Inhibition of proteoglycan synthesis by transforming growth factor β in anatomically intact articular cartilage of murine patellae

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Abstract

The effect of transforming growth factor β (TGF β) on proteoglycan synthesis and degradation in anatomically intact articular cartilage of murine patellae was studied. Exogenously added TGF β up to a concentration of 200 pmol/l had no effect on proteoglycan synthesis in intact articular cartilage. Neutralisation of endogenously produced TGF β with a specific monoclonal antibody to TGF β, however, led to stimulation of proteoglycan synthesis, indicating that TGF β itself inhibits proteoglycan synthesis in anatomically intact cartilage. Transforming growth factor β decreased the degradation of proteoglycans in intact cartilage in the absence of fetal calf serum or insulin-like growth factor 1. In the presence of fetal calf serum or insulin-like growth factor 1, TGF β had no additional effect on proteoglycan breakdown.

Transforming growth factor β (TGF β) is a multipotent regulator of cell growth and extracellular matrix synthesis. Five different subtypes of TGF β are now known from cDNA libraries or by isolation from tissues or cells. Transforming growth factor β is secreted as an inactive high molecular weight complex which has to be dissociated before functional activation of TGF β.

High levels of the active and latent forms of TGF β are seen in synovial fluid from knee joints of patients with osteoarthritis and rheumatoid arthritis.[1 2] Fava *et al* reported a concentration of 10·1 and 3·8 ng/ml active TGF β in the synovial effusions of patients with rheumatoid arthritis and osteoarthritis, respectively. In a study by Miossec *et al* a mean synovial fluid concentration of 7·2 ng/ml was found in the knee joint effusions of patients with rheumatoid arthritis.[2] A four- to fivefold increase of active TGF β levels was detected after treatment of synovial fluid with acid.[1 2]

The TGF β concentrations present in synovial fluid of patients with rheumatoid disease might have profound effects on chondrocyte metabolism. Transforming growth factor β has a biphasic effect on proteoglycan synthesis and cell proliferation of cultured growth plate chondrocytes from chicken and rabbits.[3–6] Transforming growth factor β increases proteoglycan and DNA synthesis dose dependently up to a concentration of 1 ng/ml; higher concentrations have less stimulating effects.[3–6] This biphasic effect of TGF β on growth plate chondrocytes might be related to the presence of two types of TGF β receptors on growth plate chondrocytes.[6] Binding of TGF β with the high affinity receptor results in stimulation of DNA synthesis whereas binding with the low affinity receptor leads to inhibition of DNA synthesis.[6] On articular chondrocytes only inhibitory TGF β receptors are seen.[7]

The effects of TGF β on glycosaminoglycan synthesis of isolated articular cartilage chondrocytes are contradictory. Two groups reported an inhibitory effect of TGF β on proteoglycan synthesis of articular cartilage chondrocytes[7 8] whereas another group found a stimulating effect of TGF β on articular chondrocyte glycosaminoglycan synthesis.[9] Morales and Roberts reported stimulation of proteoglycan synthesis in bovine articular cartilage explant cultures by TGF β. There was a substantial lag time, however, in the response to 5 ng/ml TGF β and maximum stimulation of proteoglycan synthesis was observed only after a six day culture period.[10]

We studied the effects of TGF β on proteoglycan synthesis and degradation in anatomically intact cartilage of murine patellae alone or in combination with fetal calf serum or insulin-like growth factor 1. Addition of TGF β to the culture medium had no effect on the proteoglycan synthesis in anatomically intact cartilage. Depletion of endogenously produced TGF β from the culture medium with monoclonal antibodies to TGF β, however, stimulated proteoglycan synthesis, indicating that TGF β itself has inhibitory effects on proteoglycan synthesis in anatomically intact cartilage.

Materials and methods

GROWTH FACTORS

Trifluoroacetic acid and acetonitrile were removed from transforming growth factor β_1 from human platelets (Calbiochem, La Jolla, CA, USA) with a vacuum concentrator (Savant Instruments, Farmingdale, NY, USA) and TGF β was resuspended in 4 mM hydrochloric acid with 0·1% bovine serum albumin (Sigma, St. Louis, MO, USA). Recombinant insulin-like growth factor 1 (Boehringer, Mannheim, Germany) was dissolved in 0·1 M acetic acid (Merck, Darmstadt, Germany) with 0·1% bovine serum albumin. All media (RPMI 1640 DM, Flow Laboratories, Irvine, UK) used in the experiments were supplemented with 0·1% bovine serum albumin.

ANATOMICALLY INTACT CARTILAGE

Patellae were obtained from seven to 10 week

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old male C57Bl10 mice in good health weighing 20–25 g. 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.*[11]

EFFECT OF TGF β ON PROTEOGLYCAN SYNTHESIS

Anatomically intact patellae were incubated in RPMI 1640 DM medium (200 μl/patella) supplemented with pyruvate (1 mmol/l, Merck) and glutamine (2 mmol/l, Merck) at 37°C in a humidified 5% carbon dioxide atmosphere for 24 or 48 hours. At the start of the incubation period, TGF β (0–5 ng/ml, 0–200 pmol/l) alone or in combination with 10% fetal calf serum (FCS, Flow Laboratories) or 0·5 μg/ml insulin-like growth factor 1 was added to the culture medium. An equivalent amount of 4 mM hydrochloric acid or 0·1 M acetic acid, or both, was added to the control cultures. An insulin-like growth factor 1 concentration of 0·5 μg/ml maintains proteoglycan synthesis on the in vivo level in murine patellae.[12] In experiments with a 48 hour incubation period, the medium and growth factors were changed after 24 hours. During the last two hours of the incubation, 1·48 MBq sodium sulphate labelled with sulphur-35 (Du Pont de Nemours, Den Bosch, The Netherlands) was added to the culture medium.

Following incubation, the patellae were washed three times with physiological saline to remove non-incorporated sulphate labelled with ^{35}S and subsequently fixed in ethanol (96%). Decalcification of the patellae with 5% formic acid was followed by stripping of the articular cartilage layer.[13] Patellar cartilage was digested by lumasolve (Perstorp Analytical, Oud-Beyerland, The Netherlands) at 60°C and the amount of incorporated sulphate labelled with ^{35}S was assayed by liquid scintillation analysis.

EFFECT OF TGF β ON PROTEOGLYCAN DEGRADATION

Directly after isolation, patellae were incubated for three hours in medium with 1·48 MBq sulphate labelled with ^{35}S and subsequently washed three times with physiological saline. The amount of incorporated sulphate in the patellae (n=6) directly after radiolabelling (t=0) was assayed as described above. The remaining prelabelled patellae were incubated for 24 hours in medium containing various concentrations of TGF β (0–5 ng/ml, 0–200 pmol/l) alone or in combination with 10% fetal calf serum or 0·5 μg/ml insulin-like growth factor 1. Following incubation, the patellae were processed as described above.

DEMONSTRATION OF ENDOGENOUS TGF β

The activity of TGF β in culture supernatants of murine patellae was based on the inhibitory effect of TGF β on the interleukin 1 induced production of interleukin 2 by NOB 1 cells.[14] The NOB/interleukin 1 assay was carried out in microtitre plates (Costar, Cambridge, MA, USA). To each well 10 μl interleukin 1 (final concentration 10 U/ml, Pfizer, Croton, NY, USA) and 5 μl culture supernatant were added. The culture supernatant was derived from a 24 hour culture of anatomically intact patellae (five patellae/ml). In addition, the wells were supplemented with either 25 μl monoclonal antibody to TGF β (1 mg/ml, 1D11.16), 25 μl purified murine IgG (1 mg/ml, Sigma), or 25 μl physiological saline. The murine monoclonal antibody 1D11.16 has neutralising activity against TGF β_1 and TGF β_2.[15] NOB 1 cells were washed twice with physiological saline and 100 μl NOB cells (1×10^5 cells/well) was dispensed in the wells. Finally, 50 μl CTLL cells (4×10^3 cells/well) was added to the wells and the cluster dish was incubated for 20 hours at 37°C in a humidified 5% carbon dioxide atmosphere. The 20 hour incubation was followed by addition of 8·5 kBq/well thymidine labelled with hydrogen-3 (Du Pont de Nemours) to the wells. After a further three hour incubation, the cells were harvested by an automatic cell harvester and the incorporated radioactivity determined by liquid scintillation counting.

The neutralising potential of the 1D11.16 monoclonal antibody was estimated by studying the neutralising effect of 1D11.16 on the inhibitory effects of known amounts of TGF β_1 in the NOB/interleukin 1 assay. The NOB/interleukin 1 assay was carried out as described earlier. Instead of culture supernatant, however, known amounts of TGF β_1 (25 μl, 0–50 ng/ml) in combination with 25 μl physiological saline or 1D11.16 (1 mg/ml) were added to the wells.

EFFECT OF MONOCLONAL ANTIBODY 1D11.16 ON PROTEOGLYCAN SYNTHESIS

Murine patellae were cultured for 24 or 48 hours in RPMI 1640 DM medium (200 μl/patella) in the presence or absence of 0·5 μg/ml insulin-like growth factor 1. At the start of the experiment, either 100 μg 1D11.16 monoclonal antibody, 100 μg purified mouse IgG, or physiological saline was added to cultures (final concentration 100 μg/ml). During the 48 hour culture period the medium and supplements were changed after 24 hours. The last two hours of the incubation were performed in the presence of 0·74 MBq labelled with ^{35}S sulphate. After incubation, patellae were processed as described earlier.

STATISTICS

Statistical significance was tested by analysis of variance in combination with Student's *t* test. A p value less than 5% was considered significant.

Results

Transforming growth factor β has been reported to have pronounced effects on the proteoglycan synthesis of articular cartilage chondrocytes but we were unable to show any significant effects of exogenously added TGF β, up to a concentration of 200 pM, on proteoglycan

synthesis in anatomically intact cartilage (fig 1). In contrast to insulin-like growth factor 1, TGF β neither stimulated nor decreased proteoglycan synthesis, as measured by sulphate incorporation, in intact murine patellae after incubation periods of 24 and 48 hours. The presence of 10% fetal calf serum or 0·5 μg/ml insulin-like growth factor 1 had no modulatory influence on the effects of TGF β on proteoglycan synthesis. As expected, insulin-like growth factor 1 stimulated the incorporation of sulphate into the murine articular cartilage.

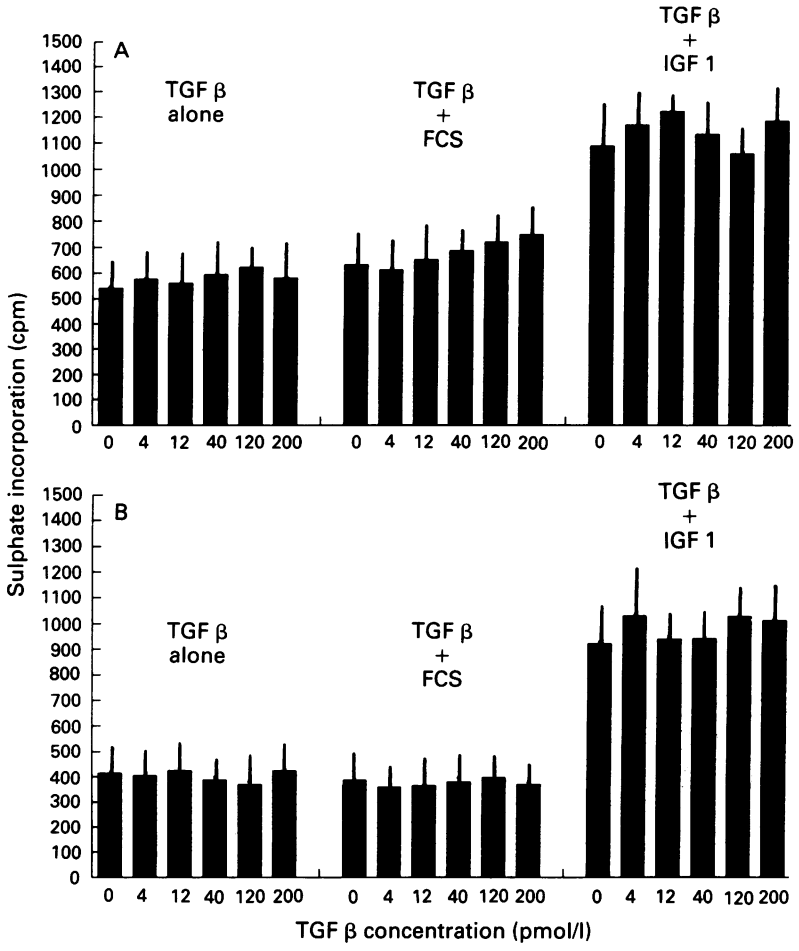

Figure 1 Effect of transforming growth factor β (TGF β) on proteoglycan synthesis in anatomically intact cartilage. Murine patellae were incubated for 24 or 48 hours with TGF β alone or in combination with fetal calf serum (FCS) (10%) or insulin-like growth factor 1 (IGF 1) (0·5 μg/ml). For the last two hours of the incubation period sulphate labelled with sulphur-35 was added to the culture media to measure proteoglycan synthesis. Results are the mean (SD) of five combined experiments. (A) 24 hours incubation; (B) 48 hours incubation.

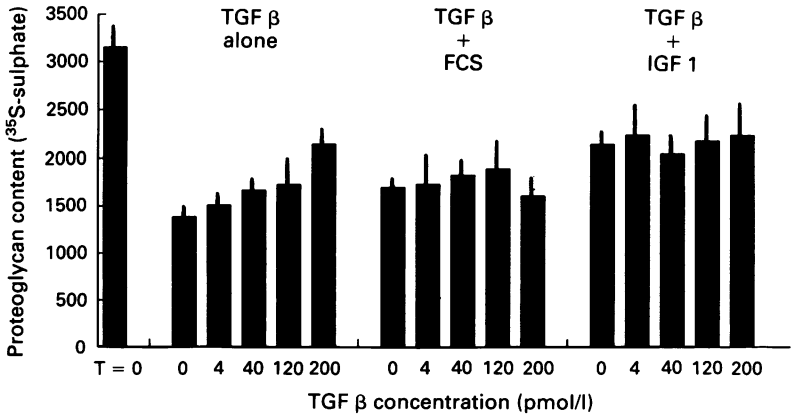

Figure 2 Effect of transforming growth factor β (TGF β) on proteoglycan breakdown in anatomically intact cartilage. Sulphate labelled with sulphur-35 was added to the patellae and incubated for 24 hours in the presence of TGF β alone or in combination with 10% fetal calf serum (FCS) or 0·5 μg/ml insulin-like growth factor 1 (IGF 1). The t=0 value is the amount of sulphate labelled with sulphur-35 incorporated directly after labelling. Results are the mean (SD) of four combined experiments.

One of our concerns was that the lack of effect of exogenously added TGF β on proteoglycan synthesis was due to the fact that TGF β did not reach the chondrocytes in the anatomically intact matrix. Exogenously added TGF β, however, had significant inhibitory, dose dependent, effects on ^{35}S labelled proteoglycan degradation in anatomically intact cartilage. In the absence of fetal calf serum or insulin-like growth factor 1, TGF β concentrations of 40 pM and higher significantly decreased the release of sulphated proteoglycans during a 24 hour culture period, measured by the reduction of ^{35}S labelled sulphate in prelabelled patellae (fig 2). Fetal calf serum and insulin-like growth factor 1 significantly decreased the breakdown of proteoglycans in the absence of TGF β. Transforming growth factor β had no additional effect on the fetal calf serum or insulin-like growth factor 1 induced reduction of proteoglycan degradation.

Another explanation of the absence of effect of exogenously added TGF β on proteoglycan synthesis could be the presence of active endogenously produced TGF β in the culture medium. Suggestive evidence was provided by the fact that the culture supernatant contained inhibitory activity when tested in the NOB/interleukin 1 assay. Addition of the monoclonal antibody to TGF β 1D11.16 totally circumvented the inhibitory effect of the culture supernatant whereas purified murine IgG had no neutralising effects (fig 3). These results show that the culture supernatant of anatomically intact patellae contains TGF β activity and that this activity can be blocked by the neutralising antibody 1D11.16 in this murine system. The culture supernatant contained approximately 40 pmol/ml active TGF β.

To test the neutralising potency of 1D11.16, the effect of 1D11.16 on various known amounts of human TGF β_1 was tested in the NOB/interleukin 1 assay. 1D11.16 (100 mg) totally neutralised the effect of 0·5 ng TGF β_1 in the NOB/interleukin 1 assay (fig 4). TGF β_1 (5 ng) was 47% neutralised by 100 μg of the monoclonal antibody 1D11.16. Addition of 100 μg/ml 1D11.16 to the patella cultures at the start of the experiment, to neutralise the endogenous TGF β in the culture medium, significantly enhanced the incorporation of sulphate into the anatomically intact murine patellae in three of four different experiments (table). Monoclonal antibody to TGF β stimulated proteoglycan synthesis in 24 hour patellae cultures in the presence of insulin-like growth factor 1 and in 48 hour cultures in the absence or presence of insulin-like growth factor 1. Although the effects were only 10–15%, significance was reached. This indicates that the endogenously produced TGF β has an inhibitory effect on the proteoglycan synthesis of articular chondrocytes in anatomically intact cartilage. The addition of purified murine IgG had no effects on proteoglycan synthesis.

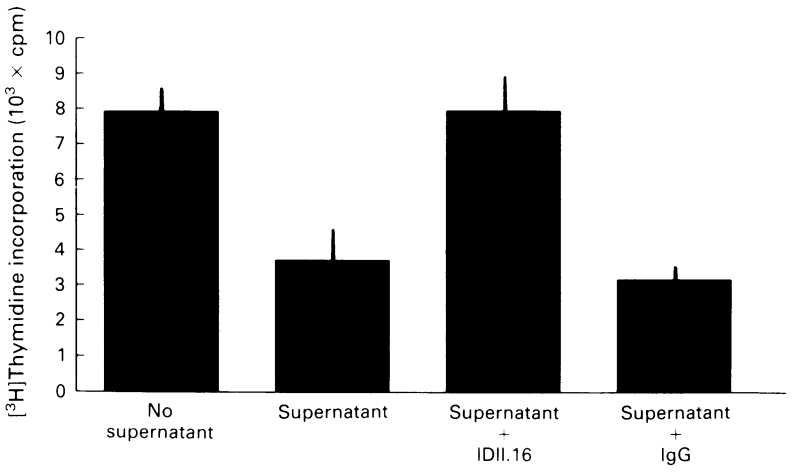

Figure 3 Inhibition of [³H]thymidine incorporation in the NOB/interleukin 1 assay by supernatant of patellae cultures. The NOB/interleukin 1 assay was performed in the presence of a standard amount of interleukin 1. The supernatant of patella cultures alone or in combination with purified murine IgG or 1D11.16 was added to the wells. Results are mean (SD) of one representative experiment out of three.

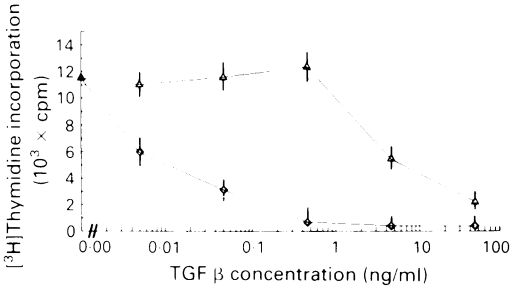

Figure 4 Neutralising effect of the monoclonal antibody to transforming growth factor β (TGF β) 1D11.16 in the NOB/interleukin 1 assay. The NOB/interleukin assay was performed in the presence of a standard amount of 1D11.16 (100 μg/ml) and interleukin 1 and various amounts of human TGF β1. Values are mean (SD) of one representative experiment out of three. (◇) TGF β; (△) TGF β in combination with 1D11.16.

Discussion

These data show that exogenously added TGF β has no effect on proteoglycan synthesis in anatomically intact articular cartilage of murine patellae but that neutralisation of endogenous TGF β leads to stimulation of proteoglycan synthesis. As a consequence of this TGF β appears to inhibit proteoglycan synthesis in anatomically intact cartilage. These data support the observations of Skantze *et al* [8] and Rosier *et al*.[16] Skantze *et al*, using agarose cultures of rabbit articular chondrocytes, showed an inhibitory effect of TGF β on chondrocyte glycosaminoglycan synthesis at a concentration of 0·2 ng/ml (8 pmol/l).[8] These results are confirmed by the observation of Rosier *et al* who found inhibitory effects of TGF β on articular cartilage chondrocytes of chicken.[16] Additionally, only inhibitory TGF β receptors are seen on articular chondrocytes.[7]

In contrast, Redini *et al* showed the stimulating effects of TGF β on isolated rabbit articular chondrocytes.[9] In our hands, TGF β inhibited proteoglycan synthesis in freshly isolated bovine chondrocytes whereas it had a stimulating effect on proteoglycan synthesis in bovine chondrocytes cultured for seven days (data not shown).

Only one other study has been published on the effects of TGF β on chondrocytes in a matrix. Morales and Roberts reported 20–60% stimulation of proteoglycan synthesis after three days and maximum stimulation after six days in bovine articular explants by TGF β.[10] This lag time in the response to TGF β appears, however, to be absent in experiments with isolated chondrocytes.[7 9 16] Inoue *et al* reported that TGF β induces morphological dedifferentiation of chondrocytes and that these cells respond to TGF β by stimulation of proteoglycan synthesis.[17] Culturing of explants for several days in the presence of large amounts of TGF β could lead to changes in the response of articular cartilage chondrocytes to TGF β.

The TGF β activity present in the culture supernatants of patellae might be derived from the articular cartilage, the damaged surrounding tissues or both. Large amounts of TGF β are present in articular cartilage[18] and part of this might be released during cartilage culture in the active form.[18] The fact that only small (10–15%), although significant, stimulating effects of 1D11.16 on glycosaminoglycan synthesis were observed might be due to the fact that IgG antibodies are unable to penetrate intact articular cartilage.[19] Decrease of active TGF β from the articular cartilage matrix will be based on depletion of TGF β from the culture medium, resulting in the increased release of TGF β from the matrix to reach a state of equilibrium between TGF β concentration inside and outside the matrix. Production of active TGF β by the surrounding tissues will be neutralised by the 1D11.16 monoclonal antibody.

In contrast to the effect of TGF β on synthesis, exogenously added TGF β decreased the degradation of proteoglycans in anatomically intact cartilage, showing that TGF β could reach the chondrocyte in the intact matrix. Transforming growth factor β only had a significant effect in the absence of fetal calf serum or insulin-like growth factor 1. The presence of the TGF β inhibitor α_2 macroglobulin in fetal calf serum might be the reason for the absence of TGF β effects on proteoglycan breakdown in the presence of fetal calf serum.[20 21] Insulin-like growth factor 1 had a strong inhibitory effect on the degradation of proteoglycans in intact cartilage which might conceal the effects of TGF β on proteoglycan breakdown. The differential effect of exogenously added TGF β on proteoglycan synthesis or breakdown may be caused by

Effect of antibodies to transforming growth factor β (TGF β) on proteoglycan synthesis in anatomically intact murine patellar cartilage. Patellae were incubated for 24 or 48 hours in the presence or absence of insulin-like growth factor 1 (0·5 μg/ml). At the beginning of the experiment antibodies to TGF β, purified IgG, or saline were added to the culture media. The last two hours of the incubation took place in the presence of radiolabelled sulphate. The results given are the combined values (mean (SD)) of three experiments (n=18)

	Incorporation of sulphate labelled with sulphur-35 (counts/min)			
	RPMI (24 h)	*Insulin-like growth factor 1 (24 h)*	*RPMI (48 h)*	*Insulin-like growth factor 1 (48 h)*
Saline	512 (101)	824 (116)	340 (66)	680 (88)
Purified IgG	446 (67)	786 (94)	368 (52)	694 (88)
1D11.16	481 (71)	898 (97)	433 (84)	793 (145)

$^{*}p<0·05$; $^{**}p<0·01$.

differences in the TGF β dose response relation of proteoglycan synthesis and degradation in anatomically intact cartilage. Neutralisation of endogenously produced TGF β with monoclonal antibodies had no effect on proteoglycan breakdown, indicating that higher concentrations than those produced endogenously are needed to inhibit breakdown (data not shown).

This study indicates that TGF β inhibits proteoglycan synthesis in normal chondrocytes in an anatomically intact matrix. However, in disease states such as osteoarthritis or rheumatoid arthritis chondrocytes might change phenotypically under the influence of cytokines and growth factors such as interleukin 1 and TGF β.[17 22] Phenotypically changed chondrocytes might have a different expression of TGF β cell membrane receptors from normal articular chondrocytes, rendering these cells susceptible to the matrix synthesis and cell proliferation promoting effects of TGF β, as seen with growth plate chondrocytes. In this way TGF β could play a part in the regulation of matrix synthesis and cell proliferation (inhibition) in normal chondrocytes, as well as in the repair response of osteoarthritic cartilage, characterised by increased proteoglycan synthesis and chondrocyte proliferation.

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