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Early Elevation of Transforming Growth Factor-β, Decorin, and Biglycan mRNA Levels During Cartilage Matrix Restoration After Mild Proteoglycan Depletion

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ABSTRACT. Objective. To elucidate the role of transforming growth factor-β (TGF-β) and the small proteoglycans biglycan and decorin in the repair of articular cartilage after proteoglycan depletion.

Methods. Limited and reversible proteoglycan depletion was induced by injection of murine knee joints with 0.5% papain. Proteoglycan content of patellar cartilage was examined by safranin O staining on histological sections and overall proteoglycan synthesis was measured by incorporation of 35S sulfate. Changes in mRNA expression of TGF-β, aggrecan, decorin, and biglycan were determined by semiquantitative reverse transcription polymerase chain reaction.

Results. Papain injection led to rapid depletion of proteoglycans, which was partly overcome 7 days after injection, while total replenishment of the cartilage matrix with proteoglycans was observed on Day 24. The incorporation of radiolabeled sulfate in patellar proteoglycans was initially decreased (up to Day 3), but significantly enhanced on Days 4 and 7 after papain injection. Upregulation of TGF-β, decorin, and biglycan mRNA in patellar cartilage was observed on Day 2, markedly before elevation of overall proteoglycan synthesis. mRNA levels were less augmented on Day 7, and on Day 24 all messenger RNA levels had returned to control values. As well, in the soft tissue adjoining the patella swift upregulation of TGF-β mRNA was observed.

Conclusion. mRNA of both TGF-β and the small proteoglycans decorin and biglycan are elevated at an early phase during cartilage repair after moderate proteoglycan depletion, implying a functional role for these molecules in this repair process. (J Rheumatol 1997;24:543-9)

Key Indexing Terms:
- CARTILAGE REPAIR
- TRANSFORMING GROWTH FACTOR-β
- BIGLYCAN
- AGGREGAN

Articular cartilage covers the ends of the long bones and makes smooth and nearly frictionless movement of the articulating surfaces possible under normal conditions. However, during pathology such as rheumatoid arthritis (RA) or osteoarthritis articular cartilage is degraded, resulting in loss of joint function. One factor contributing to the irreversible damage of articular cartilage in these diseases is the limited capability of articular cartilage for repair of the extracellular matrix. The extracellular matrix of articular cartilage consists mainly of collagen (e.g., type II, IX, XI) and proteoglycans such as aggrecan and to a lesser extent decorin, biglycan, and fibromodulin. All these matrix molecules are synthesized and degraded by the articular chondrocytes.

Factors suggested to be important in repair processes in miscellaneous tissues are the various transforming growth factor-β (TGF-β) subtypes. In mice, TGF-β is upregulated in an early phase during adult and embryonic wound repair, but temporal and spatial differences between the 3 mammalian isoforms were observed. Application of additional TGF-β appears to lead in several systems to stimulation of the repair process. Closure of skull defects, repair of macular holes, recovery of damaged heart tissue, and repair of incisional wounds can be accelerated by application of TGF-β. As well, it has been suggested that the presence of TGF-β in articular cartilage lesions as a result of RA is an indication for a role for TGF-β in tissue repair at these sites. In addition, stimulation of murine articular cartilage repair after a simulated arthritic insult, induced by intraarticular interleukin 1 injection, was found by our group.

Both the large aggregating proteoglycan of cartilage, aggrecan, and the so-called small proteoglycans like decorin, biglycan, and fibromodulin are structural and regulatory components of articular cartilage. The major function of aggrecan is hydration of the extracellular matrix, while the functions of the small proteoglycans in cartilage are largely unknown. Immunohistochemical studies have shown that biglycan has a different localization from decorin in articular cartilage, suggesting different functions. Decorin was distributed throughout the matrix,
Animals

Induction of mild proteoglycan depletion. Proteoglycan depletion was induced by TGF-β. Incubation of human fibroblast with TGF-β resulted in upregulation of biglycan mRNA, while decorin expression was reduced or unaltered. Also, in isolated human articular chondrocytes biglycan mRNA expression was increased, while decorin mRNA was diminished under the influence of TGF-β. In mesangial cells both decorin and biglycan synthesis is reported to be upregulated to 50-fold by TGF-β. Moreover, TGF-β not only interferes with the synthesis of the small proteoglycans, but these molecules also interact on the protein level. Both decorin and biglycan can be able to bind TGF-β, which can result in either inhibition of TGF-β biological activity or stimulation of TGF-β action by these molecules. In this respect biglycan as well as decorin can be considered as both negative and/or positive modifiers of TGF-β activity, regulating the potential repair stimulating ability of TGF-β.

For insight into the roles of TGF-β and the cartilage proteoglycans aggrecan, decorin, and biglycan during restoration of the articular cartilage matrix after moderate proteoglycan depletion we studied temporal expression of mRNA of these molecules during in vivo cartilage repair.

MATERIALS AND METHODS

Animals. Male C57Bl/6 mice at 10–12 weeks of age were used in all experiments. The animals were kept in cages with wood chip bedding in air-conditioned rooms at constant temperature. They were fed a standard laboratory diet and had access to tap water ad libitum.

Induction of mild proteoglycan depletion. Proteoglycan depletion was induced as described by van der Kraan, et al. The right knee joint of mice was injected once, intraarticularly along the patellar ligament, with 6 μl of 0.5% papain solution (type IV, 15 units/mg; Sigma, St. Louis, MO, USA). To activate the papain, the solution was supplemented with 0.03 M L-cysteine HCl (Sigma). The left (control) knees were injected with physiological saline. The papain injection in the patellar cartilage leads to limited, reversible proteoglycan depletion and inhibition of proteoglycan synthesis one and 2 days after the injection, followed by supranormal proteoglycan synthesis later. At several time points after papain injection, patellae were isolated and used for measurement of proteoglycan synthesis and determination of mRNA levels. Surrounding tissue was used for detection of mRNA levels only.

Determination of patellar proteoglycan synthesis. Patellar cartilage proteoglycan synthesis was measured ex vivo according to the method of van den Berg, et al. Whole patellae, with a standard amount of surrounding tissue, were dissected from the knee joints. Patellae were labeled immediately after isolation with 35S sulfate (1.1 MBq/ml) for 2 h at 37°C in RPMI 1640 DM medium (Flow Laboratories, Irvine, UK). After labeling, the patellae were washed, fixed, decalcified, and punched out of the surrounding tissue. The patellae were dissolved in lumasolve (Lumac, Groningen, The Netherlands) and the quantity of incorporated 35S sulfate was determined by liquid scintillation counting.

Whole knee joint histology. Mice were killed by cervical dislocation, and carefully dissected knee joints were fixed in phosphate buffered formalin (pH 7.4) for 5 days and subsequently decalcified in 5% formic acid for 4 days. Standard processing of the knee joints in an automatic tissue processing apparatus was followed by embedding of the knee joints in paraffin wax. Frontal knee joint sections were prepared (6 μm) and stained with safranin O and fast green.

Isolation of patellar cartilage and synovial tissue. Patellae were dissected from the murine knee joints and immediately decalcified for 4 h at 4°C in 3.5% EDTA (Sigma). After decalcification the complete articular cartilage layer was stripped from the underlying bone. The isolated cartilage was instantly put in TRizol reagent (Life Technologies) for RNA extraction. In control experiments it was shown that this procedure did not affect the RNA isolation or reverse transcription polymerase chain reaction (RT-PCR) performance negatively (data not shown).

The patellar tendon and synovium of knee joints were dissected and 2 pieces of synovial tissue were punched out from the tissue on a standard location (area 7 mm²). Directly after punching out, the synovial tissue was quickly frozen in liquid nitrogen.

RNA isolation and RT-PCR. Total RNA was isolated from patellar cartilage and synovial tissue by TRizol extraction. RNA was directly extracted from cartilage but synovial tissue was first homogenized and then put in TRizol reagent. Cartilage and synovial tissue of 5 mice were pooled. Before reverse transcription the isolated RNA was treated with DNase I (Life Technologies). The reverse transcription reaction was performed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies) using an oligo(Dt)₁₅ primer (Eurogentec, Liege, Belgium). Amplification of DNA was accomplished by using Taq DNA polymerase (Life Technologies) up to a cycle number of 35. To determine the relative mRNA levels samples were taken at different cycle numbers and the amount of amplified product was calculated as described below. All RT-PCR reactions were performed in duplicate.

The amplification products were labeled by digoxigenin labeled nucleotides (Boehringer, Mannheim, Germany). After separation on a 1.5% agarose gel the products were blotted on positively charged nylon membranes (Boehringer). Amplification products were detected by chemiluminescence using alkaline phosphatase labeled antidigoxigenin antibodies in combination with lumigen PPD according to the manufacturer (Boehringer). Spots were scanned on the photographic film using an image analyzer. Only spots lying within the linear part of the PCR were used for scanning. The results are presented as relative mRNA levels using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels as an internal control. Due to the limited amount of tissue and quantities of RNA, measurement of the amount of total RNA was not possible.

The following nucleotide primers were used in the amplification reactions. Aggrecan primers were used as described by Grover and Roughley (product 501 bp), while decorin primers were used according to Asundi and Desher (product 400 bp). The biglycan upper primer had the following sequence: 5'-AGAAGGCCCTTTACCGCCCTG-3', and the lower primer 5'-ACTTTGGCTAAGGGTGTC-3' (product 130 bp). Murine TGF-β 1 primers were derived from Clontech (Palo Alto, CA, USA) (product 525 bp), whereas TGF-β 3 primers were used as described by Mulheron, et al (product 380 bp). To detect GAPDH, the primers 5'-AATCCCTCCAAGATTGTCCAGCA-3' and 5'-TCCACACCCCGTTGCTGTA-3' were used (product 553 bp). Primer sequences were selected with the computer program Primer (Whitehead Institute, Cambridge, MA, USA) and Oligo 4.0 (National Biosciences, Plymouth, MN, USA).

RESULTS

To obtain reversible depletion of proteoglycans from the patellar cartilage 0.5% papain was injected into the right knee joints of the mice. Papain injection resulted after one day in obvious depletion of proteoglycans, measured by safranin O staining, in the patellar cartilage (Figure 1). Moderate inflammation of the joint, characterized by granulocyte infiltration, was seen at this time. Three days after papain injection the inflammation had largely vanished, but the amount of proteoglycan depletion was different from one day after papain injection. On Day 7 the proteoglycan
Figure 1. Effect of papain injection on proteoglycan content of patellar cartilage. These are frontal sections of the murine knee joint stained with safranin O and fast green (original magnification x 100). Depletion of proteoglycan can be seen on Day 1 and 7, whereas normal staining is observed at Day 24. A: control joint, B: Day 1, C: Day 7, D: Day 24 (c: cartilage, b: bone, f: femur, p: patella).

At several times after papain injection patellar proteoglycan synthesis was measured *ex vivo* by incorporation of radiolabeled sulfate. Inhibition of proteoglycan synthesis was measured up to 3 days after the injection of papain (Figure 2). It is possible that part of the newly synthesized proteoglycan is degraded by papain remaining in the cartilage. However, patellar proteoglycan content is restored within 10 days, making it unlikely that active papain persists in the patellar cartilage. On Days 4 and 7 proteoglycan synthesis was supranormal, roughly doubled compared to the control knee joints. Twenty-four days after papain injection radiolabel incorporation into the papain injected knee joints had reached control levels.

Since it has been suggested that TGF-β might play a role...
in the stimulation of matrix synthesis during repair of connective tissues such as articular cartilage, we studied the mRNA expression of TGF-β in patellar cartilage and neighboring soft tissue. Both in the surrounding tissue and the patellar cartilage there was early elevation of messenger RNA of TGF-β1 and TGF-β3 (Figure 3). Even on Day 2 after papain injection, mRNA in the surrounding tissue and patellar cartilage was elevated 3 to 6-fold. In the surrounding tissue the TGF-β1 message appeared to be upregulated more strongly than the TGF-β3. From Day 3 the mRNA levels of both TGF-β1 and TGF-β3 declined, and on Day 7 only TGF-β3 mRNA in the cartilage still appeared to be elevated. Twenty-four days after papain injection mRNA levels of TGF-β1 and TGF-β3 were normal.

To investigate if there was an association between the upregulation of TGF-β mRNA and mRNA levels of proteoglycans in the recovering patellar cartilage we measured mRNA levels of aggrecan, decorin, and biglycan in this structure. Messenger RNA levels of the small proteoglycans, decorin and biglycan, were already elevated 2 days after papain injection, while aggrecan mRNA in the papain injected knees was comparable to the control knee joints (Figure 4). Proteoglycan synthesis measured by incorporation of radiolabeled sulfate was noticeably inhibited up to Day 3. Also on Days 3 and 4 upregulation of biglycan and decorin mRNA was observed, whereas on Day 7 the levels were diminished compared to the levels on Day 2. Aggrecan mRNA levels were never strikingly altered during the period of the experiment. On Day 24 all mRNA levels were similar to the control levels.

**DISCUSSION**

Murine knee joints were injected with papain to study the repair response of articular cartilage after limited proteoglycan depletion. This model was used since papain injection in the dosage we used leads to proteoglycan depletion with a restricted inflammatory response. Our results show that patellar proteoglycan depletion after papain injection can be restored to normal by the articular chondrocytes. Even 7 days after the induction of proteoglycan depletion, part of the matrix was restored, while after 24 days no signs of proteoglycan depletion could be detected by safranin O stain of the matrix. At least one of the mechanisms leading to replenishment of the cartilage matrix with proteoglycans appeared to be enhanced synthesis of proteoglycans, starting between 3 and 4 days after papain injection.

Even at an early time point during the repair phase, several days before increased incorporation of radiolabeled sul-
fate, TGF-β mRNA levels were augmented, both in patellar cartilage and the soft tissue adjoining the patellae. This indicates that upregulation of TGF-β mRNA is an early event during the restoration of the cartilage matrix after proteoglycan depletion by papain, suggesting a role for TGF-β in the repair process. The elevation of TGF-β mRNA was not very longlasting, and 7 days after papain injection the message levels were lower than on Day 2, whereas on Day 24 mRNA levels had reached control values. TGF-β2 was measured in the surrounding tissue and patellar cartilage on Day 2 only. It also appeared to be elevated in both compartments at this time (data not shown). These observations are in accord with the data of Martin, et al, who observed in mouse embryos rapid induction and clearance of TGF-β after wounding.11

With respect to the bioavailability of TGF-β, newly synthesized TGF-β protein is only one of the sources of TGF-β. TGF-β is bound to the extracellular matrix by several binding proteins.33-36 In the matrix of bovine articular cartilage considerable amounts of TGF-β were detected that appeared to be inaccessible to the chondrocytes under normal circumstances.37 However, it can be imagined that as a result of proteolytic action the majority of matrix bound TGF-β in cartilage can become available to the chondrocytes after exposure to papain. Moreover, TGF-β being synthesized as a latent molecule might be activated by the proteolytic activity of papain and also in this way TGF-β bioactivity can be enhanced by papain. On the other hand, papain might degrade active TGF-β and in this way lower the bioavailability of TGF-β. The early upregulation of TGF-β mRNA coincided with a simultaneous increase of decorin and biglycan mRNA levels. In patellar cartilage chondrocytes, mRNA levels of decorin and biglycan were elevated about 3 to 4-fold even 2 days after papain injection, when glycosaminoglycan synthesis appeared to be diminished by about 50%. Not before Day 4 could enhanced synthesis of proteoglycans, measured by 35S sulfate incorporation, be observed in the patellar cartilage after papain injection. During the whole study period the mRNA levels of aggrecan, the proteoglycan contributing predominantly to the incorporation of sulfate in cartilage, did not change strikingly. This indicates that at least part of the proteoglycan synthesis, as measured by sulfate incorporation, is not regulated by transcription of the aggrecan core protein gene but by other factors, such as upregulation of aggrecan core protein synthesis due to posttranslational mechanisms. Increased addition of glycosaminoglycan chains to the aggrecan core protein during the repair phase seems unlikely, since no changes in the size of aggrecan could be detected using Sepharose Cl 2B chromatography (data not shown). The early elevation of the small proteoglycans decorin and biglycan can be interpreted in several ways. Since no upregulation has been found after injection of proteins that cause inflammation similarly to papain but without proteoglycan degradation, elevated mRNA levels as a reaction to the inflammatory response in the joint seem unlikely. However, TGF-β mRNA levels can be affected by the inflammatory response in the joint. Upregulation of mRNA of the small proteoglycans could occur as a result of TGF-β activity on patellar chondrocytes. This would be a consequence of release and activation of inactive TGF-β by papain or a result of enhanced TGF-β synthesis. In this way the small proteoglycans could function as enhancers of TGF-β bioactivity or function in a negative biofeedback mechanism to control the TGF-β action on the chondrocytes.25-27 The observations of Roughly, et al21, with incubation of isolated human chondrocytes with TGF-β leading to increased biglycan mRNA and decreased decorin mRNA levels, appear to contradict a role for TGF-β in upregulation of mRNA of both small proteoglycans. Partly in parallel with the studies of Roughly, et al, Collier and Ghosh observed increased biglycan synthesis and unaltered decorin synthesis after exposure of meniscal chondrocytes to TGF-β38. However, both studies were performed with chondrocytes of different species and with isolated chondrocytes, instead of an in vivo model as described here. Nevertheless, TGF-β could play a role in regulation of cartilage proteoglycan synthesis: the elevation of decorin and biglycan mRNA we observed could be independent from TGF-β action. The early elevation of biglycan and decorin mRNA suggests an intrinsic role for these proteoglycans in assembly of the cartilage matrix during the repair period, irrespective of their relation to TGF-β. Decorin is able to influence the fibrillogenesis of type I and type II collagen and has been shown to bind to collagen type VI in binding assays.39,40 In addition, decorin has been reported to interact with fibronectin and thrombospondin, both extracellular matrix components.41,42 Biglycan has a different distribution than decorin within the cartilage matrix, implicating different functions for biglycan than decorin in cartilage physiology.17,18 Upregulation of mRNA of decorin and biglycan might be a functional mechanism in the reconstruction of damaged cartilage matrix, independent of their role in control of TGF-β activity. In accord with our results, Cs-Szabo, et al found 5 to 10-fold higher mRNA levels of biglycan and decorin in human osteoarthritic cartilage compared to normal cartilage, suggesting a role for these proteoglycans in local cartilage repair foci that can be found in osteoarthritic cartilage.43

In conclusion, our results indicate that during restoration of the patellar cartilage matrix after moderate proteoglycan depletion, upregulation of TGF-β and decorin and biglycan messenger RNA in patellar cartilage and TGF-β in adjoining soft tissue is an early event. Upregulation of mRNA is initiated days before enhancement of proteoglycan synthesis, as measured by incorporation of radiolabeled sulfate. These results suggest that both TGF-β and the small proteoglycans

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decorin and biglycan might play a role in regulation of the chondrocyte metabolism, while the small proteoglycans could have a potential structural function in repair of damaged articular cartilage matrix.

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