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Quantitative analysis of gene expression in human articular cartilage from normal and osteoarthritic joints

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Summary

Objective: To quantify the expression of genes encoding extracellular matrix (ECM) proteins in human cartilage from normal and osteoarthritic (OA) joints.

Design: Human cartilage samples were classified as control (CTR) or OA according to clinical evaluation and assessed histologically and biochemically to confirm the diagnosis. mRNAs encoding collagen types I, II and X, aggrecan, versican, osteopontin and osteocalcin were quantified by real-time reverse transcription-PCR assays and normalized to a reference mRNA (GAPDH).

Results: RNA from native cartilage could be reproducibly and efficiently amplified by real-time PCR only if isolated using purification membranes. Primers and fluorescent probes for real-time PCR, endowed with comparable (<6% difference from GAPDH) and high (>91%) amplification efficiencies, were designed and validated for the selected ECM genes. The expression of most genes under investigation displayed large variations and was not significantly different in CTR and OA cartilage. Only osteopontin mRNA levels were significantly higher in OA than CTR specimens. mRNA ratios of collagen type II to I and of aggrecan to versican, defined as indexes of chondrocyte differentiation, were less variable within each population than the single genes and markedly higher (27.0 and 7.6-fold, respectively) in CTR than OA cartilage, with high statistical significance (P=0.00013 and P=0.00007, respectively).

Conclusions: Our results provide evidence that gene patterns related to chondrocyte differentiation discriminate between CTR and OA human cartilage with higher sensitivity than single ECM genes. The method described here has the potential to improve understanding of the progression of OA and could become a valuable diagnostic tool. © 2001 OsteoArthritis Research Society International

Key words: Real-time PCR, Chondrocyte, Extracellular matrix, Differentiation.

Introduction

The unique mechanical properties of articular cartilage depend on the composition and organization of its extracellular matrix (ECM). It is generally agreed that the progressive functional failure of cartilage in osteoarthrosis (OA) is not only due to enhanced degradation of the main tissue components (e.g. type II collagen and aggrecan), but also to discoordinate changes in the types and amounts of newly synthesized ECM molecules[1]. Thus, investigation of OA chondrocyte anabolic activity by quantification of the expression of mRNAs encoding specific ECM molecules should help understand the progression of the pathology, and may ultimately improve its diagnosis and treatment.

Despite the wealth of knowledge on the structure of different cartilage ECM molecules and their genes, there is relatively little and sometimes contradictory information on the quantitation of their expression in normal and OA articular cartilage. It was reported that mRNAs for collagen types I and X are absent in normal adult cartilage and that collagen types II and X, but not I, are consistently up-regulated in specific layers of OA tissue[2–6]. However, independent studies performed using the same technique (i.e. *in situ* hybridization) reported that collagen type II mRNA was detectable in only about 50% of the processed OA cartilage samples[7], and that collagen type I was abundantly expressed by chondrocytes of deep zones of OA fibrocartilaginous tissue[8]. Also the expression of different types of proteoglycans, investigated using a competitive PCR technique, was not always consistent when different sets of samples were used[9,10].

The discordance among the different studies could be due not only to variability among different individuals, but also to limitations inherent to the techniques used. Northern blotting and *in-situ* hybridization methods are not sensitive enough to detect low-level gene expression and not accurate enough to quantify the full range of expression. An amplification step may therefore be required to quantify mRNA amounts from limited tissue biopsies. However, quantification of mRNA using conventional PCR techniques may be of limited accuracy, both because of the detection method used (i.e. semi-quantitative image analysis), and because analysis is often performed using a constant number of amplification cycles, after which the system could be in a saturation phase.

Recently, real-time PCR has enabled the quantification of mRNA with high accuracy, reproducibility and sensitivity in a wide dynamic range, without the need of post-PCR processing[11]. In this paper, we developed and validated real-time PCR assays to quantify the expression of genes encoding the most abundant molecules in the ECM of normal articular cartilage (i.e. collagen type II and aggrecan) and of degenerated fibrocartilage (i.e. collagen type I and versican), as well as of typical markers of chondrocyte hypertrophy (i.e. collagen type X and

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osteopontin) and of bone cells (i.e. osteocalcin). Our hypothesis was that real-time PCR can lead to the identification of quantitative patterns of ECM gene expression, typical for normal and OA adult human cartilage tissue.

Methods

PATIENTS AND SAMPLES

Patient's characteristics

Human articular OA cartilage was obtained from femoral heads, femoral condyles, and tibial plateaus of patients operated for knee, hip or ankle replacement (10 individuals; mean age 68.4, range 55–87) in the University Hospital of Basel. The diagnosis of OA was based on clinical and radiographic evaluations, according to standard criteria[12]. Control (CTR) cartilage samples (seven individuals; mean age 59.3, range 25–85) were collected from the hip or ankle of patients with no history of joint disease, undergoing joint replacement following femoral neck fracture or foot amputation for tumor resection.

Cartilage specimens

Non-calcified cartilage (i.e. superficial, middle, and deep layers above the tidemark)[3]. was dissected from the bone immediately after surgery. Pieces of cartilage were obtained from the weight-bearing area of the joints, which was macroscopically degenerated in OA patients, and processed for the different assays as follows. Samples were fixed in 4% buffered formalin at 4°C for 24 h for histological analysis, frozen in pre-weighed vials for biochemical assessment, or homogenized in Trizol (Life Technologies, Basel, Switzerland) for mRNA quantitation. Some samples were also fixed in acetone at 4°C for 10 min, or digested with 0.15% collagenase for 22 h to isolate primary chondrocytes.

Osteoblast isolation

Bone chips from the iliac crest of patients undergoing spinal fusion were cultured in Petri dishes in medium supplemented with 10% fetal bovine serum, ascorbic acid, dexamethasone and beta-glycerophosphate. After 10 days, outgrown cells were collected and homogenized in Trizol for mRNA analysis and served as controls for the expression of bone-specific proteins.

HISTOLOGICAL ANALYSIS

Fixed samples were dehydrated, embedded in paraffin, and cut into 5 μm sections. Cartilage slices were cut vertically from the surface to the bottom. Sections, stained with safranin O for sulfated glycosaminoglycans (GAG), were evaluated based on the Mankin's score by three independent experienced observers[13].

BIOCHEMICAL ANALYSES

Samples for biochemical analyses were frozen, lyophilized, and digested with proteinase K in the presence of protease inhibitors[14]. Briefly, 10–40 mg dry weight of sample were digested with 1 ml proteinase K solution (1 mg/ml) containing 50 mM Tris, 1 mM EDTA, 1 mM iodoacetamide, and 10 μg/ml pepstatin A at 56°C for 15 h. Proteinase K was then inactivated by boiling the digest for 12 min. The GAG content was determined spectrophotometrically using dimethylmethylene blue dye and bovine chondroitin sulfate as a standard[15]. Collagen type II was quantified using a monoclonal antibody-based inhibition ELISA[14].

REAL-TIME QUANTITATIVE PCR

Theoretical basis

Real-time quantitative PCR monitors the degradation of a sequence-specific, dual-labeled fluorescent probe after each cycle of PCR amplification. During the extension phase, the 5′-exonuclease activity of Taq DNA polymerase cleaves the probe, separating the 5′-reporter fluorescent dye from the 3′-quencher fluorescent dye, resulting in an increase in the emission spectra of the reporter fluorescent dye. After subtraction of the background fluorescence, calculated during the first 15 amplification cycles, the measured fluorescence is graphed as an amplification plot. Each reaction is characterized by a value, Ct, defined as the fractional number of cycles at which the reporter fluorescent emission reaches a fixed threshold level in the exponential region of the amplification plot. The Ct value is correlated to input target mRNA amount: a larger starting quantity of mRNA target results in a lower number of PCR cycles required for the reporter fluorescent emission to reach the threshold, and therefore a lower Ct value. Thus, the method is not based on the measurement of the total amount of amplified product after a fixed number of cycles, as in conventional PCR, and does not require post-PCR processing of the product[11].

Primers and probes

Primers and probes for human GAPDH, collagen types I, II, and X, aggrecan, versican, osteopontin and osteocalcin were designed with the assistance of the Primer Express computer program (Perkin-Elmer Applied Biosystems, Foster City, CA), in order to display minimal internal structure (i.e. primer–dimer formation) and similar melting temperatures. The total gene specificity of the nucleotide sequences chosen for the primers and probes was confirmed by BLASTN searches (GenBank database sequences). To avoid non-specific fluorescent emission derived from the recognition of contaminating genomic DNA by the probe, the middle third of the probe was placed at the junction between two exons. The nucleotide sequences of the oligonucleotide hybridization primers and probes are shown in Table I. Primers were purchased from Microsynth (Balgach, Switzerland) and probes were from Perkin-Elmer Applied Biosystems or Eurogentech (Seraing, Belgium). Optimal concentrations for the designed primers and probes were determined as the lowest ones giving the highest fluorescence levels and the lowest Ct values. The efficiency of the amplification for each target gene was assessed as follows. Ct values were measured after serial dilutions of the cDNA template. The amplification efficiency was calculated as the slope of the best linear fit of the expected increase in Ct (i.e. the logarithm in base 2 of the dilution factor) plotted vs the measured increase in Ct. A slope of 1 would correspond to one additional cycle

Table I
Description of the designed primers and probes

Gene	Forward and Reverse primers (5′→3′)	Primers concentration used (nM)	Probe* (5′→3′)	Probe concentration used (nM)	Amplicon size (bp)	Amplification efficiency (1=100%)
GAPDH	ATGGGGAAGGTGAAGGTCG TAAAAGCAGCCCTGGTGACC	300	CGCCCAATACGACCAAATCCGTTGAC	150	119	0.969
Collagen I	CAGCCGCTTCACCTACAGC TTTTGTATTCAATCACTGTCTTGCC	300	CCGGTGTGACTCGTGCAGCCATC	100	83	0.985
Collagen II	GGCAATAGCAGGTTCACGTACA CGATAACAGTCTTGCCCCACTT	900	CCGGTATGTTTCGTGCAGCCATCCT	200	79	0.914
Collagen X	CAAGGCACCATCTCCAGGAA AAAGGGTATTTGTGGCAGCATATT	900	TCCAGCACGCAGAATCCATCTGA	200	70	0.945
Aggrecan	TCGAGGACAGCGAGGCC TCGAGGGTGTAGCGTGTAGAGA	900	ATGGAACACGATGCCTTTCACCACGA	200	85	0.992
Versican	TGGAATGATGTTCCCTGCAA AAGGTCTTGGCATTTTCTACAACAG	900	CTGGCCGCAAGCAACTGTTCCTTT	200	98	0.924
Osteopontin	CTCAGGCCAGTTGCAGCC CAAAAGCAAATCACTGCAATTCTC	500	AAACGCCGACCAAGGAAAACTCACTACC	200	81	0.958
Osteocalcin	GAAGCCCAGCGGTGCA CACTACCTCGCTGCCCTCC	300	TGGACACAAAGGCTGCACCTTTGCT	150	70	0.913

*The underlined bases in the probe sequence indicate the position of an intron in the corresponding genomic sequence.

required to reach the threshold after a 1:2 dilution, indicating a 100% efficiency.

Total RNA extraction and cDNA synthesis

Freshly harvested cartilage (100 mg of tissue chopped in 2–3 mm chunks) was homogenized using a power tissue homogenizer (Politron PT 1200, Kinematica AG, CH) in 1 ml of Trizol Reagent and processed according to three different protocols, as follows.

Protocol 1: RNA was isolated using the standard single-step acid-phenol guanidinium method[16].

Protocol 2: After precipitation in isopropyl alcohol according to protocol 1, RNA was resuspended in 50 μl 0.5% SDS and purified with a second extraction with Phenol/ Chloroform/Isoamyalcohol[16].

Protocol 3: After centrifugation with chloroform, the RNA phase was mixed with an equal amount of 70% ethanol, and precipitated by application to silica-gel-based membranes (RNeasy® Mini Kit, Qiagen, Basel, CH) according to the manufacturers' instructions.

All the RNA samples were finally redissolved in 30 μl water and stored at −25°C. In some cases, RNA was also isolated from formalin and acetone-fixed cartilage specimens. cDNA was generated from 2 μg of RNA by using murine MLV reverse transcriptase (BRL, Gaithersville, MD) in the presence of dNTPs and DTT, according to the manufacturers' instructions.

PCR amplification and analysis

PCR reactions were performed and monitored using a ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). The PCR master mix was based on AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems). cDNA samples (2.5 μl in a total of 25 μl per well) were analysed in duplicate or triplicate. Primers and probes were used at concentrations ranging from 50 to 900 nM. After an initial denaturation step at 95°C for 10 min, the cDNA products were amplified with 45 PCR cycles, consisting of a denaturation step at 95°C for 15 s and an extension step at 60°C for 1 min. Data analysis was carried out by using the Sequence Detector V program (Perkin-Elmer Applied Biosystems). For each sample, the Ct value was determined as the cycle number at which the fluorescence intensity reached 0.05; this value was chosen after confirming that in this range all curves were in the exponential phase of amplification. For each cDNA sample, the Ct value of each target sequence was subtracted to the Ct value of the reference gene (GAPDH), to derive ΔCt. The level of expression of each target gene, normalized to GAPDH, was then calculated as $(1+E_t)^{\Delta Ct}$, where E_t is the efficiency of amplification of the target sequence. The resulting values, although normalized to GAPDH, are still dependent on the efficiency of the mechanism of probe cleavage and fluorescence emission (PE-ABI; Sequence Detector User Bulletin 2). Therefore, the reported figures cannot be considered as absolute expression levels of the genes of interest, and can only be used for comparative analyses among samples. GAPDH was chosen as the reference housekeeping gene based on the majority of previous studies on chondrocyte gene expression. Since collagen type II and aggrecan are the typical markers of differentiated chondrocytes in hyaline cartilage, as opposed to collagen type I and versican, which are expressed by dedifferentiated chondrocytes and in fibrocartilage, we defined the ratios of mRNA levels of collagen type II to I (CII/CI) and of aggrecan to versican (Agg/Ver) as 'differentiation indexes' related to the expression of collagens and proteoglycans, respectively.

STATISTICAL ANALYSIS

Differences between CTR and OA cartilage specimens were assessed by one-way analysis of variance (ANOVA). Values of $P<0.02$ were considered to indicate statistically significant differences.

Results

DEVELOPMENT AND VALIDATION OF THE REAL-TIME PCR ASSAYS

Sequences for the designed primers and probes, the size of the amplicons, and the position of the exon-to-exon junctions within the probe are presented in Table I. The optimal concentrations of the primers and probes, as well as the specificity and efficiency of the amplifications, were determined using total RNA samples isolated from human primary chondrocytes and osteoblasts according to Protocol 1. For each target gene, the amplification product of positive controls revealed a single band at a level corresponding to the expected size of the amplicon (data not shown). The measured fluorescence after each amplification cycle showed typical profiles: the emitted signal remained at baseline levels during early cycles, then increased exponentially when sufficient hybridization probe had been cleaved, and reached a plateau phase at high cycle numbers [Fig. 1(A)]. The linear correlation between the Ct values and the initial cDNA amounts, diluted up to 128-fold, confirmed the accuracy of the method in a wide working range [Fig. 1(B)]. The amplification efficiencies for each target sequence (E_t), calculated from serial dilutions of cDNA as shown in Fig. 1(C), are reported in Table I. Since E_t values were high (>0.91) and comparable for all target genes (<6% difference with respect to GAPDH), in subsequent experiments we approximated E_t constants to 1 and therefore calculated the expression of each gene normalized to GAPDH according to the formula $2^{\Delta Ct}$ (PE-ABI; Sequence Detector User Bulletin 2).

For samples of native cartilage tissue, the amount of RNA isolated according to protocol 1 was poor and its purity not acceptable (i.e. OD_{260}/OD_{280} values lower than 1.0). Using an additional purification step according to protocol 2, the RNA yield and the purity index improved (approximately 1.5), but the amplification of target sequences by PCR was highly inefficient (data not shown). Protocol 3 (purification on silica-gel-based membranes) enabled isolation of significant amounts of RNA (approximately 20 ng per mg of tissue), with high purity index (approximately 2.0), which could be amplified by real-time PCR with high reproducibility and efficiency, yielding amplification curves comparable to those described for RNA extracted from cells.

For some cartilage specimens, in which mRNA was extracted both from the intact tissue and from cells after tissue digestion, PCR analysis showed that the expression of all the genes of interest was markedly and differentially affected by tissue digestion, with differences up to 10–15 fold. RNA isolated from formalin fixed cartilage tissue could not be amplified by PCR, whereas fixation in acetone allowed efficient RNA extraction and PCR amplification.

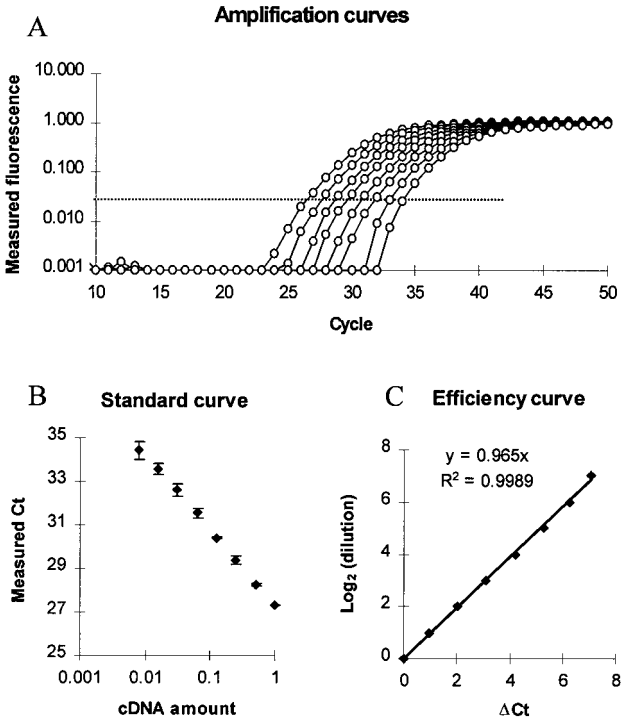

Fig. 1. Typical amplification, standard and efficiency curves obtained by real-time PCR. (A) Amplification plots for GAPDH after serial dilutions of cDNA, starting from 1 (undiluted, leftmost curve) to 0.0078 (1:128 dilution, rightmost curve). Ct is defined as the fractional cycle number at which the fluorescent signal reaches the selected threshold (indicated by a dotted line). Plots are the average of two replicates. In the graph, values lower than 0.001 were set to 0.001. (B) Standard curve for GAPDH, plotting the Ct measured as described above vs the starting cDNA amount. (C) Efficiency curve for GAPDH, plotting the measured increase in Ct after each 1:2 dilution (ΔCt) vs the expected increase in Ct (i.e. the logarithm in base 2 of the dilution factor). The amplification efficiency for each target sequence (E_t) was calculated as the slope of the best linear fit of the corresponding efficiency curve (e.g. 0.965 in this case). An efficiency of 1 (or 100%) would correspond to one additional cycle required to reach the same threshold after a 1:2 dilution.

ANALYSIS OF HUMAN CARTILAGE FROM NORMAL AND OA JOINTS

Histologic and biochemical analyses

CTR cartilage samples showed few microscopic changes, mostly related to reduction of safranin O staining, and the scores ranged from 0.3 to 3.3 [Table II(A)]. The specimens from different OA patients displayed considerable variability in their histological appearance, ranging from surface irregularities to marked fibrillation, from cell cloning to hypocellularity, and from moderate reduction to complete absence of safranin O staining. Repair tissue was never observed in the samples processed in this study. The distribution of Mankin's histological scores ranged from 4.0 to 8.3 [Table II(A)].

The wet weight fractions of water, GAG and type II collagen of the cartilage specimens are presented in Table II(B). The water content was significantly higher in OA than in CTR cartilage, whereas the GAG and type II collagen fractions of wet weight were higher in CTR cartilage samples. Histologic and biochemical data thus validated the clinical classification of the specimens as CTR and OA[14,17].

Quantification of mRNA levels

RNA for these analyses was isolated according to Protocol 3 from undigested, non-fixed cartilage samples. C_t values for GAPDH from comparable amounts of different tissue samples displayed a standard deviation lower than 7%, thus ruling out the possibility of extensive RNA degradation or reduced efficiency in cDNA synthesis for selected specimens. The levels of expression of all the target genes, normalized to GAPDH, are reported in Table II(C). The average expression of collagen types I, II, versican and osteopontin was markedly higher in OA than in CTR cartilage (3050.6, 6.3, 17.2 and 6.4-fold, respectively). However, only up-regulation of osteopontin in OA specimens was statistically significant (P<0.015). mRNA average levels for collagen type X and aggrecan were comparable in OA and CTR cartilage. Osteocalcin mRNA was detected at trace levels only in one OA sample (data not shown). In both normal and OA cartilage, collagen types I and II, aggrecan and versican mRNA levels displayed large variability. Ratios of collagen type II to I (CII/CI) and of aggrecan to versican (Agg/Ver), defined as chondrocyte differentiation indexes, displayed in both populations more limited differences than single target genes [Table II(D)]. These indexes were markedly higher in CTR than in OA cartilage (27.0 and 7.6-fold, respectively), with high statistical significance (P=0.00013 and P=0.00007, respectively).

Discussion

In this paper we describe for the first time the development and validation of real-time PCR assays to quantify gene expression in human cartilage tissue. The method allows quantification of levels of ECM gene expression averaged among all cartilage layers, and is thus complementary to *in-situ* hybridization techniques, providing semi-quantitative assessment of the spatial distribution of collagen and proteoglycan mRNAs[2,3,6]. As compared to other techniques currently available for RNA analysis from whole cartilage specimens, however, real-time PCR is more sensitive than Northern blotting, requires smaller tissue samples, possesses a wider dynamic range and has a higher sample throughput. Most importantly, this technique makes RNA quantitation more precise and reproducible than conventional PCR, because it is based on Ct values established in the early exponential phase of the PCR reaction, when none of the reagents is rate-limiting, rather than end-point measurement of the amount of accumulated product[11].

Our main finding was that expression of the genes encoding collagen types I and II and aggrecan and versican was highly variable and not significantly different in chondrocytes obtained from CTR or OA cartilage samples, whereas ratios of collagen type II to I (CII/CI) and of aggrecan to versican (Agg/Ver), defined as indexes of cell differentiation, were significantly higher in CTR than OA cartilage. The cartilage specimens included in the CTR group had relatively high Mankin's scores. In addition, the majority of them was from a different joint as compared to the OA cases (i.e. mostly from hip joints as opposed to knee joints). Nevertheless, the statistical significance for the difference in the CII/CI and Agg/Ver ratios in the two experimental groups was remarkable. Our result, then, suggests a clear general behaviour of OA chondrocytes, i.e. the tendency to repair cartilaginous injuries by forming a

Table II
Analysis of different cartilage specimens

	A		B			C						D	
Group/ joint	Age	Mankin's score	Water %ww	GAG %ww	CII %ww	CI	CII	CX	Agg	Ver	OP	CII/CI	Agg/Ver
CTR													
Hip	72	0.3	74.62	4.24	19.02	0.146	19.9	0.000	0.75	0.004	0.00	136.3	186.8
Hip	73	0.3	73.67	6.11	13.36	0.032	19.7	0.036	1.93	0.016	2.39	615.6	120.6
Hip	66	2.0	73.63	4.64	12.20	0.036	4.4	0.032	0.64	0.014	3.15	123.2	47.0
Hip	85	1.7	71.14	4.10	13.08	0.026	11.6	0.000	0.54	0.004	0.14	444.2	135.9
Hip	67	0.7	70.55	5.41	14.35	0.116	26.0	0.000	0.61	0.010	0.50	224.5	61.4
Ankle	25	1.7	74.46	5.52	17.12	0.158	44.0	0.088	1.15	0.008	1.62	278.2	144.0
Ankle	27	3.3	74.71	3.50	11.63	0.149	35.8	0.063	0.84	0.014	0.37	239.9	58.5
Mean	59.3	1.4	73.25	4.79	14.39	0.095	23.0	0.031	0.92	0.010	1.17	294.6	107.7
Stderr	8.9	0.4	0.65	0.35	1.02	0.023	5.14	0.013	0.18	0.002	0.47	66.9	20.0
OA													
Knee	87	7.7	75.50	3.74	9.05	1.63	6.5	0.029	0.16	0.022	6.13	4.0	7.3
Knee	83	7.7	83.56	2.40	5.37	0.08	1.3	0.048	0.16	0.016	7.52	16.5	9.9
Knee	83	8.3	77.46	0.97	12.96	113.40	53.6	0.069	0.46	0.200	18.57	0.5	2.3
Knee	78	4.3	81.46	2.11	4.37	8.11	58.9	0.005	1.09	0.148	0.66	7.3	7.4
Knee	61	8.0	85.19	3.23	4.59	10.52	393.4	0.003	1.48	0.037	0.82	37.4	40.2
Knee	63	8.3	80.71	3.29	7.66	1722.20	103.4	0.000	0.81	0.727	6.32	0.1	1.1
Ankle	66	7.7	80.72	2.91	10.67	453.51	233.1	0.035	1.14	0.331	9.32	0.5	3.4
Ankle	55	4.0	79.58	3.92	5.75	6.23	173.7	0.037	1.70	0.046	11.16	27.9	37.0
Hip	71	8.3	80.17	3.84	7.01	11.85	87.8	0.000	0.31	0.024	0.52	7.4	12.6
Hip	56	6.3	77.93	4.76	12.39	282.40	196.3	0.030	0.27	0.019	12.55	0.7	14.1
Mean	68.4	7.0	80.75	3.05	7.86	289.81	144.6	0.025	0.82	0.172	7.49	10.9	14.2
Stderr	3.7	0.5	0.90	0.34	1.00	169.43	38.2	0.007	0.18	0.071	1.85	4.1	4.4
P value*	0.22	**0.000001**	**0.00004**	**0.005**	**0.00057**	0.222	0.035	0.69	0.543	0.109	**0.015**	**0.00013**	**0.00007**

The mean (Mean) and standard error (Stderr) are reported for each group, along with the statistical *P*-value for the difference between the two groups.

A: Classification of cartilage samples as control (CTR) or osteoarthritic (OA) was based on clinical and radiological evaluation[12].

B: Biochemical assessment: wet weight fractions of water, glycosaminoglycans (GAG), and type II collagen (CII).

C: mRNA expression normalized to GAPDH of collagen types I (CI), II (CII), and X (CX), aggrecan (Agg), versican (Ver), and osteopontin (OP). RNA used for these experiments was extracted using silica-gel-based membranes and displayed relatively high purity (OD_{260}/OD_{280} around 2.0).

D: Ratios of mRNA expression levels of collagen type II to I (CII/CI) and of aggrecan to versican (Agg/Ver).

*Bold type indicates statistically significant difference between the CTR and OA groups ($P<0.02$).

fibrocartilaginous tissue rather than hyaline cartilage, consistent with the ultimate structural and functional degeneration of cartilage tissue observed in late stages of OA[17].

Among the markers of chondrocyte hypertrophy, we investigated the expression of collagen type X and osteopontin. In contrast to previous reports[2,5], in some of the CTR samples we detected collagen type X mRNA. This finding may be explained by the higher sensitivity of real-time PCR as compared to *in-situ* hybridization, and is consistent with the immunolocalization of collagen type X in the surface layers of normal adult cartilage[18]. We then observed that the expression of type X collagen in the OA cartilage samples was not increased as compared to the CTR group. This result, also apparently in contrast to previous reports, can be explained by the fact that our specimens did not include the calcified cartilage layer, in contact with the subchondral bone, where collagen type X expression was found increased during OA[2,5]. Interestingly, osteopontin was significantly upregulated in OA specimens. The expression of osteopontin gene during chondrocyte maturation is known to be one of the key events involved in cartilage-to-bone transition in fracture repair[19], and it was shown to be increased in chondrocytes exposed to IL-1β *in vitro*[20]. However, the expression of osteopontin gene in normal and OA human articular cartilage has not been previously reported.

Reliable and reproducible PCR amplification curves were obtained only if RNA was extracted and purified on silica-gel membranes (protocol 3). The insufficiently pure material yielded by the typical one-step RNA extraction technique (protocol 1) and the non-reproducible amplifications obtained with an additional purification step (protocol 2) can be explained by the large amounts of ECM proteins in cartilage tissue possibly interfering with the extraction procedure and/or inhibiting the DNA polymerase. We also report that cartilage incubation with collagenase induces rapid changes in chondrocyte gene expression, implying that RNA analysis should not be performed on isolated cells after tissue digestion. The finding that the method can be applied after cartilage fixation in acetone is consistent with previous reports related to lymphoid tissue[21], and opens the possibility of quantifying gene expression in specific regions of native cartilage, selected by histologic or immunohistochemical analysis.

The use of real-time PCR to quantify gene expression in cartilage tissue may be extended to the analysis of different inflammatory cytokines and degradative enzymes involved in the process of OA in order to identify additional quantitative patterns of gene expression, not only related to ECM proteins and to cell differentiation. An extension of the assays in this direction might lead to an explanation of the large variability found among OA samples in the expression

of ECM genes, particularly collagen types I and II, and possibly to correlate it with different stages/modalities of the pathology. The method described here may thus help to elucidate the complex mechanisms involved in cartilage degeneration and, given the high level of intra- and inter-laboratory standardization it can provide, it may become a valuable diagnostic tool to detect early changes in potential OA patients. The assays developed in this paper and the defined differentiation indexes can also be used to quantify ECM gene expression in cultured chondrocytes, and thus improve the understanding of the effects of different regulatory molecules in the process of chondrocyte de- and redifferentiation.

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References

1. Matyas JR, Ehlers PF, Huang D, Adams ME. The early molecular natural history of experimental osteoarthritis. I. Progressive discoordinate expression of aggrecan and type II procollagen messenger RNA in the articular cartilage of adult animals. Arthritis Rheum 1999;42:993–1002.
2. Hoyland JA, Thomas JT, Donn R, Marriott A, Ayad S, Boot-Handford RP, *et al.* Distribution of type X collagen mRNA in normal and osteoarthritic human cartilage. Bone Miner 1991;15:151–63.
3. Aigner T, Stoss H, Weseloh G, Zeiler G, von der Mark K. Activation of collagen type II expression in osteoarthritic and rheumatoid cartilage. Virchows Arch B Cell Pathol Incl Mol Pathol 1992;62:337–45.
4. Aigner T, Bertling W, Stoss H, Weseloh G, von der Mark K. Independent expression of fibril-forming collagens I, II, and III in chondrocytes of human osteoarthritic cartilage. J Clin Invest 1993;91:829–37.
5. Aigner T, Reichenberger E, Bertling W, Kirsch T, Stoss H, von der Mark K. Type X collagen expression in osteoarthritic and rheumatoid articular cartilage. Virchows Arch B Cell Pathol Incl Mol Pathol 1993;63:205–11.
6. Aigner T, Vornehm SI, Zeiler G, Dudhia J, von der M, Bayliss MT. Suppression of cartilage matrix gene expression in upper zone chondrocytes of osteoarthritic cartilage. Arthritis Rheum 1997;40:562–9.
7. Pullig O, Kladny B, Weseloh G, Swoboda B. Metabolic activation of chondrocytes in human osteoarthritis. Expression of type II collagen. Z Orthop Ihre Grenzgeb 1999;137:67–75.
8. Miosge N, Waletzko K, Bode C, Quondamatteo F, Schultz W, Herken R. Light and electron microscopic in-situ hybridization of collagen type I and type II mRNA in the fibrocartilaginous tissue of late-stage osteoarthritis. Osteoarthritis Cartilage 1998;6:278–85.
9. Melching LI, Cs-Szabo G, Roughley PJ. Analysis of proteoglycan messages in human articular cartilage by a competitive PCR technique. Matrix Biol 1997;16:1–11.
10. Cs-Szabo G, Melching LI, Roughley PJ, Glant TT. Changes in messenger RNA and protein levels of proteoglycans and link protein in human osteoarthritic cartilage samples. Arthritis Rheum 1997;40:1037–45.
11. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res 1996;6:995–1001.
12. Kellgren JH, Lawrence JS. Radiological assessment of osteoarthrosis. Ann Rheum Dis 1957;16:494–501.
13. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J Bone Joint Surg (Am) 1971;53:523–37.
14. Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, *et al*. Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. J Clin Invest 1994;93:1722–32.
15. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883:173–7.
16. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
17. Mankin HJ, Mow VC, Buckwalter JA, Iannotti JP, Ratcliffe A. Form and function of articular cartilage. In: Simon SR, Ed. Orthopaedic Basic Science. Rosemont: American Academy of Orthopaedic Surgeons 1994:1–44.
18. Rucklidge GJ, Milne G, Robins SP. Collagen type X: a component of the surface of normal human, pig, and rat articular cartilage. Biochem Biophys Res Commun 1996;224:297–302.
19. Nakase T, Sugimoto M, Sato M, Kaneko M, Tomita T, Sugamoto K, *et al*. Switch of osteonectin and osteopontin mRNA expression in the process of cartilage-to-bone transition during fracture repair. Acta Histochem 1998;100:287–95.
20. Margerie D, Flechtenmacher J, Buttner FH, Karbowski A, Puhl W, Schleyerbach R, *et al*. Complexity of IL-1 beta induced gene expression pattern in human articular chondrocytes. Osteoarthritis Cartilage 1997;5:129–38.
21. Fend F, Emmert-Buck MR, Chuaqui R, Cole K, Lee J, Liotta LA, *et al*. Immuno-LCM: laser capture microdissection of immunostained frozen sections for mRNA analysis. Am J Pathol 1999;154:61–6.