglycans were highly positive inside the cells and in the extracellular matrix (Fig. 5). This positivity was more intense compared to healthy controls (data not shown). Keratan sulfate showed a diffuse positivity (Fig. 6), as observed for chondroitin-6-sulfate, while the controls showed a light positivity (data not shown). It was possible to observe a weak positivity also in the healthy controls, mainly localized in the fundamental substance of the intermedial zone and the pericellular one (data not shown).

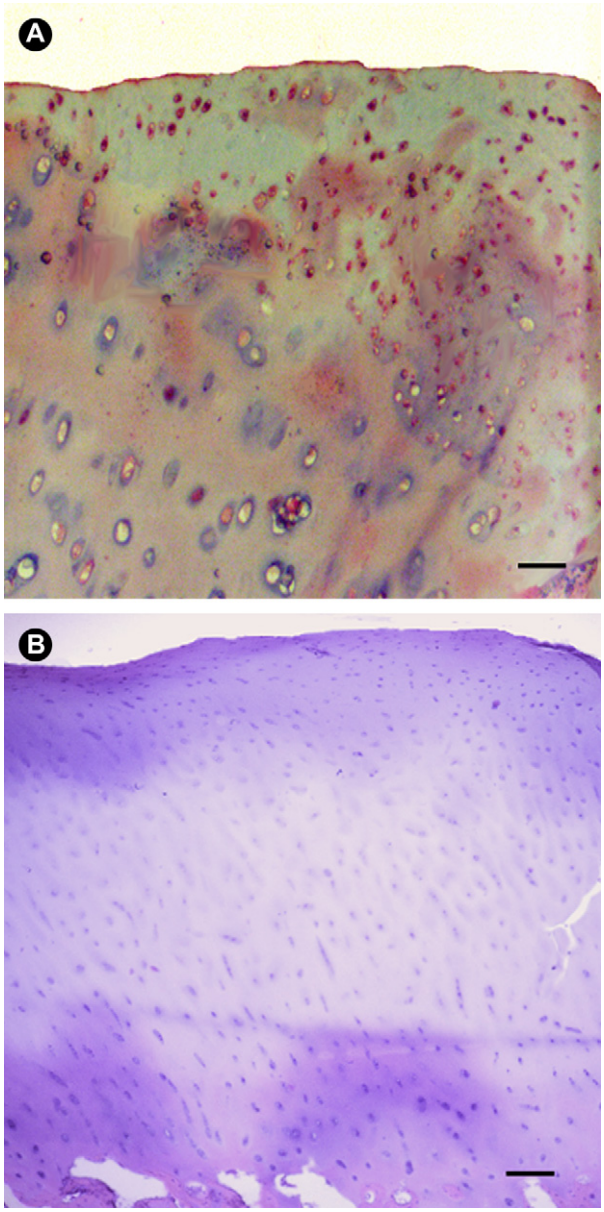

Fig. 4. Immunostaining for collagen type I shows a great positivity at cellular level both in the superficial and in the deep zones (A) (bar = 200 μm). The control tissue is negative (B) (bar = 320 μm). Collagen type I was developed using new fuchsine (red is positive stain).

Discussion

The data obtained in our study showed proteoglycan accumulation in the articular cartilage of patients suffering from Morquio disease. This was particularly evident for

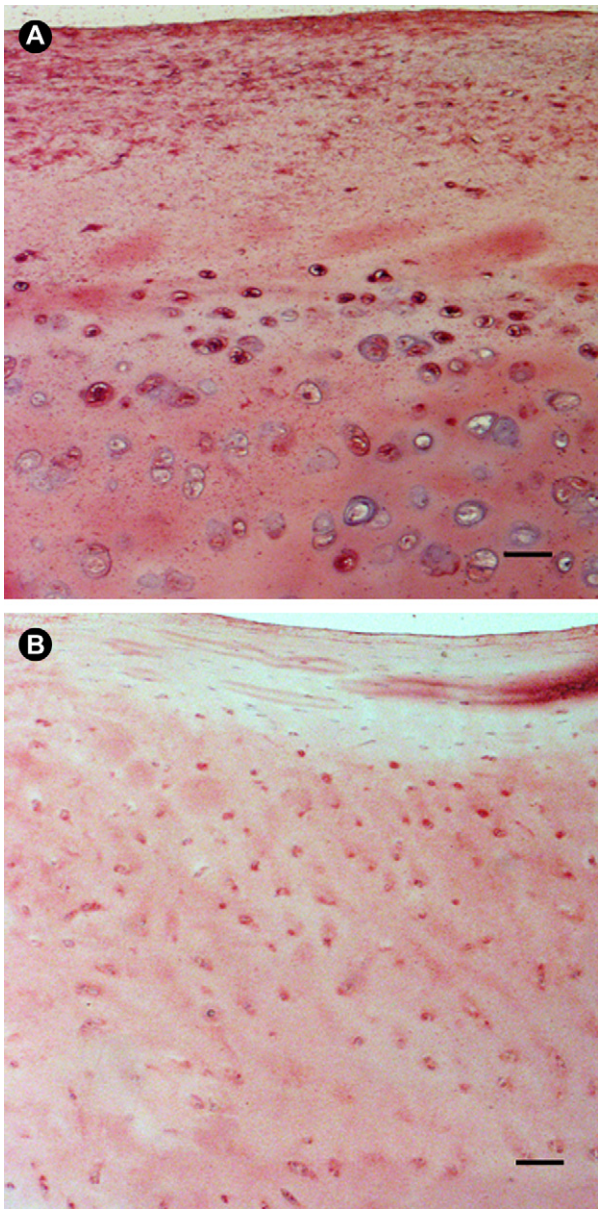

Fig. 5. Immunostaining for total proteoglycans is highly positive inside the cells and in the extracellular matrix also at the superficial layer (A) (bar = 200 μm). The control shows a normal staining and the superficial layer is almost negative (B) (bar = 200 μm). Proteoglycans were developed using new fuchsine (red is positive stain).

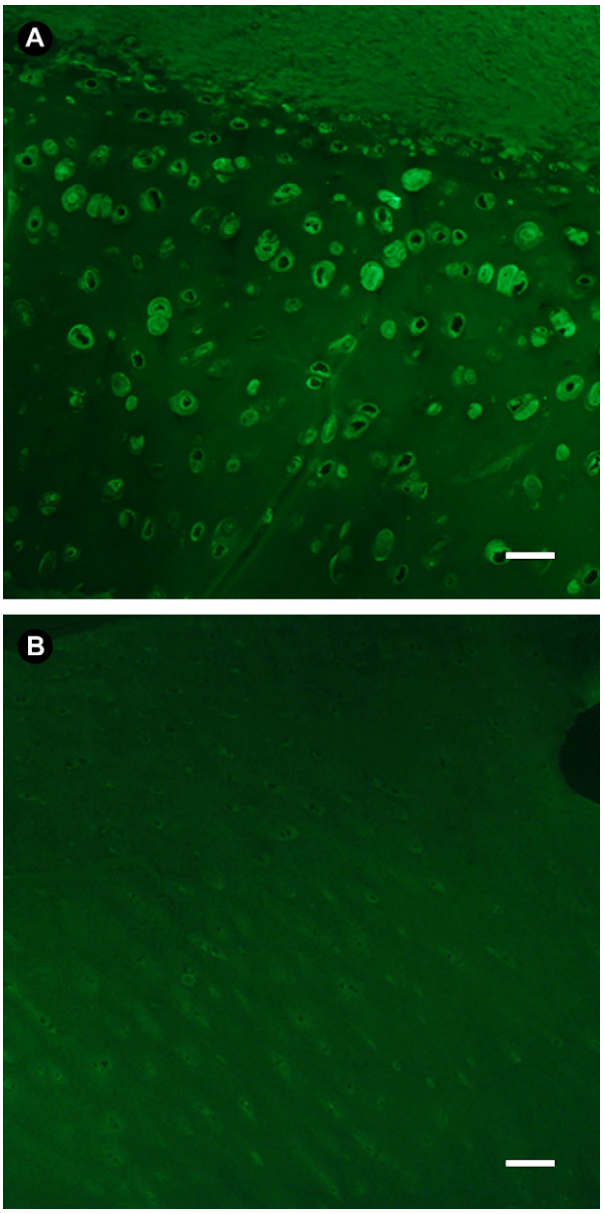

Fig. 6. Immunostaining for keratan sulfate showed a diffuse positivity (A) (bar = 200 μm), while the control is negative (B) (bar = 200 μm). The image was acquired under green fluorescence and shows only those nuclei that are positive.

keratan sulfate and chondroitin-6-sulfate, which are highly present not only in the deep zones of the tissue, but also at the superficial layers, where they are usually scarcely represented. This accumulation may be responsible for some of the problems that affect people with the severe form of the disease[20]. For a good performance of the articulation, in fact, proteoglycan concentration must be maintained constant[21]. The molecular and histological features found in our patients were in agreement with the genetic investigations that have shown the presence of a mutation of the gene which encodes for GALNS[22], the enzyme responsible for the catabolic pathway of proteoglycans. The immunohistochemical analysis, which showed that the accumulation was localized in particular inside intracellular vacuoles (the site of the GALNS enzyme), supported this hypothesis. However, molecular evaluation showed that the expression of aggrecan mRNA was lower compared to that of healthy controls. This is not unusual since the homeostasis of the cartilaginous tissue is regulated by a feedback mechanism in which chondrocytes increase the proteoglycans' production after loss of matrix[23]. On this same basis, the cells might downregulate the expression of the gene which encodes for aggrecan following an increase in proteoglycans. An interesting finding was also the low expression of collagen type II both at protein and molecular levels. As known, collagen type II is universally recognized as molecular marker of hyaline cartilage phenotype, since it is the main constituent among cartilage

collagens and gives this tissue biomechanical properties, such as tensile strength and traction forces[24]. The reduction in ankle and knee joint movement, which is observable in patients with Morquio disease, is therefore due to several factors and not only to proteoglycan accumulation. The high amount of collagen type I and the scarce presence of collagen type II fibers might be one of the causes. In Morquio disease an inversion of the characteristic cartilaginous pattern can be seen, with a higher percentage of collagen type I compared to type II.

The histological and molecular analyses performed in this study increased our knowledge about the etiopathogenesis of MPS IV, highlighting the involvement of other factors besides those already demonstrated. This could open new therapeutic approaches as an alternative to surgery, which is currently the only way to treat the skeletal abnormalities caused by this pathology. In particular, on the basis of recent significant advances in recombinant DNA technology, gene therapy, the insertion of genes into the cells or tissues to modulate gene expression[25], may represent in the future an interesting approach to treat patients affected by Morquio syndrome.

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