Protection from interleukin 1 induced destruction of articular cartilage by transforming growth factor β: studies in anatomically intact cartilage in vitro and in vivo

Henk M van Beuningen, Peter M van der Kraan, Onno J Arntz, Wim B van den Berg

Abstract
The modulation of interleukin 1 (IL-1) effects on proteoglycan metabolism in intact murine patellar cartilage by transforming growth factor β (TGF-β) was investigated in vitro and in vivo. In vitro TGF-β (400 pmol/l) had no effect on basal proteoglycan degradation. Proteoglycan degradation induced by IL-1, however, was suppressed by TGF-β in serum free medium alone and in medium supplemented with 0.5 µg/ml insulin-like growth factor 1. This suggests a specific regulatory role for TGF-β under pathological conditions. In contrast with the suppression of breakdown, synthesis of proteoglycans was stimulated by TGF-β for both basal and IL-1 suppressed proteoglycan synthesis in cultures without insulin-like growth factor. In the presence of insulin-like growth factor no extra effect of TGF-β on proteoglycan synthesis was observed. With insulin-like growth factor, however, TGF-β potentiated the ex vivo recovery of IL-1 induced suppression of proteoglycan synthesis. Analogous to the in vitro effects, TGF-β injected intra-articularly suppressed IL-1 induced proteoglycan degradation. Furthermore, TGF-β injected into the joint counteracted IL-1 induced suppression of proteoglycan synthesis. This indicates that in vivo also TGF-β can ameliorate the deleterious effects of IL-1 on the cartilage matrix.

Materials and methods

ANIMALS
C57B1/10 mice aged 12 weeks or 18 months were used. They were fed a standard diet and tap water ad libitum.

CHEMICALS
RPMI 1640 culture medium (Dutch modification) was obtained from Flow Laboratories (Irvine, UK). A 10-40 pg sample of IL-1α corresponded to one unit in the lymphocyte activating factor assay. Transforming growth factor β1, purified from human platelets (Calbiochem, La Jolla, CA, USA) was decontaminated of trifluoracetic acid and acetoniitrile with a vacuum concentrator (Savant Instruments, Formingdale, NY, USA) and resuspended in 4 mM HCl for activation. Recombinant human insulin-like growth factor 1 (Boehringer, Mannheim, Germany) was dissolved in 0.1 M acetic acid (Merck, Darmstadt, Germany). All medium used in these experiments was supplemented with 0.1% ultrapure bovine serum albumin (Sigma, Darmstadt, Germany).

Many studies have provided evidence for interleukin 1 (IL-1) being a key mediator in inflammation in general and in arthritis in particular. It causes an influx of inflammatory cells into the joints,4-6 suppresses chondrocyte proteoglycan synthesis, and stimulates cartilage degradation.5-15 Interleukin 1 concentrations in arthritic joints are increased13-15 and IL-1 could be responsible for most of the cartilage damage that is characteristic of arthritis.

It has been found that the ubiquitous and multifunctional regulatory peptide transforming growth factor β (TGF-β) can counteract the effects of IL-1 on several cell types,16-17 including chondrocytes.18-19 In arthritis it could be an important factor because of its immunosuppressive functions16-20-25 and its effects on articular cartilage. With regard to articular cartilage it was reported that TGF-β in vitro can suppress IL-1 induced proteoglycan degradation in monolayers of articular chondrocytes and in sliced cartilage.18-19 As TGF-β concentrations in arthritic joint fluids26-29 are in the range yielding optimum TGF-β effects on chondrocytes in vitro,30-32 TGF-β could play an important regulatory part in cartilage destruction during arthritis.

In the present study we investigated TGF-β modulation of IL-1 effects on proteoglycan degradation and proteoglycan synthesis in intact articular cartilage. We studied this modulation in vitro in basal medium, to confirm earlier studies,18-19 and in the presence of insulin-like growth factor, which is the major stimulatory factor, with respect to cartilage proteoglycan synthesis, in serum and synovial fluid.33-34 In addition to these more extensive in vitro experiments, we showed for the first time that TGF-β injected into the joint modulates the effects of IL-1 on proteoglycan metabolism.
St Louis, MO, USA). In vivo injections with TGF-β were performed with purified human TGF-β1. The purity of TGF-β was verified using sodium dodecyl sulfate polyacrylamide gel electrophoresis, showing only a single 25 kilodalton band. An extra band was visible after reduction. A neutralising antibody to TGF-β (1D11.16) completely blocked the IL-1 inhibiting effect of this TGF-β on IL-1 induced production of IL-2 by NOB-1 cells.

**Histology**

Histology was used to compare cartilage depletion and cellular influx into the joints treated with IL-1, TGF-β, or a combination of both. Whole knee joints were dissected and processed as described elsewhere. Semiserial frontal sections were stained with safranin O or haematoxylin-eosin to be used in studies on cartilage proteoglycan depletion and cellular influx respectively.

**IN VITRO EFFECTS OF INTERLEUKIN 1/ TRANSFORMING GROWTH FACTOR β ON CARTILAGE PROTEOGLYCAN METABOLISM**

Mice were killed by cervical dislocation and the whole patellae, with a standard amount of surrounding tissue, were dissected from the knee joints as described by van den Berg et al. In studies on proteoglycan degradation patellae were prelabelled with [35S]sulphate for three hours and rinsed to remove non-incorporated label before culture with IL-1 and growth factors. Six patellae were placed in 1 ml of incubation medium consisting of RPMI 1640 supplemented with l-glutamine (2 mmol/l), pyruvate (1 mmol/l), and gentamycin (40 μg/ml) at 37°C in a humidified 5% carbon dioxide atmosphere. At the start of the incubation, TGF-β (10 ng/ml=400 pmol/l) or IL-1 (30 U/ml), or both, were added, alone or in combination with 0.5 μg/ml insulin-like growth factor 1. Proteoglycan degradation experiments were stopped after 24 hours of culture. In proteoglycan synthesis experiments the medium was changed at 24 hours and cultures were continued for another 24 hours to obtain a maximum effect of IL-1 on proteoglycan synthesis. Patellae were then labelled with [35S]sulphate for two hours at the end of the incubation with IL-1 and growth factors. The patellae were fixed, decalcified, punched out of the surrounding tissue, and dissolved as described elsewhere. The [35S] content of each patella, which is a reliable measure of cartilage proteoglycan production, was measured by liquid scintillation analysis.

**STATISTICAL ANALYSIS**

Differences between experimental groups were tested using Student’s t test, unless stated otherwise.

**Results**

**EFFECT OF TRANSFORMING GROWTH FACTOR β ON INTERLEUKIN 1 INDUCED PROTEOGLYCAN DEGRADATION IN VITRO**

Although basal proteoglycan turnover rates in murine cartilage are already relatively high, markedly enhanced proteoglycan degradation was observed in patellae cultured without growth factors. In one day cultures of patellae in medium without insulin-like growth factor 1 (table 1) 52% of the [35S]-labelled proteoglycans was released from the patellar cartilage. Interleukin 1 caused a small but significant extra release of 27% of the label compared with control cultures. In IL-1 treated patellae the release of labelled sulphate was significantly suppressed by 400 pmol/l TGF-β. This regulation was also present and of the same order of magnitude in cartilage derived from old animals (18 months old; data not shown).

Similar studies were performed in the presence of insulin-like growth factor 1 (0.5 μg/ml). In these cultures the release of [35S]-labelled proteoglycans was 13% (table 1). This is comparable with the proteoglycan degradation measured in patellar cartilage in vivo. Interleukin 1 caused a significant extra release of 35% of the label compared with control cultures. Also under these conditions (with insulin-like growth factor 1), TGF-β diminished only the IL-1 induced proteoglycan release (37% reduction), not the IL-1 independent release. This indicates that suppression of

<table>
<thead>
<tr>
<th>Addition</th>
<th>No addition</th>
<th>Insulin-like factor growth 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td>522 (p&lt;0.01)</td>
<td>849 (164) (p=0.10)</td>
</tr>
<tr>
<td>IL-1</td>
<td>849 (164) (p=0.10)</td>
<td>1023 (224) (p&lt;0.05)</td>
</tr>
<tr>
<td>IL-1 + TGF-β</td>
<td>596 (104) (p&lt;0.05)</td>
<td>1023 (224) (p&lt;0.05)</td>
</tr>
</tbody>
</table>

*Patellae were prelabelled with [35S]sulphate (three hours) and the [35S] content was measured immediately (1800 counts/minute), or after 24 hours of culture in medium with the various additions.
†TGF-β (400 pmol/l) and IL-1 (30 U) effects were compared with the corresponding controls.
§IL-1 plus TGF-β effects were compared with the effects of IL-1 alone.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>In vitro modulation of interleukin 1 (IL-1) induced proteoglycan degradation by transforming growth factor β (TGF-β)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition</td>
<td>No addition</td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
</tr>
<tr>
<td>TGF-β</td>
<td>760 (164) (NS)</td>
</tr>
<tr>
<td>IL-1</td>
<td>922 (119) (p&lt;0.01)</td>
</tr>
<tr>
<td>IL-1 + TGF-β</td>
<td>596 (104) (p&lt;0.05)</td>
</tr>
</tbody>
</table>

*Patellae were prelabelled with [35S]sulphate (three hours) and the [35S] content was measured immediately (1800 counts/minute), or after 24 hours of culture in medium with the various additions.
†TGF-β (400 pmol/l) and IL-1 (30 U) effects were compared with the corresponding controls.
§IL-1 plus TGF-β effects were compared with the effects of IL-1 alone.

**IN VIVO EFFECTS OF INTERLEUKIN 1/ TRANSFORMING GROWTH FACTOR β ON CARTILAGE PROTEOGLYCAN METABOLISM**

**Intra-articular injections**

Transforming growth factor β (40, 200, or 1000 ng/6 μl) was injected into the joint space of the right knee, and the contralateral joint received an equal volume (6 μl) of phosphate buffered saline (PBS). One hour after the TGF-β injections IL-1 (30 U) was injected into the right knee while the left knee received a second injection of PBS. In controls for the effects of IL-1 alone or TGF-β alone, respectively, the first or the second injection into the right knee was performed with PBS. In vivo experiments on proteoglycan degradation were preceded by a prelabelling of articular cartilage with 74 kBq [35S]sulphate/g body weight, injected subcutaneously 24 hours before the intra-articular injections.
Modulation of effects of interleukin 1 on proteoglycan metabolism by TGF-β

Table 2 In vitro modulation of interleukin 1 (IL-1) induced suppression of proteoglycan synthesis by transforming growth factor β (TGF-β)

<table>
<thead>
<tr>
<th>Addition</th>
<th>[35S] sulphate incorporation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>459 (78)</td>
</tr>
<tr>
<td>TGF-β</td>
<td>594 (90) (p&lt;0.001)</td>
</tr>
<tr>
<td>IL-1</td>
<td>224 (78) (p&lt;10^-9)</td>
</tr>
<tr>
<td>IL-1 + TGF-β</td>
<td>325 (112) (p&lt;0.001)</td>
</tr>
</tbody>
</table>

*Patellae were pulse labelled as a measure of proteoglycan synthesis with [35S]sulphate either directly (1120 (198) counts/minute), or after 48 hours of culture in medium with the various additions.

In cultures without insulin-like growth factor 1 each experimental group consisted of 36 patellae, and in cultures with insulin-like growth factor 1 consisted of 18 patellae.

IL-1 induced release by TGF-β is not a general suppression of degradation, but a specific regulation that counteracts IL-1 effects.

EFFECT OF TRANSFORMING GROWTH FACTOR β ON INTERLEUKIN 1 INDUCED SUPPRESSION OF CHONDROCYTE PROTEOGLYCAN SYNTHESIS IN VITRO

As cartilage homeostasis is the result of a balance between degradation and synthesis, in addition to proteoglycan breakdown we also studied proteoglycan synthesis. In the absence of insulin-like growth factor 1 (table 2), proteoglycan synthesis rapidly decreased during culture. Suppression of [35S]sulphate incorporation after two days was 59% compared with synthesis immediately after the dissection of patellae. Interleukin 1 caused 52% extra suppression of proteoglycan synthesis compared with synthesis after parallel cultures without the cytokine. Addition of TGF-β (400 pmol/l) led to a stimulation of proteoglycan synthesis, both with and without IL-1. In the presence of insulin-like growth factor 1 (table 2) the proteoglycan synthesis was stimulated to 140% of synthesis levels measured immediately after dissection of patellae. Interleukin 1 caused 70% suppression of patellar proteoglycan synthesis compared with cultures without the cytokine. Under insulin-like growth factor conditions, TGF-β had no extra effect on proteoglycan synthesis in both IL-1 treated and non-treated patellae.

RESTORATION OF INTERLEUKIN 1 SUPPRESSED PROTEOGLYCAN SYNTHESIS

To conserve articular cartilage extracellular matrix integrity it is of utmost importance that IL-1 induction of matrix proteoglycan depletion is downregulated, but repair of established depletion is also a crucial mechanism. Therefore, we studied TGF-β effects in a phase of ex vivo recovery of IL-1 induced suppression of proteoglycan synthesis. Transforming growth factor β clearly enhanced restoration of the proteoglycan synthesis after IL-1 induced suppression (figure). In these experiments TGF-β caused an extra stimulation, superimposed on stimulating effects of insulin-like growth factor or serum. More specifically, TGF-β prolonged insulin-like growth factor stimulation of proteoglycan synthesis and caused an accelerated stimulation of proteoglycan synthesis in cultures with 30% murine serum. Effects of TGF-β on proteoglycan synthesis in control patellae (no IL-1 treatment) in four day cultures in the presence of insulin-like growth factor 1 or serum were minimal (data not shown).

EFFECTS OF INTRA-ARTICULARLY INJECTED TRANSFORMING GROWTH FACTOR β

To answer the question of whether modulation of IL-1 effects by TGF-β is also possible in
IL-1, cartilage inflammatory leucocytes. In combination was treatment and was studied in vivo, we studied patellar proteoglycan metabolism after injection of both IL-1 and TGF-β into the knee joint. Cartilage proteoglycans were prelabelled with a single subcutaneous injection of [35S]sulphate at day -1. Interleukin 1 (30 U) injected intra-articularly at day 0 caused 23% release of labelled proteoglycans within 24 hours with saline injection (table 3). When TGF-β was injected one hour before IL-1 into the murine knee, the release of labelled proteoglycans in a 24 hour chase period was reduced. This suppression of IL-1 induced proteoglycan degradation was significant in the 40 ng and the 1000 ng TGF-β doses. No dose response effect was seen in this dose range. Surprisingly, TGF-β injected alone (40 or 200 ng) significantly stimulated proteoglycan degradation, which may mask part of the protection against IL-1 induced proteoglycan degradation offered by TGF-β.

We also studied proteoglycan synthesis after intra-articular injections of TGF-β or IL-1, or both. Proteoglycan synthesis was measured ex vivo in patellae isolated one day after injection. Interleukin 1 caused 57% suppression of proteoglycan synthesis compared with normal synthesis (table 4). The combination of IL-1 with 40 or 200 ng TGF-β significantly decreased this suppression. Again, no dose response effect of TGF-β was seen, suggesting that the 40 ng dose already yields the maximum effect. Transforming growth factor β alone did not influence proteoglycan synthesis in vivo.

Histology (not shown) showed that the injected TGF-β had a clear chemoattractant capacity. At six hours and to a lesser extent at 24 hours after injection, considerable infiltration and exudation of inflammatory cells was seen. The predominant cells were polymorphonuclear leucocytes. In combination with IL-1, the injected TGF-β had an additive effect in mediating the attraction of inflammatory cells. Our histology was not suited to confirm the protective effect of TGF-β because single IL-1 injections caused no visible proteoglycan depletion in articular cartilage in the mouse, as determined with safranin O staining. Studies using repeated injections of IL-1 alone or in combination with TGF-β are in progress.

### Discussion
To study the effects of combinations of cytokine and growth factors on articular cartilage proteoglycan synthesis we cultured anatomically intact patellar cartilage embedded in a minimum area of surrounding tissue. Other workers have used sliced cartilage or isolated chondrocytes in the absence of underlying bone and synovial tissue. We used an IL-1 concentration yielding the maximum effects on patellar cartilage proteoglycan metabolism. The chosen TGF-β concentration (10 ng/ml) has been reported to have an optimum effect on chondrocytes and is physiologically relevant because comparable concentrations of active TGF-β have been found in arthritic joints. For the first time we tested the effects of TGF-β in the presence of insulin-like growth factor 1. In an attempt to create a physiologically relevant situation we used an insulin-like growth factor 1 concentration equal to the levels measured in murine serum samples.

We found that in the presence of insulin-like growth factor 1 the spontaneous release of proteoglycan from cultured cartilage was reduced, reaching levels comparable with basal proteoglycan release in vivo. The protective potency of insulin-like growth factor in cartilage degradation has been reported previously. Transforming growth factor β inhibited IL-1 induced proteoglycan degradation but had no effect on the spontaneously occurring loss of newly formed proteoglycans. In an earlier report describing in vitro TGF-β effects on proteoglycan loss from sliced cartilage TGF-β also inhibited spontaneously occurring proteoglycan loss. The importance of our observation is that for the first time we found evidence indicating that TGF-β modulation of IL-1 induced proteoglycan degradation is specific, independent of TGF-β effects on basal degradation. Possibly IL-1

### Table 3
<table>
<thead>
<tr>
<th>Treatment</th>
<th>35S proteoglycan content of cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>R 178 (51) (77%)</td>
</tr>
<tr>
<td>IL-1 + 40 ng TGF-β</td>
<td>R 200 (50) (88%); p=0.0116*</td>
</tr>
<tr>
<td>IL-1 + 1000 ng TGF-β</td>
<td>R 223 (36) (91%); p=0.0092*</td>
</tr>
<tr>
<td>40 ng TGF-β</td>
<td>R 217 (50) (99%); p=0.0242†</td>
</tr>
<tr>
<td>200 ng TGF-β</td>
<td>R 208 (59) (99%); p=0.0015†</td>
</tr>
</tbody>
</table>

*Effect of the combination of IL-1 and TGF-β on release of labelled proteoglycans was compared with the release induced by IL-1 alone.
†Treated (R) and contralateral (L) patellae of each individual animal were compared by Student's t test for paired samples.

### Table 4
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proteoglycan synthesis in cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>R 486 (191) (43%)</td>
</tr>
<tr>
<td>IL-1 + 40 ng TGF-β</td>
<td>R 629 (76) (56%); p=0.007*</td>
</tr>
<tr>
<td>IL-1 + 200 ng TGF-β</td>
<td>R 646 (119) (59%); p=0.006*</td>
</tr>
<tr>
<td>40 ng TGF-β</td>
<td>R 1116 (170) (103%); NS†</td>
</tr>
<tr>
<td>200 ng TGF-β</td>
<td>R 1204 (247) (106%); NS†</td>
</tr>
<tr>
<td>L 1135 (224) (n=18)</td>
<td></td>
</tr>
</tbody>
</table>

*Proteoglycan synthesis after treatment with the combination of IL-1 and TGF-β was compared with proteoglycan synthesis after treatment with IL-1 alone.
†Treated (R) and contralateral untreated (L) patellae were compared.
Modulation of effects of interleukin 1 on proteoglycan metabolism by TGF-β

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Induces a separate set of proteases, different from the proteases taking part in normal turnover, that can be modulated specifically by TGF-β. Whether TGF-β mainly influences the production of proteases or protease inhibitors remains to be investigated. Another possible mechanism for specific TGF-β modulation of IL-1-induced proteoglycan degradation could be that TGF-β, in contrast with other growth factors, downregulates IL-1 receptor expression. Induction of an IL-1 receptor antagonist protein by TGF-β in peripheral blood monocytes has also been reported.

The only previous report describing the effects of TGF-β on proteoglycan synthesis in articular cartilage was that by Morales and Roberts. They used long term cultures of sliced bovine cartilage. In our studies we cultured for only two days, the time necessary to obtain a maximum IL-1 effect on proteoglycan synthesis. Again, the presence of insulin-like growth factor appeared to be important for stabilisation of proteoglycan metabolism in our patella culture system. In the absence of insulin-like growth factor, TGF-β (400 pmol/l, 48 hours) stimulated both IL-1 suppressed and spontaneously occurring proteoglycan synthesis. Chandrasekhar and Harvey found the same trend in monolayers of articular chondrocytes. In earlier studies performed in our laboratory, TGF-β (0–200 pmol/l) was found to have no effect on proteoglycan synthesis in short term experiments. In addition, also in vivo, we saw no effect of TGF-β on basal proteoglycan synthesis 24 hours after intra-articular injections. A trend is noted that stimulating TGF-β effects only appear when cartilage slices have been in culture for some time. A plausible explanation seems to be that TGF-β has no stimulating effect on proteoglycan synthesis of normal articular chondrocytes, but becomes effective when the phenotype of articular chondrocytes has changed owing to prolonged culture or exposure to, for example, cytokines. Phenotype dependent receptor expression could be an explanation for the late onset of stimulating responses to TGF-β. Moreover, it has been reported that TGF-β induces phenotype changes itself. This could indicate that after 48 hours of culture in the presence of TGF-β and without insulin-like growth factor, the effects of TGF-β on proteoglycan synthesis no longer reflect the in vivo situation. In our studies, when we cultured for two days in the presence of insulin-like growth factor, which is known to stimulate proteoglycan synthesis on its own and to protect against phenotype changes, TGF-β had no extra effect on either IL-1 suppressed or basal proteoglycan synthesis.

Conditions do exist where TGF-β influences proteoglycan synthesis in spite of the presence of growth factors. During a phase of ex vivo recovery of IL-1 suppressed proteoglycan synthesis, TGF-β can stimulate this process, whatever the mechanism involved.

In accordance with previously published work, TGF-β injected into the knee joint had a clear chemoattractant capacity. The predominant inflammatory cells were polymorphonuclear leucocytes. This was also noted in studies performed in the knee of rats, whereas in the ankle joints of rats most infiltrating leucocytes were of monocytc lineage.

In vivo modulation of basal and IL-1 affected proteoglycan metabolism in articular cartilage by TGF-β has not been described previously. Transforming growth factor β injected into the joint caused a considerable reduction of IL-1-induced proteoglycan degradation. This is in accordance with the in vitro observations. In contrast with the in vitro treatments, where TGF-β had no significant effect, basal proteoglycan degradation was stimulated after TGF-β injections. A possible explanation for this difference could be the inflammatory potential of TGF-β. It clearly induces inflammation and may therefore be indirectly responsible for enhanced degradation. The proinflammatory effect of TGF-β could thus mask part of the protection against IL-1-induced proteoglycan degradation that is offered by TGF-β. The fact that TGF-β injected intra-articularly caused marked effects, namely extra proteoglycan degradation and an influx of inflammatory cells, indicates that the endogenous TGF-β in the non-inflamed joint is either in a latent form or present in too low amounts, or controlled by inhibitors.

In vivo studies on proteoglycan synthesis show that TGF-β had different effects on basal proteoglycan synthesis and suppressed proteoglycan synthesis after IL-1 treatment. The observation that basal proteoglycan synthesis is not influenced by TGF-β is in agreement with its in vitro effects in the presence of a physiological insulin-like growth factor concentration, maintaining in vivo proteoglycan metabolism at levels similar to the normal in vivo conditions. In contrast with insulin-like growth factor cultures, TGF-β in vivo decreased the suppression of proteoglycan synthesis induced by IL-1. Maybe this specific TGF-β modulation of IL-1 effects acts via TGF-β downregulation of IL-1 receptors. Whatever is the mechanism of TGF-β inhibition of IL-1-induced suppression of proteoglycan synthesis in vivo, histology shows that TGF-β did not inhibit the IL-1-induced influx of inflammatory cells into the joint; TGF-β might, however, suppress the production of cytokines, oxygen metabolites, or proteases by these cells. It has been reported that TGF-β can cause defective phagocytic function of polymorphonuclear leukocytes. Moreover, TGF-β can also regulate the production of other growth factors or their receptors, or both. We conclude that TGF-β is able to block a considerable part of the IL-1 induced proteoglycan degradation and suppression of proteoglycan synthesis in articular cartilage. In the light of the destructive effect of IL-1 during arthritis we suggest an important protective function of TGF-β for articular cartilage extracellular matrix.
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