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Osteoarthritis and Cartilage (1998) **6**, 341–350
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1063–4584/98/050341 + 10 $12.00/0

OSTEOARTHRITIS and CARTILAGE

Influence of pregnancy on gene expression in rabbit articular cartilage

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Summary

Objective: Articular cartilage is known to be influenced by estrogen and the pregnancy-associated hormone, relaxin, *in vitro*. Such observations have raised the possibility that articular cartilage in females may be subjected to unique regulatory influences by such hormones *in vivo*. The purpose of this study was to evaluate mRNA levels for several relevant molecules in the articular cartilage of pregnant and non-pregnant rabbits.

Design: Total RNA was extracted from New Zealand White rabbit knee articular cartilage using the TRIspin method. The total RNA was reverse transcribed and analysed by the sensitive molecular technique of semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) using rabbit specific primer sets.

Results: Total RNA yield from articular cartilage from primigravida rabbits was reduced to 65% of age-matched control values ($P = 0.0003$); however the yield from multiparous animals was not significantly depressed. In both cases, DNA yields were not affected by pregnancy. There was a general tendency for depressed mRNA levels for most genes investigated in cartilage from pregnant animals. Articular cartilage from multiparous rabbits showed a significant decrease in mRNA levels for relevant molecules such as type II collagen, biglycan, collagenase and tissue inhibitors of metalloproteinases (TIMP)-1, as well as necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) and cyclo-oxygenase 2 (COX-2). Transcripts for collagenase and lumican were significantly lower in cartilage from primigravida rabbits. Transforming growth factor β1 (TGF-β1) transcript levels were significantly decreased in both pregnant groups. In contrast, basic fibroblast growth factor (bFGF) and insulin-like growth factor-2 (IGF-2) mRNA levels were significantly decreased in cartilage from primigravida rabbits, whereas transcripts for these molecules were upregulated in the cartilage of multiparous rabbits.

Conclusions: The present study demonstrates that regulation of RNA levels in articular cartilage during pregnancy is complex and is influenced by the parity and/or the skeletal maturity of the animals.

Key words: Articular cartilage, Extracellular matrix gene expression, Cytokines, Growth factors, Proteinases and inhibitors, Nitric oxide, Cyclo-oxygenase 2, Pregnancy.

Introduction

Articular cartilage (AC) is composed of a highly organized extracellular matrix in which are embedded chondrocytes. The integrity of articular cartilage is maintained by the ability of chondrocytes to synthesize and degrade matrix macromolecules, and any perturbation in chondrocyte metabolism may alter tissue function [1]. Articular cartilage has been the focus of intense study in inflammatory and degenerative diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA) [2, 3].

In RA, there appears to be gender differences in the development of the disease, which then change with age and the onset of menopause [4–8]. Such indications have led to studies investigating the effects of sex hormones on articular cartilage. First, estrogen receptors have been detected in cartilage of various species [9–11], supporting the concept that cartilage should be responsive to this hormone. From *in vivo* models of arthritis, evidence has been obtained that sex hormones protect cartilage from inflammation-induced

Received 22 December 1997; accepted 7 May 1998

This work was supported by The Arthritis Society and the Medical Research Council of Canada.

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degradation [12, 13]. Interestingly, there is also evidence that estrogen and progesterone reduce interleukin-1 (IL-1)-induced cartilage breakdown *in vitro* [12]. In addition, Estradiol treatment has been shown to suppress proteoglycan synthesis in both normal and experimental OA cartilage [14]. However, the concentration of proteoglycan was not diminished, suggesting that proteoglycan catabolism was not altered [14]. As OA usually develops later in life, the effect of pregnancy on OA has not been as well studied. However, few clinical studies are available on the relationship between development of OA and pregnancy history that suggest an overall modest but protective effect of parity [15]. Moreover, *in vitro* experiments have shown that rabbit cartilage metabolism is influenced by the pregnancy-associated hormone, relaxin [16]. Beside such opposing effects of sex hormones on rheumatic diseases, surprisingly, very little is known about the influence of pregnancy on normal articular cartilage.

The data regarding the regulation of mRNA levels in connective tissues such as articular cartilage remain limited, particularly during pregnancy. Pregnancy is a physiological state that involves major hormonal changes. Therefore, in an attempt to further develop our understanding of the influence of pregnancy on connective tissue, we have analyzed RNA isolated from articular cartilage from pregnant and non-pregnant rabbits for the presence of transcripts for estrogen receptors, matrix molecules, proteinases and inhibitors, growth factors, cytokines, cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) by the very sensitive molecular technique of reverse transcription-polymerase chain reaction (RT-PCR). As the quantity of cartilage available from the rabbit knee is limited and biochemical analysis not readily feasible for many molecules, this method allows an accurate, reproducible assessment of cell activity. While not all inclusive, the molecules assessed are representative of cellular activities known to influence cartilage function. The results indicate that the method can be readily used to analyze RNA from rabbit articular cartilage, and we demonstrate that alterations in total RNA levels, as well as mRNA levels for a subset of relevant molecules, are detectable during pregnancy.

Materials and Methods

TISSUE PREPARATION

Primigravida (5 months of age), multiparous (11–12 months of age; total number of pregnancies = 3) and age-matched non-pregnant control New Zealand white rabbits (Reimans Fur Ranch; St. Agatha, ON, Canada) were used as the source of animal tissue. Pregnant animals were purchased at 21 days gestation and were sacrificed 8 days later (29 days gestation). Age-matched non-pregnant rabbits were sacrificed at the same time. Pregnancy was confirmed at the time of sacrifice. There was no overt difference in cage activity between pregnant and non-pregnant animals. However, the activity before shipment was not controlled. Gestation in the rabbit is 31–32 days. All animals were killed by Euthanyl overdose (MTC Pharmaceuticals; Cambridge, ON, Canada) intravenously through the lateral ear vein. The articular cartilage was removed aseptically from the femoral condyles and tibial plateau. For DNA content, the fresh wet weight was immediately determined, followed by lyophilization to constant weight and dry weight determination. These values were also used to define the percentage of water content of the tissue samples. For RNA extraction, a separate set of fresh cartilage tissue samples were immediately weighed before freezing in liquid nitrogen andstorage at −80°C before processing.

RNA EXTRACTION

Total RNA from AC was extracted using the TRIspin method as described previously [17]. Briefly, frozen tissues were powdered using a Braun Dismembrator (B. Braun Biotech; Inc. Allentown, PA, U.S.A.). TRIzol reagent (Life Technologies Inc.; Gaithersburg, MD, U.S.A.) was added to the powder (1 ml/100 mg wet weight tissue) and the sample allowed to thaw before being transferred to 1.5 ml Eppendorf tubes. Chloroform was added (0.2 ml/ml TRIzol), the tubes were mixed by vortexing and centrifuged at 12 000 **g** for 15 min to separate the aqueous and organic phases. One volume of 70% ethanol was added to the aqueous phase and total RNA subsequently isolated using the RNeasy Total RNA isolation kit (Qiagen Inc.; Chatsworth, CA, U.S.A.) as directed by the manufacturer. Total RNA was quantified using the SyBr green II reagent (FMC BioProducts; Rockland, ME, U.S.A.) and a Perkin Elmer fluorimeter. All samples were stored at −70°C until analyzed.

SEMIQUANTITATIVE RT-PCR

RT was carried out with 1 μg total RNA using the StrataScript (tm) RT-PCR kit (PDI BioScience; Aurora, ON, Canada). Aliquots of cDNA were amplified by PCR as described previously [17–19]

using the primer sets depicted in Table I. PCR products were separated in a 2% agarose gel at 60 V/cm in TAE buffer, the gels stained with ethidium bromide, destained and then photographed. The negatives were then analyzed using the Masterscan Interpretive Densitometer (CSPI Inc.; Billerica, MA, U.S.A.) and the RFLP Scanalytics software. For all reported experiments, conditions were determined to be in the linear range for both the PCR amplification and the image analysis system. An example of the method is shown in Fig. 1. This process was repeated for each transcript. Briefly, for each group of samples (i.e., RNA from the cartilage of primigravida animals or multiparous animals and age-matched control animals) all of the samples were subjected to RT at the same time and subsequently, all samples of cDNA in a group amplified by PCR at the same time to avoid any potential experiment to experiment variation in efficiency. Each RT sample was assessed for GAPDH (housekeeping gene) cDNA using 3 μl of the 50 μl total volume of RT.

Following 22–25 PCR cycles, previously shown to yield results in the linear range of the method (Fig. 1), the volumes were normalized and the PCR repeated to yield very similar GAPDH integrated density values. Once the GAPDH values were determined to be similar, and in the linear range of detection, the same volumes of each sample in a group were then used to assess the cDNA levels for the remaining molecules of interest. Such an experimental construct allowed for comparisons between samples within a group (i.e., primigravida vs controls; multiparous vs controls). For each

Table I
Polymerase chain reaction primer sequences and sources used in this study

Gene	Basepairs	Primer Sequences	Primer Source
Biglycan	406	GATGGCCTGAAGCTCAA GGTTTGTTGAAGAGGCTG	human, rat, mouse, cow[a]
Collagen II	366	GCACCCATGGACATTGGAGGG GACACGGAGTAGCACCATCG	Metsaranta, M., Vuorio, E.[b]
Decorin	419	TGTGGACAATGGTTCTCTGG CCACATTGCAGTTAGGTTCC	Zhan, Q. *et al.*[c]
Lumican	576	CTGCAGTGGCTCATTCTA GACCTCCAGGTAATAGTT	Human, rat, cow[a]
Aggrecan	313	GAGGAGATGGAGGGTGAGGTCTTT CTTCGCCTGTGTAGCAGCTG	Bayne, E. K. *et al[r,].*[d]
Urokinase	392	TGGTTTGCAGCCATCTAC TCCAAAGCCAGTGATCTC	Human, rat, cow, pig, baboon[a]
Collagenase	322	TCAGTTCGTCCTCACTCCAG TTGGTCCACCTGTCATCTTC	Fini, M. E. *et al.*[e]
PAI-1	263	TGGAACAAGGATGAGATCAG CCGTTGAAGTAGAGGGCATT	Human, cow, mink[a]
TIMP-1	326	GCAACTCCGACCTTGTCATC AGCGTAGGTCTTGGTGAAGC	Horowitz, S. *et al.*[f]
IGF-2	205	GACCGCGGCTTCTACTT GGAAGAACTTGCCCACG	Dull, T. J. *et al.*[g]
TGF-β1	271	CGGCAGCTGTACATTGACTT AGCGCACGATCATGTTGGAC	Taylor, J. K. *et al.*[h]
bFGF	282	TACAACTTCAAGCAGAAGAG CAGCTCTTAGCAGACATTGG	Abraham, J. A. *et al.*[i]
IL-1β	354	TACAACAAGAGCTTCCGGCA GGCCACAGGTATCTTGTCGT	Mori, S. *et al.*[j]
TNF-α	297	GGCTCAGAATCAGACCTCAG GCTCCACATTGCAGAGAAGA	Ito, H. *et al.*[k]
COX-2	282	TCAGCCACGCAGCAAATCCT GTGATCTGGATGTCAGCACG	Guan, Y. *et al.*[l]
iNOS	262	CGCCCTTCCGCAGTTTCT TCCAGGAGGACATGCAGCAC	Charles, I. G.[b]
GAPDH	293	TCACCATCTTCCAGGAGCGA CACAATGCCGAAGTGGTCGT	Applequist, S. E. *et al.*[m]
Estrogen receptor	341	CTCCATGATCAGGTCCAC GTGTCTGTGATCTTGTCC	Human, bovine, rat[a]

[a]Consensus sequences for these species were found from Genbank sequences; [b]Personal communication; [c]Genbank accession number S76584; [d]Genbank accession number L38480; [e]Genbank accession number M17821; [f]Genbank accession number J074112; [g]Genbank accession number X03562; [h]Genbank accession number AF000133; [i]Genbank accession number X04432, X04433; [j]Genbank accession number D21835; [k]Genbank accession number M12845; [l]Genbank accession number U97696; [m]Genbank accession number L23961.

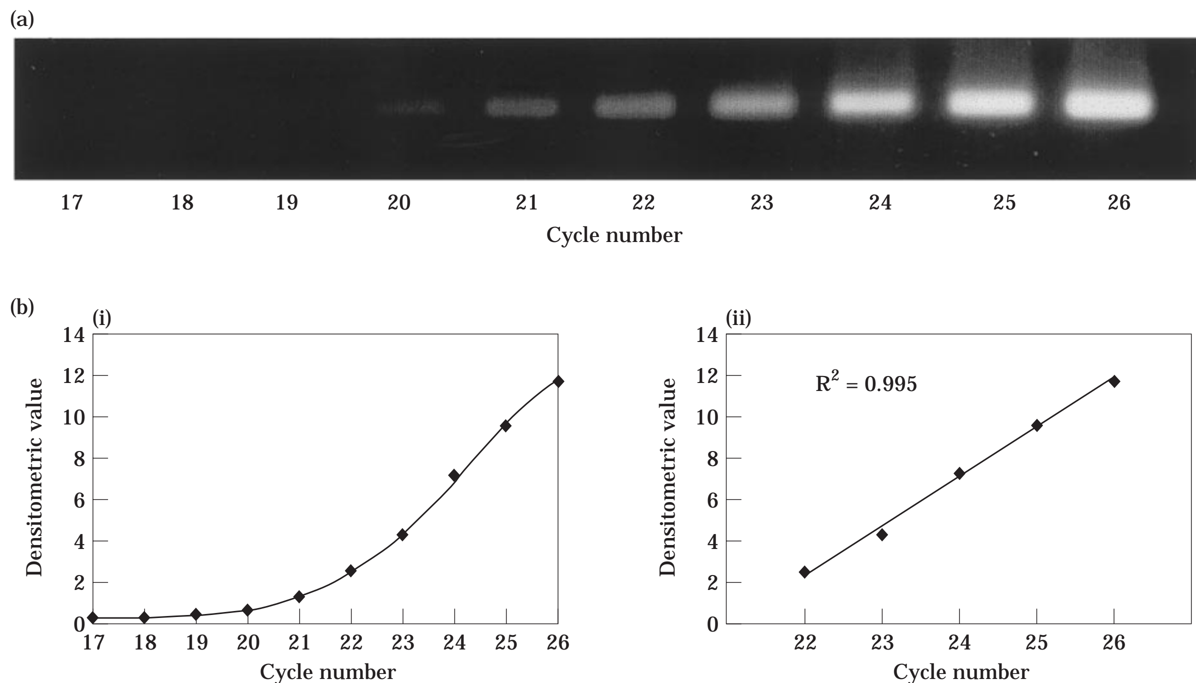

Fig. 1. Determination of the linear range of PCR amplification of GAPDH. (a) Twenty microliters of the GAPDH PCR product were subjected to agarose gel electrophoresis following PCR amplification ranging from 17–26 cycles. (b) (i) The densitometric values obtained by scanning of the negative of the photograph shown above were plotted vs cycle number. A linear portion of the curve is evidenced between 22–26 cycles of PCR. (ii) The linearity of the points between cycles 22–26 was assessed by straight line regression. The R2 value of 0.995 indicates a high degree of linearity along these points within this PCR cycle range.

primer set, the optimal cycle number was determined and the resulting amplified bands analyzed by densitometry. Integrated density values for the genes in question were normalized to the GAPDH values to yield a semi-quantitative assessment. An example of the raw data and the analysis for type II collagen (COL2) is shown in Fig. 2. Two independently isolated clones of each amplified cDNA fragment have been sequenced to verify identity of each cDNA product.

DNA CONTENT ANALYSIS

The DNA content was measured using a fluorophotometric assay according to the method reported by Lipman *et al.* [20]. Briefly, powdered cartilage samples were suspended in 0.2 ml of 1% sodium dodecyl sulfate (SDS), incubated for 10 min in a boiling water-bath, and cooled to room temperature before the addition of 0.7 ml of 0.01 M Tris–HCL (pH 8.0), along with 0.1 ml Proteinase K (5 mg/ml). Samples were then incubated at 60°C for 18 to 20 h. After cooling to room temperature, a 100 μl aliquot was added to each cuvette containing 2 ml of 0.154 M NaCL/0.015 M Na citrate and 75 μl of Tris–HCL buffer. One milliliter of Hoechst 33258 dye was added to each cuvette, mixed, and maintained in the dark for 10–15 min along with DNA standards (salmon sperm DNA in the range of 0–10 μg/ml). Fluorescence was measured in a Perkin–Elmer fluorimeter with an excitation and emission wavelength of 350 nm and 450 nm, respectively.

STATISTICAL ANALYSIS

Values are given as mean ± standard error of mean of six rabbits. Comparisons were made using analysis of variance (ANOVA). Differences with *P*-values of less than 0.05 were considered significant.

Results

CARTILAGE MASS

No cartilage lesions were observed at the time of necropsy. Only a small volume (< 0.2 ml) of synovial fluid was present in control joints from non-pregnant rabbits, whereas the volume of synovial fluid was increased to ~ 1 ml in joints from pregnant rabbits. The cartilage wet and dry masses of the knee joint were similar in both pregnant and non-pregnant rabbits. The water content of cartilage from primigravida rabbits and

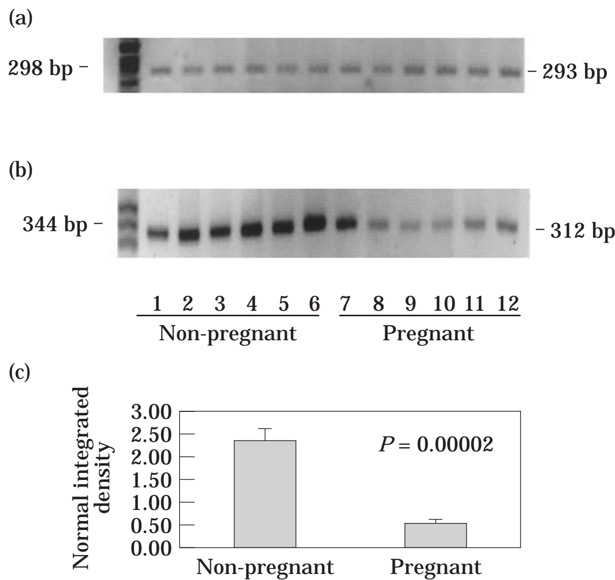

FIG. 2. PCR analysis of mRNA for GAPDH and type II collagen from the knee articular cartilage of multiparous and control rabbits. RNA (1 μg) from the knee articular cartilage of multiparous and control rabbits was reverse transcribed and then subjected to PCR using rabbit-specific primer sets depicted in Table I. (a) Gel electrophoresis of amplified GAPDH cDNA; (b) equivalent volumes of the same samples used in (a) were subjected to PCR with type II collagen primers; (c) densitometric analysis and normalization of type II collagen to GAPDH values expressed as percent of values in cartilage from non-pregnant rabbits.

multiparous rabbits were 70.8 and 71.9%, respectively. No statistically significant difference in the water content was found between cartilage from pregnant and non-pregnant rabbits ($P=0.42$ and $P=0.73$, respectively).

CONCENTRATIONS OF DNA

The mean concentration of DNA per milligram dry weight of tissue are given in Table II. The mean values for cartilage from multiparous and primigravida rabbits did not differ from those in their respective age-matched control groups ($P=0.525$, and $P=0.924$ respectively).

CONCENTRATIONS OF RNA

The mean concentration of total RNA per milligram wet weight of tissue are given in Table III. There was no significant difference between multiparous and age-matched control joints ($P=0.376$). In contrast, the concentration of RNA per milligram of tissue was significantly decreased in cartilage from primigravida joints. Compared with an age-matched control group, cartilage from primigravida animals contained 65% of age-matched control values for total RNA per milligram of cartilage ($P=0.0003$).

RT-PCR ANALYSIS

Transcript levels for a number of genes in cartilage from primigravida, multiparous and the two groups of non-pregnant control rabbits were analyzed by semiquantitative RT-PCR. Semiquantitive RT-PCR was performed for 29 day pregnant animals, and for non-pregnant, age-matched controls. Transcripts levels of estrogen receptor (ER), structural matrix macromolecules (COL2, aggrecan, biglycan, decorin and lumican), the proteinase urokinase and its inhibitor (PAI-1) as well as matrix metalloproteinase-1 (MMP-1 collagenase) and its inhibitor [tissue inhibitor of metalloproteinase (TIMP-1)], growth factors [basic fibroblast growth factor (bFGF), insuin-like growth factor 2 (IGF-2), transforming growth factor-β (TGF-β)], cytokines [IL-1β, tumour necrosis factor-α (TNF-α)] and enzymes whose products (prostaglandin E2, nitric oxide) have been implicated in cartilage metabolism (COX-2 and iNOS, respectively) [21, 22] were analyzed and are presented in Fig. 2 and 3, Tables IV and V.

ESTROGEN RECEPTOR

The mean level of ER mRNA in cartilage from multiparous rabbits was significantly elevated (180% of control) compared with control cartilage ($P=0.002$). In contrast, in cartilage from

Table II
DNA concentration per milligram of dry weight of articular cartilage

	Control 1 ($N=6$)	Primigravida ($N=6$)	Control 2 ($N=6$)	Multiparous ($N=6$)
DNA μg/mg tissue	3.72	3.63	3.5	3.46
S.D.	0.24	0.38	1.2	1.6
P-value	0.525		0.924	

Control 1 and Control 2 were groups of age-matched control rabbits for primigravida and multiparous, respectively.

Table III
Total RNA concentration per milligram of wet weight of articular cartilage

	Control 1 ($N=6$)	Primigravida ($N=6$)	Control 2 ($N=6$)	Multiparous ($N=6$)
RNA μg/mg tissue	0.26	0.16	0.14	0.18
S.D.	0.03	0.02	0.08	0.09
P-value	0.0003		0.376	

Control 1 and Control 2 were groups of age-matched control rabbits for primigravida and multiparous, respectively.

primigravida rabbits levels of ER mRNA were unchanged during pregnancy (Fig. 3).

MATRIX MACROMOLECULES, PROTEINASES AND INHIBITORS

COL2 (Fig. 2, Table IV) and biglycan (Table IV) mRNA levels were dramatically lower in cartilage from multiparous animals. The mRNA levels were, respectively, 25 and 20% of age-matched control values in cartilage from multiparous rabbits ($P=0.00002$ and $P=0.0002$, respectively). In cartilage from primigravida rabbits, the mRNA levels for COL2 and biglycan followed the same pattern, but the differences were not statistically different from their age-matched controls. Intriguingly, lumican expression was discoordinate (Table IV): compared to control values, mRNA levels in cartilage from multiparous animals were 130% of control values ($P=0.018$), whereas in cartilage from primigravida rabbits, RNA levels were 30% of control values ($P=0.0003$). In contrast, aggrecan and decorin mRNA levels did not change with pregnancy (Table IV).

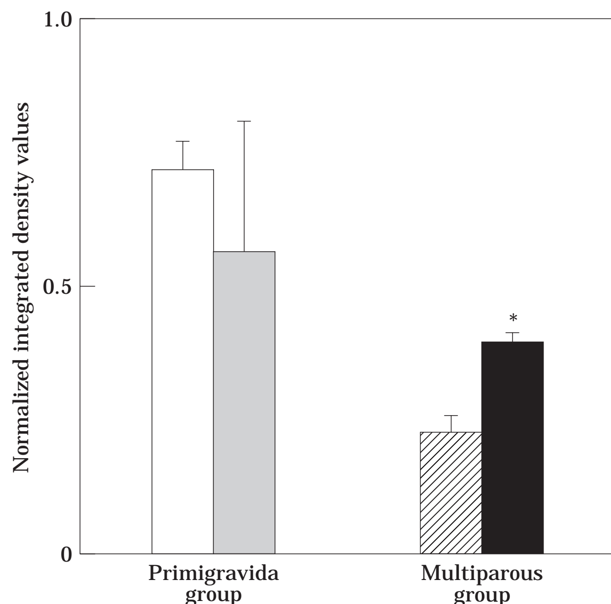

FIG. 3. Estrogen receptor mRNA levels in articular cartilage. Primigravida group: (■) pregnant rabbits; (□) age-matched controls. Multiparous group: (■) pregnant rabbits; (▨) age-matched controls. Values presented epresent the mean ± S.E.M. for each group ($N=6$). $^*P<0.05$ compared with controls.

Collagenase mRNA levels were lower in cartilage from both pregnant groups compared with their respective non-pregnant controls (Table IV). mRNA levels from cartilage from primigravida or multiparous animals were, respectively, 53 and 17% of control values ($P=0.045$ and $P=0.015$, respectively). TIMP-1 mRNA expression exhibited a similar pattern in both groups, however, compared to cartilage from control animals, the change was only significant in cartilage from multiparous animals ($P=0.018$) (Table IV). Urokinase mRNA levels were lower in both pregnant groups compared with controls, however the differences in transcript levels were only significant in cartilage from primigravida animals ($P=0.017$) (Table IV). PAI-I mRNA expression was not altered during pregnancy (Table IV).

GROWTH FACTORS

TGF-β mRNA levels were significantly lower in tissue from both primigravida and multiparous animals compared with tissue from their respective nonpregnant controls ($P=0.002$ and $P=0.009$, respectively) (Table V). Intriguingly, for bFGF and IGF2 (Table V), cartilage from primigravida and multiparous animals exhibited opposite patterns: mRNA levels in cartilage from primigravida animals were significantly lower than control values whereas cartilage from multiparous animals showed a significant elevation in mRNA levels. bFGF and IGF-2 mRNA levels were dramatically lower than control values in cartilage from primigravida animals ($P=0.005$ and $P=0.009$, respectively), whereas bFGF and IGF-2 RNA levels were elevated in cartilage from multiparous animals ($P=0.021$ and $P=0.017$, respectively).

Table IV
mRNA levels for matrix structural molecules, proteinases and inhibitors

Gene	Control	Primigravida	*P*-value
(a) Cartilage from primigravida versus control rabbits			
COL2	3.016 ± 0.483	2.284 ± 0.267	0.215
Aggrecan	0.587 ± 0.107	0.415 ± 0.206	0.234
Decorin	0.765 ± 0.047	0.629 ± 0.074	0.177
Lumican	5.61 ± 0.437	1.698 ± 0.336	**0.0003***
Biglycan	1.701 ± 0.513	1.488 ± 0.293	0.726
Collagenase	1.734 ± 0.322	0.962 ± 0.094	**0.045***
TIMP-1	0.907 ± 0.079	0.678 ± 0.098	0.099
Urokinase	0.682 ± 0.125	0.282 ± 0.062	**0.017***
PAI-1	0.472 ± 0.114	0.84 ± 0.174	0.109
(b) Cartilage from multiparous versus control rabbits			
COL2	2.388 ± 0.221	0.585 ± 0.091	**0.00002***
Aggrecan	0.606 ± 0.058	0.595 ± 0.065	0.899
Decorin	0.539 ± 0.047	0.590 ± 0.024	0.356
Lumican	0.995 ± 0.059	1.252 ± 0.069	**0.018***
Biglycan	0.246 ± 0.035	0.049 ± 0.005	**0.0002***
Collagenase	2.667 ± 0.763	0.434 ± 0.02	**0.015***
TIMP-1	1.78 ± 0.435	0.486 ± 0.138	**0.018***
Urokinase	0.299 ± 0.042	0.205 ± 0.042	0.14
PAI-1	0.383 ± 0.07	0.993 ± 0.346	0.114

Values represent averages of normalized integrated density for six rabbits and errors of mean. Significance was determined by analysis of variance ($P < 0.05$ was considered statistically).

Cytokines

Both IL-1β and TNF-α RNA levels were depressed in both pregnant groups compared with their respective controls (Table V). However, for TNF-α the differences were only statistically significant in cartilage from multiparous animals ($P = 0.003$).

cox-2 and inos

iNOS expression was dramatically lower in both pregnant groups compared to their respective control cartilage (Table V). The mRNA levels were 36% of control values in cartilage from primigravida animals ($P = 0.004$) and 45% of control values in cartilage from multiparous

Table V
mRNA levels for cytokines, growth factors and iNOS and COX-2

Gene	Control	Primigravida	*P*-value
(a) Cartilage from primigravida versus control rabbits			
IL-1β	1.02 ± 0.092	0.416 ± 0.044	**0.0001***
TNF-α	0.916 ± 0.172	0.585 ± 0.097	0.124
TGF-β	1.377 ± 0.147	0.598 ± 0.111	**0.002***
bFGF	7.637 ± 1.026	4.623 ± 0.403	**0.021***
IGF2	3.461 ± 0.373	1.944 ± 0.289	**0.009***
iNOS	6.851 ± 1.166	2.506 ± 0.16	**0.004***
COX-2	1.354 ± 0.109	1.203 ± 0.327	0.669
(b) Cartilage from multiparous versus control rabbits			
IL-1β	1.126 ± 0.304	0.64 ± 0.179	**0.0001***
TNF-α	1.603 ± 0.297	0.441 ± 0.053	**0.003***
TGF-β	0.687 ± 0.93	0.303 ± 0.075	**0.009***
bFGF	0.163 ± 0.034	0.297 ± 0.018	**0.005***
IGF2	0.835 ± 0.045	1.246 ± 0.136	**0.017***
iNOS	4.826 ± 1.074	2.235 ± 0.359	**0.045***
COX-2	0.885 ± 0.101	0.491 ± 0.092	**0.016***

Values represent averages of normalized integrated density for six rabbits and standard errors of mean. Significance was determined by analysis of variance ($P < 0.05$ was considered statistically significant).

rabbits ($P=0.045$). COX-2 followed the same pattern in both pregnant groups compared with controls, however the differences in transcript levels between pregnant and respective control values were only significant in cartilage from multiparous animals ($P=0.016$) (Table V).

Discussion

The results presented in this report indicate that pregnancy can exert both general and specific effects on RNA levels in articular cartilage of the rabbit knee, dependent on the skeletal maturity and/or the parity of the animals. No variations in DNA content were found during pregnancy, suggesting that the number of cells did not change. Given the reported similarities in cartilage weights between joints of non-pregnant and pregnant rabbits, it would appear that the observed alterations in total RNA levels during primigravida pregnancy reflect a real decrease in both ribosomal RNA (rRNA) and mRNA levels (as total RNA is approximately 80% rRNA + mRNA + tRNA). Thus, cells in articular cartilage from primigravida animals contained 67% of the synthetic activity of control cartilage, indicating that during pregnancy, chondrocyte metabolism is likely depressed. In contrast, RNA (rRNA + mRNA + tRNA) levels in skeletally mature cartilage from multiparous animals were similar to those in controls. However, as total RNA levels are 80% rRNA plus mRNA and tRNA, the findings indicate both a general and a specific effect on metabolism during pregnancy in skeletally immature primigravida females, but only specific effects in skeletally mature multiparous rabbits. While the reported studies do not address the mechanism for these differences, perhaps the mediator(s) of the observed changes interfere with growth conditions in the adolescent animals more effectively than homeostatic conditions in the skeletally mature animals. Alternatively, the differences could be due to parity, with the primigravida responding to pregnancy for the first time rather than the third time. Possibly, the post-partum environment does not revert to pre-pregnancy conditions and thus influences the outcome.

In the present study, none of the mRNA species assessed were over-expressed in cartilage from primigravida animals. In addition, in cartilage from multiparous animals, with the exception of ER, bFGF, IGF2 and lumican, the overall influence of pregnancy was to decrease steady-state mRNA levels. This general decrease in mRNA levels was particularly marked for cytokines such as IL-1β, TNF-α, and TGF-β. Inducible NOS and COX-2 mRNA levels were also depressed. All of these molecules are reported to regulate cartilage metabolism [21–26]. Because these molecules play such a major role, variations in levels of these factors could influence the regulation of articular cartilage homeostasis and response to injury or stresses.

Furthermore, transcript levels for the matrix molecules COL2 and biglycan, but not aggrecan, were dramatically decreased in articular cartilage from multiparous animals. COL2 is the major collagen component of the extracellular matrix of articular cartilage. The collagen framework provides tensile strength to the tissue. Biglycan is a small non-aggregating proteoglycan that has been shown to interact with COL2 *in vitro* [27], however, biglycan function in articular cartilage is presently poorly understood. Aggrecan is the major aggregating proteoglycan of articular cartilage. Interestingly, aggrecan mRNA levels were not modified during pregnancy. Moreover, transcript levels for lumican a small nonaggregated proteoglycan were elevated. Such a potential dysregulation of proteoglycan homeostasis during pregnancy coupled with alterations in expression of mediators which can influence proteoglycan catabolism (i.e. IL-1) could lead to changes in cartilage function.

The structural integrity of articular cartilage is maintained because of the ability of chondrocytes to synthesize and degrade the extracellular matrix molecules. Collagenase is a MMP that is secreted by chondrocytes and activated in the extracellular matrix as a proteolytic enzyme [28]. TIMPs are the most important physiological inhibitors of the activity of active metalloproteinases (i.e., collagenase). TIMP-1 is known to inactivate active metalloproteinases by forming a high-affinity complex [29]. We found a significant decrease in mRNA levels during pregnancy for both collagenase and its inhibitor. UK is a proteinase which can activate plasminogen to plasmin, an enzyme known to be capable of degrading some matrix molecules [30] and activating MMPs [31]. Interestingly, mRNA levels for UK were also decreased during pregnancy, but mRNA levels for its inhibitor PAI-1 were again unchanged. Therefore, if the mRNA levels reflect protein and activity levels, such a situation could lead to decreased enzyme activity due to enhanced enzyme-inhibitor complex formation [30]. While such a state may not favor cartilage degradation, the combination of altered matrix synthetic capabilities plus depressed matrix remodeling activities (i.e., proteinases) could lead to

modification of repair function in the tissue. Whether, the mRNA changes are reflected by alterations in enzyme activity must await further studies.

In the present report, pregnancy appears to lead to an increase in the steady-state mRNA levels for the growth factors bFGF and IGF2. These findings suggest that the influence of pregnancy on mRNA levels in articular cartilage from multiparous rabbits is gene specific, as we reported both increases and decreases in mRNA levels. In contrast, articular cartilage from primigravida rabbits exhibited a change leading to lower mRNA levels for matrix molecules (lumican and collagenase) as well as IL-1β, iNOS and the GFs: TGF-β, bFGF and IGF2. These results are similar in some respects to those of Rosner *et al.* [14] who found that estrogen treatment reduced proteoglycan synthesis as well as proteoglycan catabolism in both normal and OA cartilage. These authors further demonstrated that estrogen treatment worsened OA lesions, probably because of an alteration of the capacity of repair of AC in relation with the hypometabolism induced [32].

The results presented in this report with regard to estrogen receptors are consistent with previous reports that demonstrated the existence of ER at the protein level in canine and rabbit articular cartilage [9–11]. We observed that ER transcripts are present in RNA isolated from articular cartilage derived from both pregnant and non-pregnant rabbit knees. Such findings imply that articular cartilage should potentially respond to estrogens if the ligand is supplied in sufficient concentration. Another interesting finding in the present report is the observation that in articular cartilage from multiparous rabbits, message levels for the ER are influenced by pregnancy. The mechanism(s) by which levels for ER transcripts are regulated during pregnancy are unknown at the present time. However, many endocrine and immunologic changes occur during pregnancy so it may be difficult to pinpoint the exact mediator responsible for influencing ER expression. Nevertheless, we found that ER mRNA levels were increased in cartilage from multiparous animals. Assuming such increases are translated to functional receptors, such an increase of ER mRNA levels during pregnancy may have physiological consequences.

While estrogen and ERs may play a role in alterations in cartilage metabolism during pregnancy, the peptide hormone relaxin may also be involved in the observed changes. Relaxin is a member of the IGF family which is expressed during pregnancy [33]. Serum levels of relaxin increase during pregnancy, reaching their maximum levels by 15 days gestation in the rabbit which then decline rapidly 1–2 days post-partum [34]. The hormone has been shown to decrease collagen deposition in animal models of fibrosis [35] and reported to decrease collagen synthesis by rabbit chondrocytes *in vitro* [16]. Furthermore, relaxin has also been reported to upregulate the nitric oxide pathway in other tissues [36]. As neither collagenase nor iNOS were upregulated in cartilage from primigravida or multiparous rabbits in response to pregnancy, either the changes observed were due to a variety of stimuli or the pattern of responsiveness is due to intrinsic characteristics of cartilage, compared to other tissues.

In summary, a complex set of alterations in mRNA levels for a number of genes occur during pregnancy. Further analysis of the influence of pregnancy on articular cartilage metabolism at the protein level may provide new clues to the regulation of cartilage function, as well as new insights into gender-specific differences in the maintenance of this tissue.

Acknowledgments

The authors acknowledge the support of the Medical Research Council of Canada, The Arthritis Society and La Société Francaise de Rhumatologie for these studies. MPHLG is supported by a Fellowship from The Arthritis Society and DAH is the Calgary Foundation-Grace Glaum Professor in Arthritis Research. The authors thank Dr E. Vignon for reviewing the manuscript. The authors thank also Ray Boykiw and Paul Sciore for assistance in molecular aspects of the study.

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