Osteoarthritis and Cartilage (1999) **7,** 498–501
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Article No. joca.1999.0245, available online at http://www.idealibrary.com on IDEAL®
1063–4584/99/090498+04 $12.00/0

Osteoarthritis and Cartilage
Journal of the OsteoArthritis Research Society International

Short Communication

Type X collagen in rabbit and human meniscus

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Key words: Meniscus, Osteoarthritis, Type X collagen.

Introduction

In the knee, menisci are important fibrocartilaginous structures contributing to distribute the weight across the joint. The major collagen of the meniscus is type I, but types II, III, V and VI collagens are minor constituents.[1] In addition, a recent study has shown type X collagen deposition in mouse articular cartilage and menisci, particularly in degenerating menisci.[2] Type X collagen was originally thought to be restricted to hypertrophic chondrocytes of the growth plate,[3] but more recent studies have demonstrated the occurrence of type X collagen at the surface of normal human, porcine and rat articular cartilage,[4] in the fracture callus of normal canine articular cartilage[5] and in human osteoarthritic cartilage.[6] Moreover, type X collagen has been identified in human intervertebral disc, particularly during degeneration.[7] Taken together, these results suggest that type X collagen may contribute to the structural maintenance of articular and fibrocartilage.

Osteoarthritis (OA) is a common disease that affects the joints. It occurs frequently in the knee, where both articular cartilage and meniscus show degenerative lesions. So far, most studies on OA have focused on articular cartilage; only a few attempts have been made to survey evolution of meniscus, particularly in animal models. In the first place, this report provides the evidence that type X collagen is a natural component of human and rabbit meniscus and extends the recent identification of this protein in mouse meniscus.[2] To supplement our knowledge of type X collagen in human non hypertrophic cartilage, and its possible involvement in OA, we used Western blotting and RT-PCR analyses to study type X collagen expression in normal menisci and in degenerative menisci from OA patients. Indeed, our data revealed the presence of this protein in normal and OA menisci. Furthermore, RT-PCR analysis performed on RNA extracted from normal and OA menisci showed an increase in type X collagen expression in the degenerative menisci.

Received 28 October 1998; accepted 4 March 1999.
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Methods

RABBIT AND HUMAN SAMPLES

Rabbit samples: Rabbits *Gris de Champagne* were 8 months of age and skeletally mature. Animals were killed and menisci that looked macroscopically normal were analysed.

Human samples: three menisci (A, C, D) were obtained from individuals (aged 42, 27, 36 years, respectively) operated for an anterior cruciate ligament surgery. These menisci looked macroscopically normal. One normal meniscus in appearance (E1) and one meniscus displaying degenerative lesions (E2) were taken from the same articulation of a 74-year-old patient undergoing total knee replacement. Three menisci with degenerative lesions (B, F, G) were collected from patients aged >70 years undergoing total knee replacement. The articular cartilages of these patients were osteoarthritic in appearance.

The rabbit and human samples were frozen into liquid nitrogen and powdered with mortar and pestle, for protein or RNA extraction.

REVERSE TRANSCRIPTION-PCR (RT-PCR)

Total RNA was isolated by using a modification of the guanidinium isothiocyanate (GIT) procedure.[8] After phenol-chloroform extraction and precipitation, the pellet was resuspended in 1M GIT with 200 μg/ml Proteinase K, and incubated at 40°C until complete dissolution. The GIT concentration was then adjusted to 4M and the sample was layered on a cushion of cesium trifluoroacetate (CsTFA) with a density of 1.6 g/ml, for ultracentrifugation. For the reverse transcription, a 40 μl reaction contained 1.5 μg of total RNA, 24 μM oligo-dT primers, 125 μM each dNTP and 400 units of reverse transcriptase (SuperScript II RNAse H-; GIBCO BRL). The reaction was carried out at 42°C for

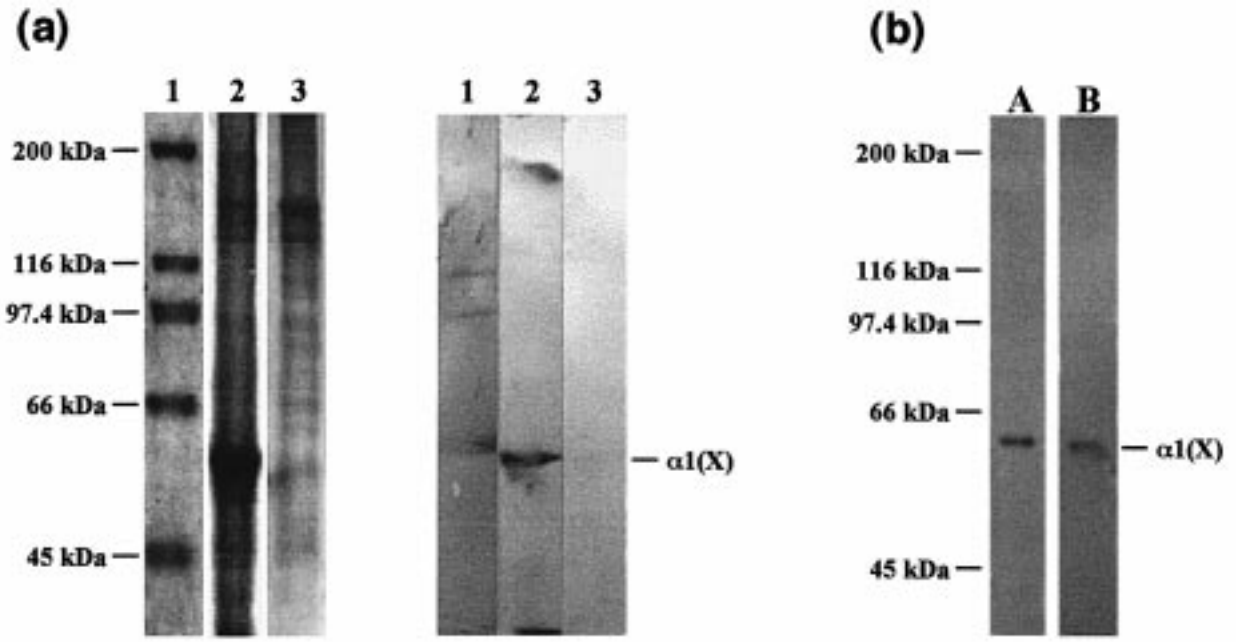

Fig. 1. (a) On the left: SDS-8% polyacrylamide gel electrophoresis of rabbit meniscus proteins stained with Coomassie blue. A molecular weight marker is shown on lane 1. After GuCl extraction, solubilized proteins were precipitated with 3M NaCl (lane 2). The unsolubilized residue was pepsinized, collagens were separated by salt fractionation and the 0.86M NaCl fraction is shown on lane 3. On the right: Western-blotting of a gel equivalent to that shown on the left, with a polyclonal antibody against deer type X collagen. In the GuCl fraction (lane 2), note the presence of a unique band reactive with the antibody and migrating with a molecular weight corresponding to α1(X) chains. The same results were obtained with menisci from four different rabbits. (b) Western-blotting of proteins solubilized in GuCl and isolated from a normal human meniscus (A) and from a degenerative meniscus of an OA patient (B), with a monoclonal antibody against human type X collagen.

50 min, and 70°C for 15 min. For the PCR amplification, a 50 μl reaction contained 5 μM each dNTP, 0.8 μM each specific primer, 2.5 units of Taq DNA polymerase (Perkin Elmer), 10 μl RT reaction for MMP-1 and 2 μl RT reaction for the other markers. Specific primers were designed for amplification of type X and type II collagen, MMP-1, MMP-3, TIMP-1 and GAPDH (see Fig. 2a). Thirty to forty cycles were employed, with denaturation at 94°C for 1 min, annealing at optimal temperature (see Fig. 2a) for 30 s, and extension at 72°C for 45 s. The PCR products were electrophoresed in a 2% agarose gel and the bands were stained with ethidium bromide and visualized under ultraviolet light. The PCR products eluted from the gels were cloned in a TA cloning vector (Invitrogen, San Diego, CA) and sequenced.

COLLAGEN EXTRACTION AND WESTERN BLOTTING

Frozen and powdered tissues were extracted in 4M guanidinium chloride (GuCl) buffer. The remaining residue was washed and treated with pepsin at 1:10 (w/w pepsin to tissue wet weight). After 24 h with stirring at 4°C, the pepsin digests were centrifuged and the collagens recovered in the acidic solution were separated by NaCl fractionation.[9]

The proteins were resolved by SDS-PAGE. Gels were stained with Coomassie blue or transferred to nitrocellulose membrane for Western blotting with the anti-type X collagen antibodies: (i) rabbit fractions were allowed to react with a polyclonal antibody raised in mice against deer type X collagen, a gift of Dr G. J. Gibson (Breeche Research Laboratories, Detroit, MI); (ii) human fractions were allowed to react with a mouse monoclonal antibody (X53) against human type X collagen.[10] The membranes were then incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA). Following several PBS-Tween washes, the membranes were treated with an avidin–biotin horseradish peroxydase complex (Vectastain ABC kit, Vector Laboratories), washed with PBS-Tween and developed with a 4-chloro-1-naphtol/H_2O_2 solution.

Results/Discussion

Previous observations have shown the presence of types I, II, III and V collagens in menisci of 8-month-old rabbits.[1] Our Western-blotting analysis revealed that at the same stage, type X collagen is also a normal constituent of rabbit meniscus (Fig. 1a). Experimental OA is commonly performed in rabbit and this reproducible model should help to clarify the role of this collagen in the normal and degenerative meniscus. Furthermore, we detected by immunoblotting type X collagen in one normal and in one OA human meniscus (Fig. 1b), and this prompted us to analyse type X collagen expression in other human menisci.

For our preliminary analyses of gene expression in human menisci, we used classical protocols described in the litterature for RNA extraction from cartilage. We obtained extremely low yields of RNA and our PCR amplification reactions were poorly reproducible, most likely because of contamination by remaining proteoglycans. We found that a treatment by proteinase K in 1M GIT prior to CsTFA ultracentrifugation, was crucial to increase the yield and purity of RNA. This led indeed in reproducible patterns of PCR after reverse transcription of minute amounts of

(a)

Molecule	Primers	Strand	Location	Product size	Annealing temperature	Source
Type X collagen	GCCTGAGGGTTTTATAAAGG	+	1505–1525	501 bp	53°C	emb X72580
	TTAGCTCTGTGGGGTGTAC	−	2006–1988			
Type II collagen	ATCGACATGTCCGCCTTTGCT	+	130–150	618 bp	53°C	emb X06268
	TGCCCGGATCTCCACGTCATT	−	747–727			
MMP-1	GGTATGATGAATATAAACG	+	1264–1282	205 bp	47°C	emb X54925
	CTGCAGTTGAACCAGCT	−	410–394			
MMP-3	GGCCATCTCTTCCTTCAG	+	1041–1058	400 bp	52°C	gb J03209
	GTCACTTTCTTTGCATTTGG	−	1440–1421			
TIMP-1	TACACCCCCGCCATGG	+	315–330	260 bp	49°C	emb X03124
	GTCCACAAGCAATGAGTG	−	574–557			
GAPDH	ATCACTGCCACCCAGAAGAC	+	601–620	443 bp	57°C	gb M33197
	ATGAGGTCCACCACCCTGTT	−	1043–1024			

(b)

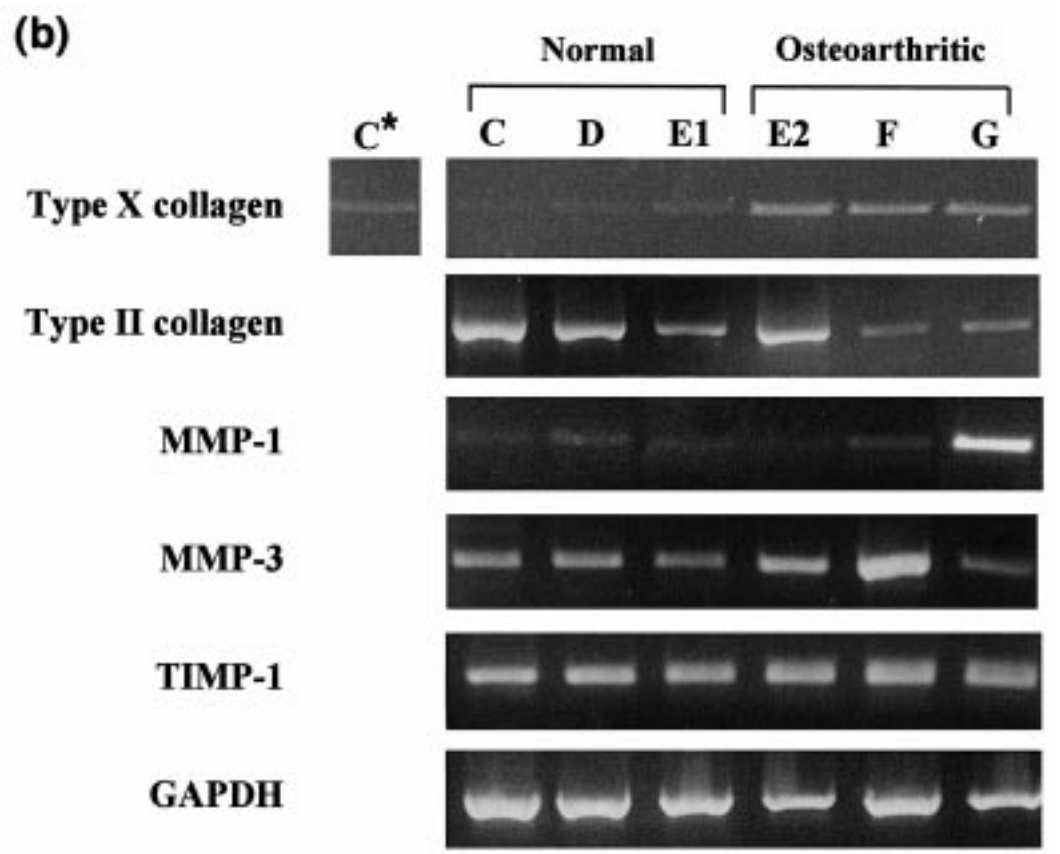

Fig. 2. (a) Oligonucleotide primers used for comparative PCR analysis. Primers are presented in a 5′ to 3′ orientation, with that for the coding strand (+) being above that for the non-coding strand (−). The location of each primer within the corresponding cDNA sequence is indicated, together with the product size generated by reverse transcription and PCR amplification of the mRNA. The annealing temperature used for each primer set, and the source of the databank used for designing the primers, are also listed. (b) RT-PCR analysis of mRNA expression in normal human menisci (C, D) and in degenerative menisci from OA patients (F, G). E1 represents a normal meniscus and E2 a degenerative meniscus isolated from the same articulation of an OA patient. After ultracentrifugation, total RNA was digested with RNAse-free DNAse, phenol-chloroform extracted, precipitated and reverse-transcribed into cDNA, followed by a PCR reaction using specific primer pairs listed in Fig. 2a. The gene products analyzed are indicated on the left. The specificity of the amplified products was confirmed by automatic nucleotide sequencing (ESGS, France). Type X collagen expression was barely detectable in C, C* represents a PCR reaction started with a 5 fold volume of RT reaction in comparison with C.

RNA, which is particularly convenient for human samples difficult to obtain in high quantities.

In the view of determining variation of a given message between normal and degenerative menisci, message for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used to ascertain that an equivalent amount of cDNA was synthesized from the different tissues. This is based on the assumption that the message for this housekeeping gene remains relatively constant on a per cell basis. We indeed obtained nearly the same level of message for the GAPDH gene, as judged by ethidium bromide staining (Fig. 2b). The PCR signals obtained after amplification of the type X collagen message were stronger in the OA menisci in comparison with the normal menisci, and interestingly, this difference was observed between two menisci of the same articulation (see Fig. 2b, samples E1 and E2). Although our RT-PCR reactions were performed under qualitative rather than quantitative conditions, our results suggest that type X collagen expression increases with degeneration of meniscus, and this is in accordance with the strong immunohistochemical staining of type X collagen observed in menisci of mice harboring OA.[2] At the same time, type II collagen was clearly detected in all samples, attesting the cartilage nature of these tissues. In order to gain more information on the phenotype of the menisci under study, we further compared gene expression of two matrix metalloproteinases (MMPs) thought to be involved in cartilage degradation. MMP-3 was expressed in all samples whereas MMP-1 gene expression was barely detectable, except in one OA meniscus where the PCR signal was clearly higher (see Fig. 2b, sample G). These results are in accordance with other observations showing that these MMPs can be differentially regulated in human OA articular cartilage.[11,12] Furthermore, we found that the level of gene expression of the tissue inhibitor of metalloprotease-1 (TIMP-1) was equivalent in the normal and degenerative meniscus (Fig. 2a). The relatively higher expression of MMP-1 in one OA meniscus (sample G) suggests that matrix degradation in degenerative meniscus could be promoted by an imbalance between the level of MMPs and their inhibitors, like it was hypothesized in the case of human OA articular cartilage.[13]

MMPs and most likely other mediators contribute to the heterogenous background of OA: molecular genetic analyses of human patients or transgenic mice have revealed that mutations in types II, IX and XI collagens can predispose to OA.[14] In regard of type X collagen, further studies need to be done to investigate the role of this protein in the structure of the meniscus in normal and pathological conditions.

Acknowledgments

We are grateful to Prof. B. Moyen (Hôpital Jules Courmont, Lyon Sud) and to Dr P. Bauchu (Polyclinique des Minguettes, Vénissieux) for providing the human samples and to Dr G. S. Gibson for the generous gift of the anti-type X collagen antibodies. We also thank Prof. M. Richard, Prof. E. Vignon and M.-J. Peschard for close continuous support. This work was financially supported by a BIOMED 2 grant (BMH4-CT95-0396) and by an EMERGENCE grant with a fellowship to G. B. from the Rhône-Alpes Region.

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