

Prothrombin Gene Expression in Articular Cartilage with a Putative Role in Cartilage Degeneration Secondary to Joint Immobility

GUY TRUDEL, HANS K. UHTHOFF, and ODETTE LANEUVILLE

ABSTRACT. *Objective.* To test the hypothesis that thrombin is expressed by chondrocytes from human and animal articular cartilage and to monitor its levels of expression during cartilage degeneration induced by joint immobility in a rat model.

Methods. Rat knees were immobilized for periods of 2 or 4 weeks, after which the articular cartilage was harvested, total RNA extracted, and the differential display (ddPCR) protocol applied to identify differentially expressed genes. One differentially expressed fragment showed 100% homology with the prothrombin gene. Results were verified by RT-PCR, Northern and Western blot analysis, and immunohistochemistry in human, rat, and rabbit articular cartilage.

Results. In our rat model of cartilage degeneration induced by joint immobilization, increases in the levels of prothrombin mRNA, thrombin protein, and fibrin deposition were observed. Expression of the prothrombin gene by chondrocytes was confirmed by ddPCR (rat), RT-PCR (rat and human), and by Northern blot analysis (rabbit). In addition, thrombin-like immunoreactivity was increased in chondrocytes after a 4 week immobilization period compared with rat knees receiving sham surgery. Thrombin activity was reflected by the presence of fibrin immunoreactivity in operated rat knee joints.

Conclusion. Articular chondrocytes express the prothrombin gene and its local expression in joints is translated into thrombin protein. Prothrombin expression is increased in response to joint immobility. Our results support generation of thrombin locally in joints and an upregulation of thrombin expression in cartilage degeneration secondary to immobility. These results may provide information on the source of increased thrombin activity in various animal models and in clinical forms of arthritis. (J Rheumatol 2005;32:1547–55)

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The sequence of cellular and molecular events leading to cartilage degeneration is unknown, and is likely to involve both external factors from the synovium as well as factors intrinsic to chondrocytes residing in the articular cartilage¹. In rheumatoid arthritis (RA), the initial steps of the inflammatory reaction in the synovium are attributed to an immunological reaction, while its maintenance has been

postulated to depend on the coagulation and fibrinolysis pathways^{2,3}. Evidence for the activation of the coagulation pathway in RA originated from the observation of frequent and persistent intraarticular fibrin deposits in the synovium, synovial fluid (SF), and articular surface in joints from patients with RA⁴⁻⁶. Fibrin formation is the final step of the coagulation cascade, and results from the conversion of fibrinogen to fibrin by the action of thrombin⁷. Evidence for the importance of thrombin in RA consists of reports of increased thrombin activity in the joints from numerous experimental arthritis models and in human RA⁸⁻¹¹. In addition, neutralizing thrombin activity led to an improvement of markers of inflammatory arthritis in a collagen induced arthritis model^{12,13}.

In addition to maintaining the inflammatory reaction in the synovium, thrombin participates directly in degradation of the cartilage matrix by inducing the release of proteoglycans¹⁴. When prothrombin plus its physiological activator, factor Xa, were incubated with normal human cartilage, proteoglycan degradation occurred¹⁴. There are few data on the source of elevated thrombin found in the arthritic joints.

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Leakage of prothrombin, its activating factors, and fibrinogen from microvessels of the synovial membrane is the current theory to explain increased thrombin activity and presence of fibrin in the arthritic joint. To test this theory, a study was conducted to compare plasma levels and SF levels of thrombin-antithrombin III and of thrombin fragments in RA patient samples⁹. These indicators of the presence of thrombin were increased in the plasma and SF of RA patients compared to controls or to patients with osteoarthritis (OA). The extent of the increase was very different: 10-fold higher levels in plasma and a 200-fold elevation in the SF⁹. Finding higher thrombin activity in the SF than in plasma suggested synthesis by local tissues. To our knowledge, this has never been tested directly.

Our group and others have demonstrated that joint immobility leads to cartilage degeneration sharing histological features with RA and OA¹⁵⁻¹⁹. Changes include increase in cartilage surface irregularity¹⁹, loss of chondrocytes^{20,21}, and a reduction in proteoglycan synthesis and content²². Articular cartilage from immobilized joints was used to perform genetic analysis of gene expression. Using the method of differential display polymerase chain reaction (ddPCR), we identified the prothrombin gene as being upregulated after 2 and 4 weeks of complete joint immobility when compared to sham operated animals (Figure 1). This observation, along with reports from others^{4,6,8-11} indicating that thrombin was increased in arthritic joints, prompted us to test the hypothesis that thrombin is generated by the chondrocytes of the articular cartilage. Our results indicated that both prothrombin mRNA and thrombin protein were generated in chondrocytes from human, rat, and rabbit articular cartilage. In a rat model of cartilage degeneration induced by joint immobility, levels of prothrombin mRNA and thrombin protein were increased in comparison to non-oper-

ated or sham operated joints. In addition, fibrin immunoreactivity was detected in operated animals, and is indicative of the presence of thrombin activity in degenerating articular cartilage.

MATERIALS AND METHODS

Cartilage samples and rat knee joint immobilization. Adult male Sprague-Dawley rats with an average weight of 355 g underwent surgery. One knee joint was immobilized in flexion using an internal fixation system we have developed^{23,24}. Briefly, a rigid DelrinTM plastic plate was fixed at one end to the proximal femur and at the other end to the distal tibia with one screw inserted in each bone. The knee joint space, capsule, and cartilage were untouched. Sham operated animals had holes drilled and screws inserted but none of these specimens were plated. For each series of experiments, the number of rats used for each timepoint in each group of sham operated and immobilized joints are: 3 for ddPCR, 3 for RT-PCR, 3 for Western blotting, and 30 for immunohistochemistry. Twelve non-operated age matched rats were also used. Animals were housed individually and articular cartilage was harvested after 2 or 4 weeks for mRNA analysis, and whole knees processed after 2, 4, 8, 16, or 32 weeks for histological analysis. Three adult male New Zealand White rabbits were anesthetized and articular cartilage was obtained from the knee joint. Human articular cartilage was obtained from the calcaneus bone of 3 consecutive cadavers (males aged 46, 48, and 81 yrs) at autopsy.

Total RNA isolation. Articular cartilage was peeled off the tibial condyles and femur with a scalpel and stored in RNA Later solution (Qiagen, Mississauga, ON, Canada) at -80°C. Total cellular RNA was extracted from the cartilage samples with Trizol reagent according to the manufacturer's protocol. Total RNA pellets were dissolved in DEPC water and RNA integrity was confirmed by ethidium bromide staining after fractionation on a 1% agarose gel. Total RNA concentrations were determined by spectrophotometry. The same procedure of RNA isolation was used for animal and human cartilages.

Differential display PCR. The profile of expression of genes in the articular cartilage was analyzed by the ddPCR method. To identify differentially expressed genes, we compared samples from sham operated animals to immobilized joints. Purified total RNA was reverse transcribed using the RNImage kit (GenHunter Corp., Nashville, TN, USA)²⁵ and anchored oligo-dT 3' primers in a mixture that consisted of 200 ng total RNA, 100

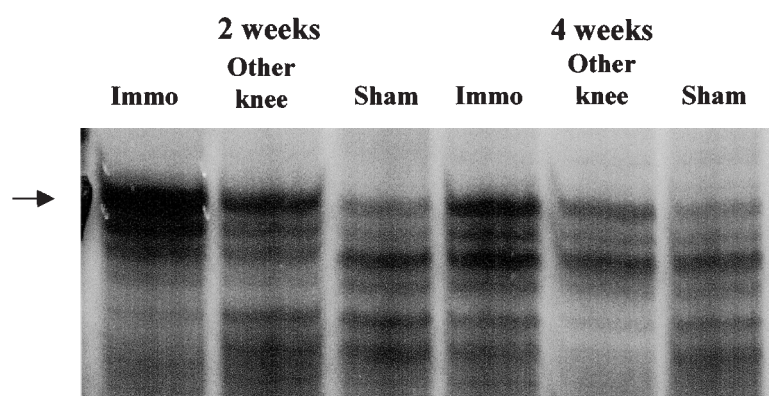


Figure 1. Differential display-PCR (ddPCR) detection of prothrombin. Prothrombin mRNA is expressed in the rat articular cartilage. ddPCR was performed using total RNA prepared from articular cartilage obtained from immobilized (Immo) or the nonoperated leg contralateral to the immobilized (Other knee) or sham operated rat knees. The 150 bp prothrombin cDNA fragment (arrow) was amplified by PCR using H-AP3 sense and H-T₁₁C anchor antisense primers and subcloned in the TA vector for sequence analysis. Three separate clones were sequenced with the T3 and T7 primers and showed 100% homology to the rat prothrombin cDNA (accession number M81397).

units of MMLV reverse transcriptase, 20 μ M dNTP, 1 μ M 3' primer, 25 mM Tris HCl, 37.6 mM KCl, 1.5 mM MgCl₂, and 5 mM dithiothreitol (DTT) in a final volume of 20 μ l for 1 h at 37°C. Separate reactions were performed for each of the 3' primers: H-T₁₁G, H-T₁₁A, and H-T₁₁C. The resulting cDNA was amplified by PCR using arbitrary 5' primers (H-AP1 to H-AP8) in conjunction with the 3 original oligo-dT primers in the presence of ³³P-dATP. A 2 μ l aliquot of the reverse transcription reaction was amplified in a 20 μ l final reaction volume using 1 unit of Taq DNA polymerase (Qiagen, Mississauga, ON, Canada) in the presence of 2 μ M dNTP, 0.2 μ M 5' primer, 1 μ M 3' primer, 10 mM Tris HCl, 50 μ M KCl, 1.5 mM MgCl₂, and 0.4 μ M ³³P-dATP. The PCR cycling parameters were: 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s for 40 cycles, followed by a 5 min period at 72°C. The resulting 24 sets of amplified cDNA fragments were displayed on a 6% polyacrylamide sequencing gel. The polyacrylamide gels were transferred onto Whatman 3MM paper, dried without fixation, and exposed to autoradiographic films overnight. Films were visually inspected for differentially expressed cDNA bands.

Cloning and sequencing of PCR products. Upon identification of differentially expressed cDNA bands on the gel, we proceeded with cloning and sequencing experiments to determine their identity. Differentially expressed cDNA bands were excised from the dried gel and reamplified by PCR using the corresponding original set of primers. The final reaction products were affinity purified using the QIAquick PCR Purification Kit (Qiagen) and subcloned in the TA vector using T4 DNA ligase (Qiagen). Plasmid DNA was introduced into the JM-109 bacterial strain and grown overnight. Plasmid DNA was isolated, digested with *Eco* RI, and resolved on a 1% agarose gel to confirm the integrity and size of the insert DNA. Sequencing was then performed using SP6 and T7 primers and the Sequenase v2.0 kit (Amersham, Baie d'Urfe, Quebec, Canada). Sequences were determined on both strands and then analyzed against the GenBank database through the BLAST alignment program.

RT-PCR amplification. A second method was used to confirm the differential expression of the prothrombin gene by chondrocytes of the articular cartilage. Increase of expression of the prothrombin gene was confirmed by RT-PCR using primers annealing in the open reading frame of this gene. RNA samples were treated with DNase (Qiagen) to eliminate genomic contamination. First-strand synthesis was carried out using 250 ng RNA in a final volume of 20 μ l containing 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 250 μ M each dNTP, 0.5 μ l of hexanucleotide mixture, and 100 U MMLV reverse transcriptase at 37°C for 2 h. PCR was performed using 2.5 μ l of the first-strand reaction in a 50 μ l reaction volume containing 0.3 μ M prothrombin primers, 10 mM Tris-HCl, 75 mM KCl, 1.5 mM MgCl₂, 200 μ M each dNTP, and 1 U Taq DNA polymerase. The reaction mixture was heated at 95°C for 5 min to denature the DNA and then subjected to 28 cycles of PCR (94°C for 1 min, specific annealing temperature for 1 min, 72°C for 2 min) followed by a final extension at 72°C for 5 min. The rat prothrombin primers (sense 5'-CAA GGC CTC TAC CCG GAT ACG-3' and antisense 5'-GTG CGT GTA GAA GCC ATA TTT CC-3') generated a 208 bp fragment as described²⁶. The human prothrombin primers (sense 5'-GGT GCG CAT TGG CAA GCA C-3' and antisense 5'-AGG GTC CCC CAC TGT CAC-3') generated a 446 bp fragment as described²⁷. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level was used to control for variations in loading the gels. Primers used to generate the rat GAPDH probe were as described and corresponded to: 5'-TCC TTG CAC CAC CAA CTG CTT A-3' sense and 5'-ACC ACC CTG TTG CTG TAG CCA-3' antisense and generated a 523 bp fragment. Primers used to generate the human GAPDH probe were as described and corresponded to: 5'-CCA CCC AAT GGC AAA TTC CAT GGC A-3' sense and 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3' antisense and generated a 598 bp fragment. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining. The identity of the PCR products was confirmed by digestion with restriction enzymes (human prothrombin digested with *Pst* I generates 303 bp and 143 bp fragments; human GAPDH digested with *Sac* II generates 449 bp and 149 bp fragments; rat prothrombin digested with *Kpn* I generates 142 bp and 65 bp

fragments; rat GAPDH digested with *Apa* I generates 355 bp and 168 bp fragments) and analysis of fragments on agarose gels. Primers were synthesized by the Biotechnology Center at the University of Ottawa and restriction enzymes were obtained from New England BioLabs (Mississauga, ON, Canada).

Northern blotting. The identity and increase of expression of the prothrombin gene were confirmed by Northern blotting. This alternative method reveals differences in the steady-state levels of the transcripts as well as the size of the transcript. Total RNA was extracted from rabbit articular cartilage or liver using Trizol reagent and the protocol provided by the supplier. Poly(A)⁺ RNA was purified from 250 μ g total RNA with a poly(A)⁺ extraction kit (Oligotex mRNA kit, Qiagen) in accord with the supplier's instructions. Briefly, mRNA were resolved on a 1% agarose/formaldehyde gel at 120 V for 3 h. After electrophoresis, mRNA were transferred from the gel to a nylon Bodine membrane by capillarity. Membranes were baked under vacuum for 2 h at 80°C to immobilize the mRNA. Hybridization was carried out with a ³²P-radiolabelled cDNA probe corresponding to rat prothrombin or to rat GAPDH and generated by RT-PCR as described in the previous section. Bands were detected using autoradiography (Kodak X-OMAT, Perkin Elmer, Mississauga, ON, Canada).

Western blotting. Expression of the prothrombin gene at the protein level was confirmed by Western blotting and an antibody specific for thrombin. Articular cartilage was peeled radially with a scalpel, preserved in 0.1 M Tris, pH 7.4, and frozen at -20°C before processing. Samples were sonicated twice (20,000 Hz, 15 s each time) and total protein concentration was measured using the bicinchoninic acid assay (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as the protein standard. Fifty micrograms of total protein from cartilage homogenates were denatured in boiling Laemmli buffer for 3 min, and proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel as described²⁸. Following electrophoresis, proteins were transferred onto nitrocellulose membranes. Membranes were blocked in 3% milk/Tris-buffered saline (TBS)/0.1% Tween-20 overnight at 4°C and then incubated with primary antibodies. A primary sheep anti-human purified polyclonal thrombin antibody (1:200) was used (antibodies specific for thrombin and mild crossreactivity to fibrinogen; Affinity Biologicals, Ancaster, ON, Canada). Immunoreactive protein bands were detected by incubation with goat anti-sheep IgG conjugated with horseradish peroxidase (Promega, Madison, WI, USA) followed by incubation with the chemiluminescence reagents (Roche Diagnostics, Laval, QC, Canada). The membranes were photographed with a Polaroid camera. The antithrombin antibody was then stripped off using a solution of TBS/ β -mercaptoethanol (100 μ mol/l)/2% SDS (incubation at 60°C for 30 min). The same membranes were washed with TBS/0.1% Tween 20 and reprobed with a rabbit polyclonal anti- β -actin antibody (Sigma, St. Louis, MO, USA) to ensure equal loading of total protein in wells. Purified rat and human thrombin (Sigma, Oakville, ON, Canada) and Kaleidoscope prestained standards (Bio-Rad, Mississauga, ON, Canada) were used as standards.

Immunohistochemistry. The presence of thrombin protein in the articular cartilage was also confirmed by immunohistochemistry. Rats were euthanized. Immobilized and sham operated knees were harvested and fixed in Bouin for 18 h at 4°C. Then specimens were decalcified in 10% EDTA for 2 mo at 4°C, changing the EDTA solution every second day. The specimens were then embedded in low melting-point paraffin (51–54°C; Oxford Labware, St. Louis, MO, USA). Embedded tissues were cut into 7 μ m-thin sagittal sections from the lateral to the medial side of the joint. Standardized serial sections of the medial mid-condylar regions of the knee were stored. The slides were processed to water, then transferred to phosphate buffered saline (PBS). All slides from immobilized and sham operated animals were stained in one session using the standard protocol as follows: endogenous peroxidase blocking was done with 3% H₂O₂ for 30 min at room temperature, followed by PBS rinse for 20 min. The slides were incubated with 0.3% BSA in normal goat serum (Sigma, St. Louis, MO, USA) at room temperature for 20 min to block nonspecific protein and non-

immune immunoglobulin affinities. The slides were washed again in PBS for 20 min. The slides were first incubated with the same primary antibody as for the Western blot, at a dilution of 1:59 in PBS overnight at room temperature, then rinsed in PBS. The slides were then incubated with a biotinylated rabbit anti-goat antibody (Dako, Glostrup, Denmark) for 30 min and then rinsed in PBS. A third incubation was done with streptavidin conjugated horseradish peroxidase solution for 45 min (BioGenex/Esbe, Markham, ON, Canada). Chromogene marking was performed with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.1 M imidazole, 0.03% H₂O₂ in PBS for 2–7 min. Counterstaining of slides was done with Mayer's hematoxylin for 10 min. For the negative controls, the primary antibody was omitted. For fibrin, we randomly selected a total of 10 slides from normal, 2 week immobilized and sham operated, and 4 week immobilized and sham operated. We used a similar protocol as for thrombin. A mouse anti-human fibrin monoclonal antibody was used as primary. Secondary was a biotinylated goat anti-mouse antibody (BioGenex, San Ramon, CA, USA) and the same streptavidin conjugated horseradish peroxidase.

Statistical analysis — immunohistochemistry. The intensity of thrombin-like immunohistochemical staining was measured with a light microscope by conventional histological analysis, where a grade of 0 designated absence of staining, 1 described weak staining, 2 moderate staining, and 3 intense staining. On a sagittal knee joint histological section, we determined one anterior and one posterior site on each of the femur and tibia, and the staining intensity of both the chondrocytes and the matrix were assessed at these 4 locations. Fifteen chondrocytes were analyzed at each articular cartilage site and the average staining of all 4 sites constituted chondrocyte staining intensity. Similarly, average matrix staining of the 4 sites constituted matrix staining intensity. Two investigators, blinded to which knee was studied, viewed each slide simultaneously to standardize the sites of measurement and recorded their staining scores independently. Data from both investigators were averaged to obtain the final grade given at each site.

SPSS for Windows v. 11.0 (SPSS Inc., Chicago, IL, USA) was used to create the database and perform statistical testing. Nonparametric statistics were used to account for unequal group sizes, ordinal variables, and small final sample sizes in each subgroup. We analyzed the database to find an effect of intervention (immobilization, sham operation, or normal) on thrombin staining intensity in chondrocytes and in cartilage matrix by Kruskal-Wallis tests at each timepoint. Post-hoc analyses of the statistically significant comparisons were carried out at each timepoint to detect the variables responsible for the initial difference in staining using Mann-Whitney tests. A *p* value < 0.05 was interpreted as statistically significant.

RESULTS

Prothrombin gene expression by chondrocytes. The use of 8 different pairs of arbitrary primers in a differential display protocol led to the identification of several differentially expressed genes in the articular cartilage from immobilized knees as compared with sham operated knees. Figure 1 shows a differential display gel, with an arrow indicating upregulation at 2 and 4 weeks of knee immobilization compared to cartilage samples from sham operated joints and from the leg contralateral to the immobilized one. The upregulated band was excised from the gel and reamplified by PCR using the same pairs of primers; H-T₁₁A and AP3. The amplified band was 150 bp in size and was subcloned in the TA vector, sequenced, and compared with the GenBank database through the BLAST alignment program to obtain identity. The clone showed 100% sequence homology with the 3' end of the rat prothrombin mRNA (accession number M81397).

Expression of the prothrombin gene by cartilage was confirmed by RT-PCR using primers in the open reading frame; for the rat, sequences 1596 to 1616 and 1781 to 1803, and for the human, sequences 1250 to 1268 and 1678 to 1695 corresponding to amino acids Lys533–His601. Amplified products were of expected sizes based on their migration in agarose gels — 208 bp for rat and 446 bp for human prothrombin and 523 bp and 598 bp for rat and human GAPDH, respectively (Figure 2). No PCR products were detected when the RT step was omitted (Figure 2A, lines 5 and 7; Figure 2C, lines 15 and 17) or in the absence of cDNA template (Figure 2A, lines 4 and 6; Figure 2C, lines 14 and 16). The identity of the PCR product as prothrombin and GAPDH sequences was confirmed by the presence of restriction sites at expected locations (Figure 2B and 2D). Expression of the prothrombin gene was confirmed by RT-PCR in the articular cartilage from human calcaneus bone and from nonoperated rat knees. Expression of the prothrombin gene was also confirmed by Northern blots of poly(A)+ selected RNA from rabbit articular cartilage (Figure 3). Prothrombin transcripts of 2.3 kb were detected in rabbit articular cartilage and liver. Expression of the prothrombin gene by chondrocytes of the articular cartilage was confirmed by 3 approaches in 3 different species: rat, human, and rabbit.

Thrombin protein detected in articular cartilage.

Expression of the prothrombin gene at the protein level was evaluated by Western blotting using thrombin antibodies, as described in Materials and Methods. In the rat knee articular cartilage, thrombin was detected as a 37 kDa protein migrating the same distance as the purified thrombin protein standard (Figure 4). Cartilages from nonoperated, immobilized, and sham operated rats all contained thrombin at comparable levels. Human cartilage obtained post-mortem also included immunoreactive thrombin comigrating with the purified human thrombin standard (Figure 4).

Thrombin protein localized to the cytosol of chondrocytes, present in the matrix, and elevated after immobilization. Positive thrombin-like immunoreactivity was detected in 7 µm-thin paraffin embedded sagittal sections of rat knees using the same antithrombin antibody used for Western blotting (Figure 5). The staining in chondrocytes was cytosolic (Figure 5D) and was also present in the extracellular matrix (Figures 5B, 5C). Increased staining was observed both in the chondrocytes at 4 weeks (Figures 5D, 5E) and in the matrix at 2 weeks (Figures 5B, 5C). Thrombin-like immunoreactivity in cartilage matrix showed no tropism, being present in all layers of uncalcified and calcified cartilage (Figure 5D). In some sections, there was a clear band (minor staining) in the intermediate zones of chondrocytes, with more intense matrix staining in the deep and superficial zones (sandwich staining). Increase in thrombin staining was observed in both sham operated and immobilized animals 2 weeks after surgery. The use of sham operated

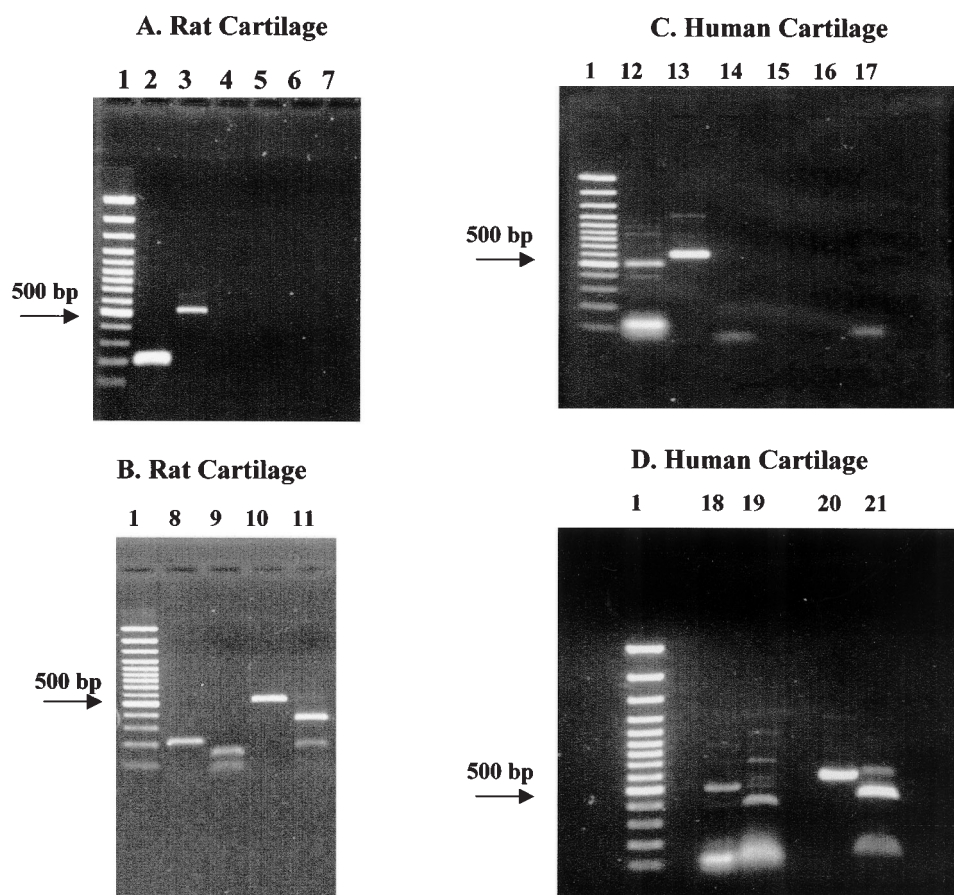


Figure 2. RT-PCR detection of prothrombin. Prothrombin mRNA is expressed in articular cartilage from human and rat joints. RT-PCR was performed on 200 ng total RNA extracted from rat (A, B) or human (C, D) articular cartilage obtained from nonoperated animals and from 3 cadavers at autopsy. Prothrombin primers amplified regions of the open reading frame for the rat and human sequences. GAPDH was used as a control. Resulting PCR products were resolved through 1.2% agarose gel stained with ethidium bromide. PCR products migrated at the expected sizes based on the size marker (lane 1); for prothrombin: 208 bp (rat, lanes 2, 8) and 446 bp (human, lanes 12, 18); for GAPDH: 523 bp (rat, lanes 3, 10) and 598 bp (human, lanes 13, 20); no products were detected in the absence of mRNA (lanes 4, 6, 14, 16) or in absence of RT (lanes 5, 7, 15, 17). Identity of bands was confirmed by restriction analysis of PCR products generating fragments migrating at expected sizes (lanes 9, 11, 19, 21). Gels are representative of 3 independent experiments run with samples from 3 different rats or cadavers.

legs as controls allows one to distinguish the effect of immobilization from that of the surgical procedure.

Thrombin staining intensity was quantified (Table 1). Immobilization significantly increased thrombin-like staining intensity in chondrocytes and extracellular matrix: chondrocytes showed higher levels of staining compared to sham operated animals at all times after 2 weeks, significant at 4 weeks ($p < 0.05$; Figure 5). Staining was greater in chondrocytes of immobilized knees compared to normal cartilage at all timepoints, significantly so at 2, 4, 8, and 32 weeks after immobilization (all $p < 0.05$). Sham surgery showed no significant effect on chondrocyte staining compared to normal specimens (all $p > 0.05$).

Immobilization and sham operation both led to more intense thrombin-like immunoreactivity in the cartilage matrix compared to controls (Table 1). Immobilized speci-

mens showed statistically higher values at 2, 4, 16, and 32 weeks and sham operated specimens after 2, 4, 8, and 16 weeks compared to normal (all $p < 0.05$). Despite this sizable modulation, no statistically significant difference was found between immobilized and sham operated specimens in the matrix.

DISCUSSION

The main findings of our study are: (1) that chondrocytes express the prothrombin gene; and (2) that increased levels of thrombin protein are found in the cartilage of joints subjected to immobilization. Previous observations indicate that thrombin acts directly on the cartilage matrix to release proteoglycan, suggesting a direct role in the process of cartilage degeneration¹⁴.

Several lines of evidence suggest that the presence of

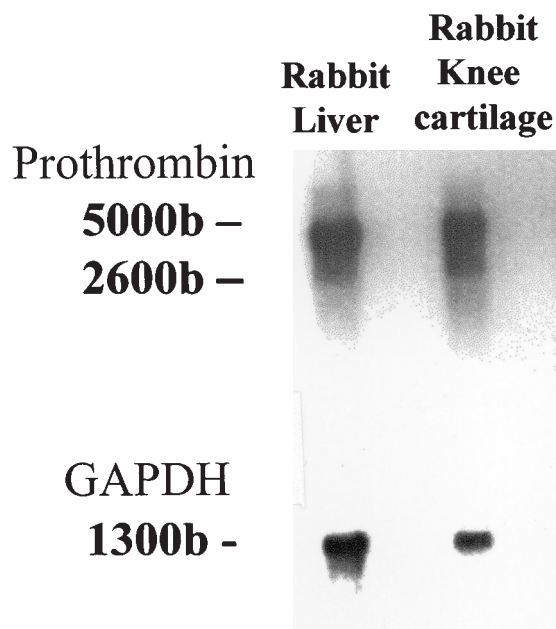


Figure 3. Northern blot detection of prothrombin. Prothrombin mRNA is expressed in rabbit articular cartilage and liver. Total RNA was isolated from rabbit knee cartilage or liver. Five micrograms were subjected to Northern blot analysis and probed with the 208 bp rat prothrombin probe generated by PCR. GAPDH was detected with the 523 bp rat probe and used as a control. Autoradiographs are representative of 3 independent experiments run with samples from 3 different animals.

thrombin in the joint is deleterious. Perhaps the most convincing substantiation is that fibrin deposits in the joint are one of the histological hallmarks of inflammatory arthritis^{3,6}. The mechanisms by which thrombin exerts deleterious effects on cartilage have been identified and are 2-fold. The first mechanism is the catalytic conversion of fibrinogen into fibrin, which is proinflammatory for the synovium³. A single injection of a low dose of thrombin into the rat knee joint space induced an inflammatory response²⁹. The mechanism proposed involves the activation of synovial fibroblasts by their contact with intraarticular fibrin³⁰. Because fibrin is an insoluble protein, clearance is reduced and its persistence would participate in the chronicity of the disease³¹. *In vivo* experiments support the involvement of thrombin in the inflammation of the synovium. Hirudin, a thrombin inhibitor, reduced joint inflammation and intraarticular fibrin¹². The second mechanism for deleterious activity of thrombin on articular cartilage is the degradation of the matrix through the release of cartilage proteoglycan¹⁴.

While markedly heightened activity of elements of the coagulation pathway leading to fibrin formation has been described in OA and RA joints, the origin of prothrombin remains unknown. The suggestion that plasma fibrinogen, prothrombin, and factor Xa diffuse into the joint cavity implies a blood origin for thrombin³. To assess the blood circulation as a potential source of procoagulant molecules in SF, plasma and SF were compared in patients with arthritis⁹.

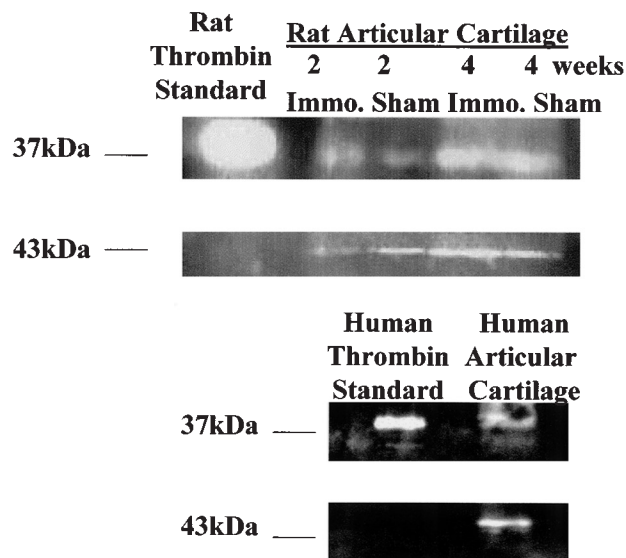


Figure 4. Western blot analysis of thrombin. The thrombin antibody was specific and detected a 37 kDa protein in both human and rat articular cartilage. Actin (43 kDa) was used as a control for variations in loading between lanes. Cell extracts were prepared from articular cartilage isolated from rat (upper panel) or human joints (lower panel) from 3 cadavers at autopsy. Fifty micrograms protein were loaded for 10% SDS-PAGE and then transferred onto nitrocellulose membrane. Polyclonal antibodies against rat or human thrombin were used, and horseradish peroxidase conjugated secondary antibody was revealed by enhanced chemiluminescence. Purified rat or human thrombin protein was used as standard. Detection of actin was used as a control. Photographs are representative of 3 independent experiments run with samples from 3 different animals.

In those samples, thrombin was found complexed to its endogenous inhibitor, as thrombin-antithrombin (TAT) complexes, or was detected by the levels of degradation fragments *F1 + 2*. In RA patients, indicators of thrombin activity, TAT and *F1 + 2*, were both increased, but less so in the plasma compared to SF by factors of 11-fold and 133-fold, respectively. The extremely high thrombin activity measured in the SF supports the view that thrombin is locally produced rather than simply diffusing from an increased circulatory pool. Our finding of thrombin mRNA and protein in the articular cartilage provides the first direct evidence for the production of thrombin locally in the joint by chondrocytes *in vivo*.

Our data indicate that expression of prothrombin by chondrocytes occurs not only in animal models but also in human articular cartilage. The increased levels of mRNA and protein in the cartilage during the degeneration caused by joint immobility suggest that chondrocytes participate in the degradation of the cartilage matrix by thrombin production. Chondrocytes have the ability to modulate the degradation of their surrounding matrix through the synthesis of proteases. Proteolytic enzymes capable of proteoglycan degradation that also break down the collagen network include 4 classes: matrix metalloproteinases (MMP), cysteine proteases, serine/threonine proteases, and aspartic pro-

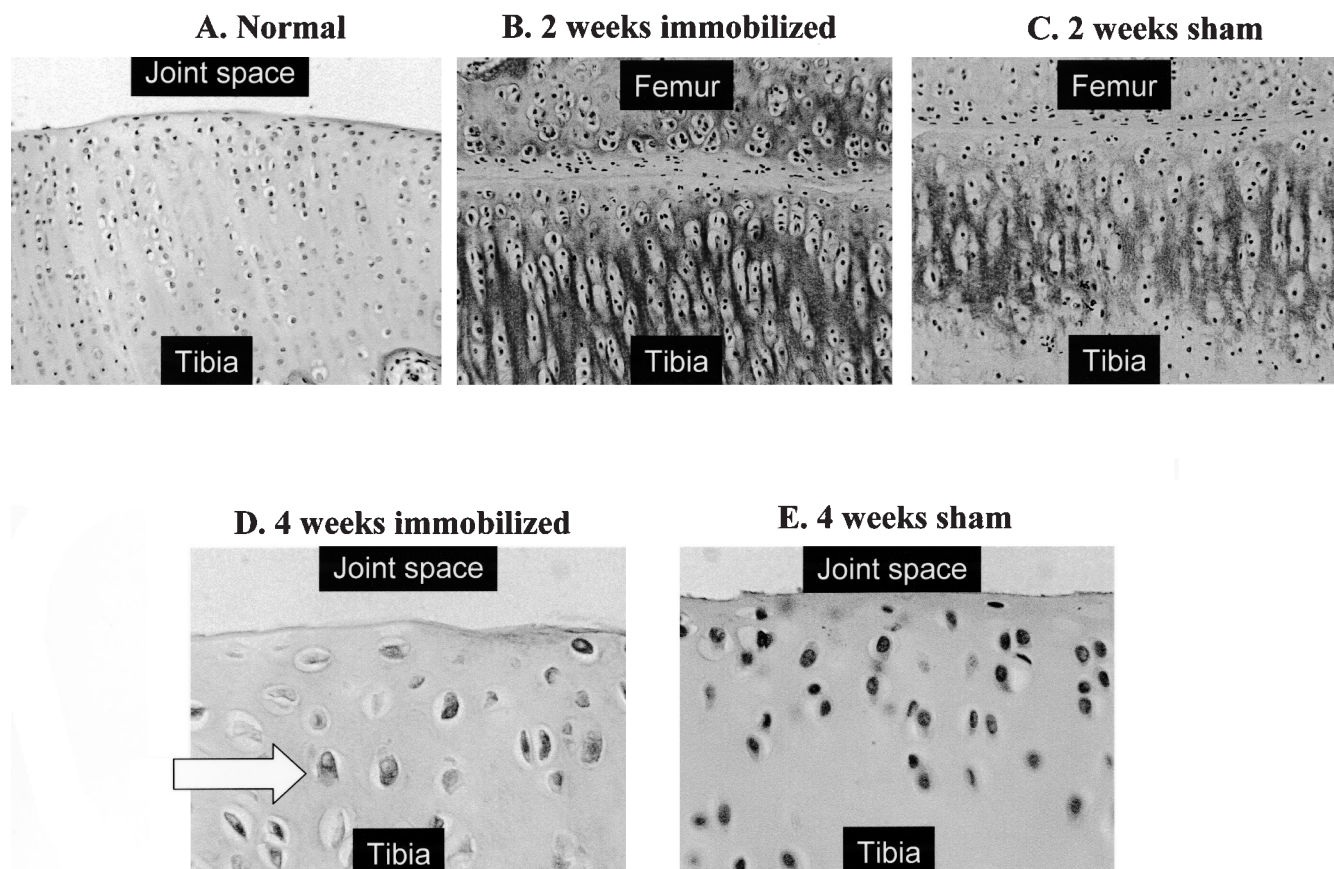


Figure 5. Immunohistochemistry of thrombin. Micrographs of knee sections stained with thrombin antibody. Note the dramatic increase in matrix staining 2 weeks after surgery (B, C). After 4 weeks, there is elevated chondrocytic staining compared with sham and decreased matrix staining (D, E). Normal animals displayed very low levels of thrombin staining (A). Arrow indicates positively stained chondrocyte.

Table 1. Thrombin-like staining intensity on immunohistochemistry (staining intensity in units from 0: no staining to 3: strong staining).

Time	Chondrocyte		Cartilage Matrix	
	Sham Operated, median (range) [†]	Immobilized, median (range)	Sham Operated, median (range)	Immobilized, median (range)
2 weeks, n = 3, 8 ^{††}	1.25 (0.06–1.38)	0.75 (0.53–1.11) [#]	2.25 (1.69–2.31) [#]	2.31 (1.97–2.47) [#]
4 weeks, n = 4, 6	0.38 (0.08–0.67)*	1.67 (0.66–1.98)* [#]	0.16 (0.16–1.00) [#]	1.27 (0.56–1.58) [#]
8 weeks, n = 2, 5	0.34 (0.19–0.33)	0.58 (0.34–1.5) [#]	0.41 (0.09–0.52) [#]	0.17 (0.00–0.78)
16 weeks, n = 3, 5	0.25 (0.06–0.38)	0.81 (0.04–1.59)	0.25 (0.25–0.34) [#]	0.25 (0.19–0.83) [#]
32 weeks, n = 4, 6	0.56 (0.16–1.62)	1.70 (0.90–2.42) [#]	0.00 (0.00–0.33)	0.43 (0.09–1.31) [#]
Normal, n = 12	0.12 (0.12–0.41)		0.00 (0.00–0.00)	

[†] Shown as median and 25% to 75% interquartile range. ^{††} Numbers shown as sham operated immobilized.

* Difference $p < 0.05$ between immobilized and sham, [#] $p < 0.05$ between immobilized or sham and normal.

teases³². Significant efforts have been devoted to elucidating the pathways of MMP expression in the context of cartilage degeneration. Control of MMP synthesis, activation, and activity are tightly regulated and are altered in arthritis³³. The first step of regulation is at the level of gene expression, and some changes in the levels of transcripts encoded by MMP genes at different phases of human OA have been documented^{34,35}. Our work indicates that the ser-

ine protease thrombin, similar to MMP, is regulated at the level of gene expression in chondrocytes from joints subjected to immobility. Increased expression of the prothrombin gene, indicated by the presence of prothrombin mRNA, was found in the cartilage of immobilized joints compared to sham operated and to nonoperated joints.

Thrombin generation by articular cartilage emphasizes the importance of serine proteases produced by chondro-

cytes in the destruction of the matrix. Another member of the serine protease family capable of degrading the cartilage matrix is granzyme B³⁶. Granzyme B is a serine protease synthesized by chondrocytes and able to degrade the proteoglycan component of cartilage *in vitro*³⁷. Although its origin was initially suspected to be the cytotoxic lymphocytes from RA synovial tissue, recent data indicate that chondrocytes do express the granzyme B gene^{36,38,39}. Expression of the granzyme B gene by chondrocytes was confirmed by *in situ* hybridization and by RT-PCR³⁶. A larger number of granzyme B-positive chondrocytes were found in RA cartilage compared to OA cartilage. These observations and our current data on the expression of prothrombin gene by chondrocytes indicate that the family of serine proteases plays an important role in the degradation of articular cartilage by chondrocytes.

The mechanisms that contribute to elevated levels of thrombin expression in chondrocytes of the articular cartilage are unclear. Control of thrombin synthesis is likely to involve steps additional to gene expression. Thrombin is synthesized as an inactive proenzyme and released by the action of factors Xa and V⁴⁰. In addition, the activity of thrombin is regulated by endogenous antithrombin factors capable of binding thrombin with a high affinity⁴¹. The lack of motion and loading of a joint alters the dynamic balance between synthesis and degradation of the extracellular matrix components elaborated by the chondrocytes, and shifts it toward degradation. While downstream matrix targets are being identified, upstream mediators also need to be identified. Whether prothrombin gene expression by chondrocytes is increased in other models of cartilage degeneration such as OA and RA remains to be investigated.

We describe expression of the prothrombin gene and detection of thrombin protein in chondrocytes of the articular cartilage. Increased levels of both mRNA and protein after joint immobility suggest a direct role of thrombin in cartilage degeneration.

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