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Osteoarthritis-like changes in the murine knee joint resulting from intra-articular transforming growth factor-β injections

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Summary

Objective: To examine the impact of prolonged TGF-β exposure on cartilage and ligamentous joint structures *in vivo*, to investigate involvement of TGF-β in osteoarthritis pathology.

Methods: TGF-β was injected into murine knee joints once or repeatedly, whereafter articular cartilage proteoglycan (PG) synthesis and content, and histological changes in knee joints were studied over a 2-month period.

Results: A single injection of TGF-β stimulated patellar cartilage PG synthesis for 3 weeks and PG content for 2 weeks. Triple TGF-β injections prolonged the increase in PG content to 3 weeks. Patellar cartilage showed no histological abnormalities at 1 and 2 months after the last injection. In contrast, 2 months after triple TGF-β injections the superficial layer of tibial cartilage still had an increased proteoglycan content, while severe PG depletion was found in deeper layers of the posterior part of the lateral tibia in particular. Eventually, lesions occurred at the level of the tide-mark, exactly the site where cartilage is torn off in experimental and spontaneous osteoarthritis in mice. Additionally, multiple TGF-β injections induced formation of chondroid structures along the margins of articular cartilage. These chondroid structures were transformed into osteophytes via endochondral ossification. Formation of chondroid tissue was also observed in collateral ligaments.

Conclusion: Multiple intra-articular injections of TGF-β induce changes in articular cartilage and surrounding tissues that have strong resemblance to features of experimental and spontaneous osteoarthritis in mice, suggesting a role for TGF-β in the OA process. © 2000 OsteoArthritis Research Society International

Key words: TGF-β, Focal PG loss, Chondrogenesis, Osteoarthritis.

Introduction

In the search for mediators involved in osteoarthritis (OA) pathology, transforming growth factor-β (TGF-β) has been considered a likely candidate. TGF-β is a multipotent regulator of cell growth and differentiation, and of extracellular matrix production. Three different isoforms have been found in mammalian species: TGF-β1, -β2, and -β3. The mature TGF-β's are all 25 kDa homodimers, with each monomer consisting of 112 amino acids containing nine cysteine residues. TGF-β is normally secreted as an inactive high molecular weight complex which has to be dissociated before activation. High levels of active TGF-β have been found in synovial fluids of rheumatoid arthritis and OA patients,[1,2] indicating that this factor is produced and activated during joint pathology. Enhanced expression of TGF-β mRNA has been found in articular cartilage of the STR/ort mouse which develops natural OA.[3]

In earlier studies[4,5] we have shown that intra-articular injections of TGF-β1 or -β2 stimulated articular cartilage proteoglycan (PG) synthesis, counteracted suppression of PG synthesis by interleukin-1, and accelerated PG replenishment of depleted articular cartilage, suggesting that TGF-β protects articular cartilage during pathology. On the other hand, evidence is present that excessive/prolonged exposure to this factor can have adverse effects. TGF-β is a growth factor which is activated after tissue damage, a condition which may apply to OA development after ligament rupturing or cartilage lesions. Possibly, TGF-β is involved in enhanced articular cartilage PG synthesis as found in early experimental osteoarthritis[6,7] and after a period of attempted repair it could be a trigger for cartilage degeneration. Excessive levels of TGF-β also induce fibrosis in joint tissues,[4] as well as in kidney and liver diseases and excessive scar formation in skin healing, and this has been termed the dark side of tissue repair.[8,9] In the present study we investigated changes in cartilage and other joint structures, over a 2-month period after single and repeated injections of TGF-β into the murine knee joint, and compared the findings with spontaneous OA lesions in aged mice.

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Materials and methods

ANIMALS

Male C57Bl/10 mice aged 12 weeks or 18 months were used. They were fed a standard diet and tap water *ad libitum*.

INTRA-ARTICULAR INJECTIONS

After anaesthetizing the mice with ether, 200 ng recombinant human TGF-β1 (Genentech Inc., San Francisco, CA, U.S.A.) dissolved in 6 μl of physiological saline (0.9% NaCl)+0.1% ultrapure bovine serum albumin (Sigma, St Louis, MO, U.S.A.) was injected into the joint cavity of the right knee once, or three times at alternate days. Because of the short half-life of TGF-β in the joint, we had to use high-dose injections. The 200 ng dose has been shown effective in earlier in-vivo studies[4,5] Simultaneously, the contralateral joint received an equal volume of saline+0.1% bovine serum albumin. To study whether the effects of triple TGF-β injections can be augmented, 12 mice received six injections, at alternate days. Moreover, to find out whether the joint would still react to TGF-β after intense exposure, a group of six mice received, besides these six injections, two extra injections 2 weeks after the last of six injections. Experimental osteoarthritis was induced by intra-articular injection of 10 units of highly purified collagenase (type VII, Clostridium histolyticum, Sigma, St Louis, MO, U.S.A) dissolved in 6 μl physiological saline.[10,11]

DETERMINATION OF PATELLAR CARTILAGE PROTEOGLYCAN SYNTHESIS

PG synthesis was measured *ex vivo*. Whole patellae, with a standard amount of surrounding tissue, were dissected from the knee joints. Patellae were then pulse-labeled (2 hours at 37°C) with ^{35}S-sulfate. Subsequently, they were washed, fixed, decalcified, punched out of surrounding tissue, and dissolved as described before.[12] The ^{35}S-content of each patella, which is a reliable measure of patellar cartilage PG synthesis,[13] was measured by liquid scintillation counting.

HISTOLOGY

Whole knee joints were dissected at different time points after repeated intra-articular injection of 200 ng TGF-β and processed as previously described.[15] Semiserial frontal sections (6 μm) were mounted on gelatin-coated slides and stained with hematoxylin/eosin or safranin O/fast green for examination of cells and cartilage matrix, respectively. In addition to TGF-β-induced changes, we also studied histology of naturally occurring murine OA in joints of 18-month-old mice, and of experimentally induced OA in collagenase-injected mice.

DETERMINATION OF PATELLAR CARTILAGE PROTEOGLYCAN CONTENT

Articular cartilage PG content is reflected by safranin O staining intensity in histological sections. This was measured, as described before,[14] using an automated image analysis system (VIDAS, Kontron Elektronik GMBH). Microscopic images were recorded by a CDD video camera (Sony) and stored and processed by a personal computer. Optical density was measured by integral measurement in a 20 μm layer, along the cartilage surface. The total zone of non-calcified cartilage was approximately 30–40 μm in width. Fast green staining was neutralized by use of a green filter. Measurements were corrected for lacunae. PG content in articular cartilage of TGF-β injected joints was compared to content in contralateral vehicle-injected joints.

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 (Life Technologies). Cartilage of 10 patellae was pooled and synovium punches were taken from the same mice. 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 40. To determine the relative mRNA levels, 5 μl samples were taken at increasing cycle numbers. The PCR products were electrophorised in 1.6% agarose gels containing ethidium bromide. The cycle number at which the product was first detected on the gel was taken as a measure for the amount of specific mRNA present in the originally isolated RNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control. This method was validated by van Meurs *et al.*[16] All RT-PCR reactions were performed in duplicate. The following primers were used in the amplification reactions. To detect GAPDH, the primers 5′-AACTCCCTCAAGATTGT CAGCA-3′ (upper) and 5′-TCCACCACCCTGTTGCTG TA-3′ were used (product 553 bp). Murine TGF-β1 primers were derived from Clontech (Palo Alto, CA, U.S.A.) (product 525 bp), whereas murine TGF-β2 (product 489 bp) and -β3 (product 380 bp) were used as described by Mulheron *et al.*[17] For each time-point studied the RNA extracts obtained from at least two different experiments were analyzed.

Statistical analysis

Differences between groups were tested using the Student's *t*-test. Differences were considered significant if $P<0.05$.

Results

PROTEOGLYCAN SYNTHESIS

Intraarticular injection of 200 ng TGF-β stimulated articular cartilage PG synthesis after a lag time of about two days. Maximum synthesis (>200%) was reached after 4 days. Even three weeks after a single injection PG synthesis was still significantly increased (Fig. 1A). Stimulation of PG synthesis was not further increased or prolonged if 200 ng TGF-β was injected three times, at

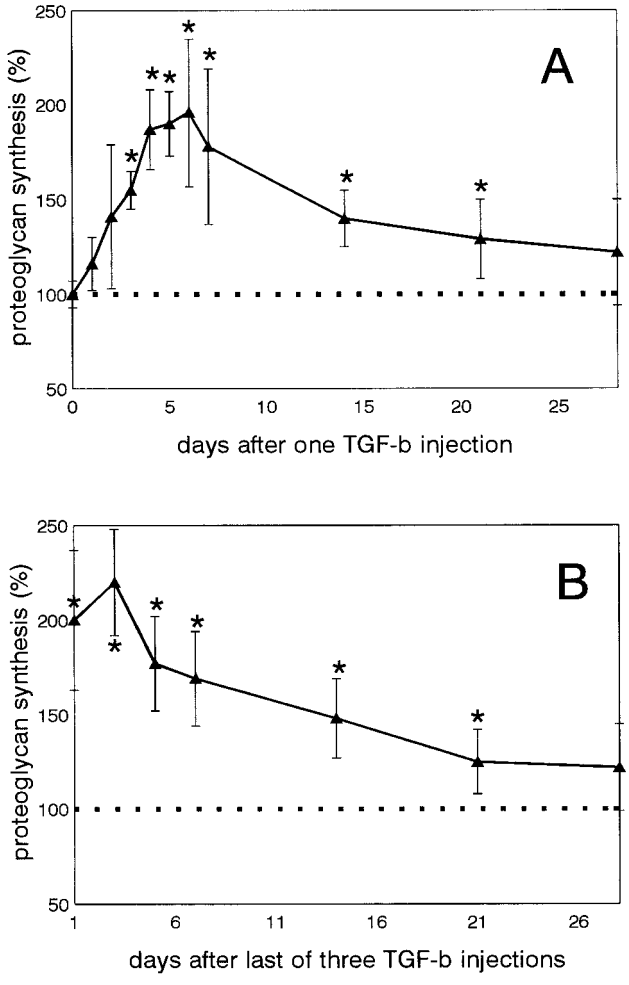

Fig. 1. Long-lasting stimulation of PG synthesis in patellar cartilage after one injection with 200 ng human rec TGF-β1 (A), or after three such injections at alternate days (B). PG synthesis (^{35}S-sulfate incorporation) is expressed as percentage of that in the contralateral, vehicle-injected joints. The experiment was performed twice, with six patellae per experimental group each time. Therefore the values represent the mean of 12 patellae. *=significant increase.

alternate days, as compared with a single injection of 200 ng TGF-β (Fig. 1B).

PROTEOGLYCAN CONTENT

Densitometric analysis of safranin O staining intensity in histological sections showed that the GAG content in the 20 μm superficial layer of the non-calcified articular cartilage of the patella had significantly increased one week after injection of 200 ng of TGF-β1 (Table I). This increase was still present after two weeks (not shown), but after three weeks it was lost. As compared with a single injection, three injections of 200 ng TGF-β did not augment the enhancing effect of TGF-β on patellar cartilage PG content one week after the last injection, but the period of increased PG content was prolonged with one extra week, to three weeks after the last injection. One month after the last injection, the patellar cartilage PG content was not different from normal, even if six injections were given instead of three. Moreover, at this point of time, the patellar cartilage showed no striking histologic changes. Besides patellar cartilage, TGF-β temporarily increased the PG content in all articular cartilage surfaces of the injected joint. In the tibia the disturbance of PG content of the superficial cartilage was even more prolonged, and was found over the whole two-months period studied (Table I).

Table I
Increase of safranin O staining in superficial articular cartilage after TGF-β injections

Group	Day*	Patella		Lateral tibia	
		N†	Increase (% of control)	*N*†	Increase (% of control)
3×vehicle	D7	8	−11±9		
1×TGF-β	D7	8	19±10‡		
3×TGF-β	D7	12	21±6‡		
1×TGF-β	D21	6	7±6		
3×TGF-β	D21	6	11±5§		
3×TGF-β	D28	12	15±11	12	18±8§
6×TGF-β	D28	12	18±18	12	11±5§
3×TGF-β	D56	8	9±7	8	12±6§

Safranin O staining of articular cartilage in histological sections was measured using an automated image analyzer. The increase in staining intensity is expressed as percentage of the contralateral, non-injected control knee joint.

*Days after the last injection.

†Number of joints measured: at least three sections of each joint were measured.

Statistical significance of the TGF-β-induced increase was tested using the Student's *t*-test. §$P<0.05$, ‡$P<0.01$.

LESIONS IN TIBIAL CARTILAGE

Simultaneously with these supranormal GAG contents in the 20 μm superficial layer, focal areas with decreased safranin O staining developed deeper in the cartilage of load bearing areas of the lateral tibia (Fig. 2). These areas with low chondrocyte density, and low extracellular matrix PG content were found at sites just above the tide-mark. In the adjacent superficial part of the cartilage, occasional surface roughening and more often, clusters of chondrocytes with high pericellular PG concentration were found. In sections of several knee joints, a crack along the tide-mark was found at the site of the PG depletion (Fig. 2D). Interestingly, in early stages of natural murine OA, in aging mice, similar areas of PG depletion were found. In further advanced stages of murine OA noncalcified cartilage was lost, appearing to have been torn off from the tide-mark (Fig. 2E).

TGF-β-induced focal PG loss was already present one week after the last injection of TGF-β and the depleted areas became larger with time. They were not found in superficial frontal sections of the knee, but emerged in deeper sections of the posterior part of the joint. In a small percentage of control joints, some PG loss was found in the same area, but the extent of spontaneous PG depletion was marginal. No PG loss was ever found in patellar cartilage. To study whether additional administration of TGF-β further augmented the formation of TGF-β-induced

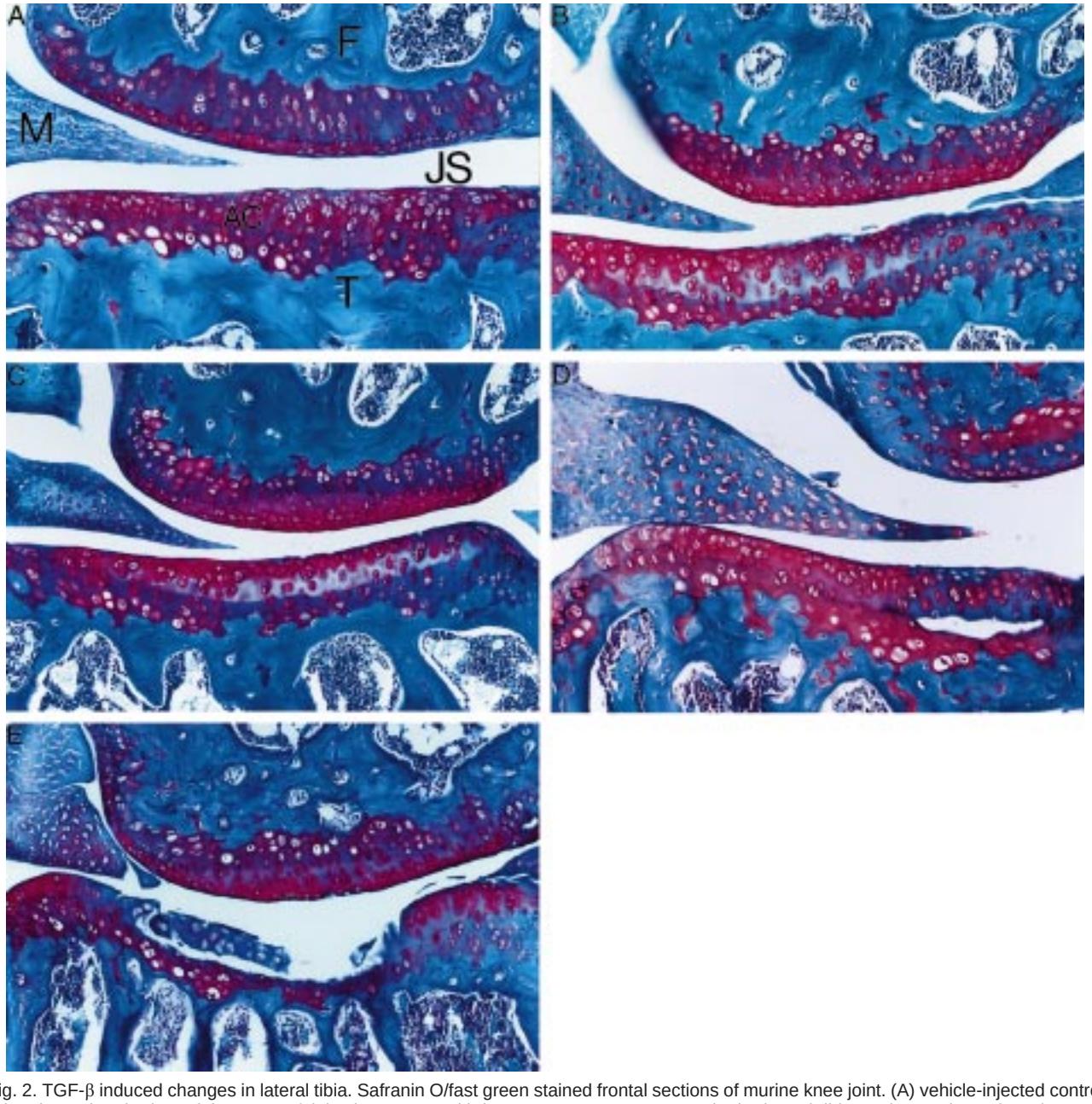

Fig. 2. TGF-β induced changes in lateral tibia. Safranin O/fast green stained frontal sections of murine knee joint. (A) vehicle-injected control (B) 7 days after the last of three TGF-β-injections areas with low PG content are present in the lateral tibia, and sometimes there is some surface roughening (C) another TGF-β injected animal, showing abnormal distribution of chondrocytes, with high PG concentration around superficial chondrocytes (D) crack at the level of the tide-mark in TGF-β-injected animal with lesion (E) for comparison, spontaneously occurring crack at the level of the tide-mark in 18-month old mouse. M=meniscus, F=femur, T=tibia, AC=articular cartilage, JS=joint space. Original magnification: ×200.

lesions, experiments were performed in which six or eight injections of TGF-β were given. However, these additional injections did not affect number or size of the areas with PG depletion (data not shown).

OSTEOPHYTES

A single injection of 200 ng TGF-β induced transient fibrosis in the synovium, but did not lead to changes in the appearance of articular cartilage and other joint structures. In contrast, triple injections of 200 ng TGF-β induced development of cartilage-like, PG-containing tissue, at the margins of articular cartilage (Fig. 3B, C). These new structures, which we named ‘chondrophytes’, seemed to originate from the periosteum. Chondrophytes were not found after a single TGF-β injection. A week after the last injection, the new tissues had reached their maximum size and thereafter they calcified via endochondral ossification and developed into mature osteophytes, containing bone

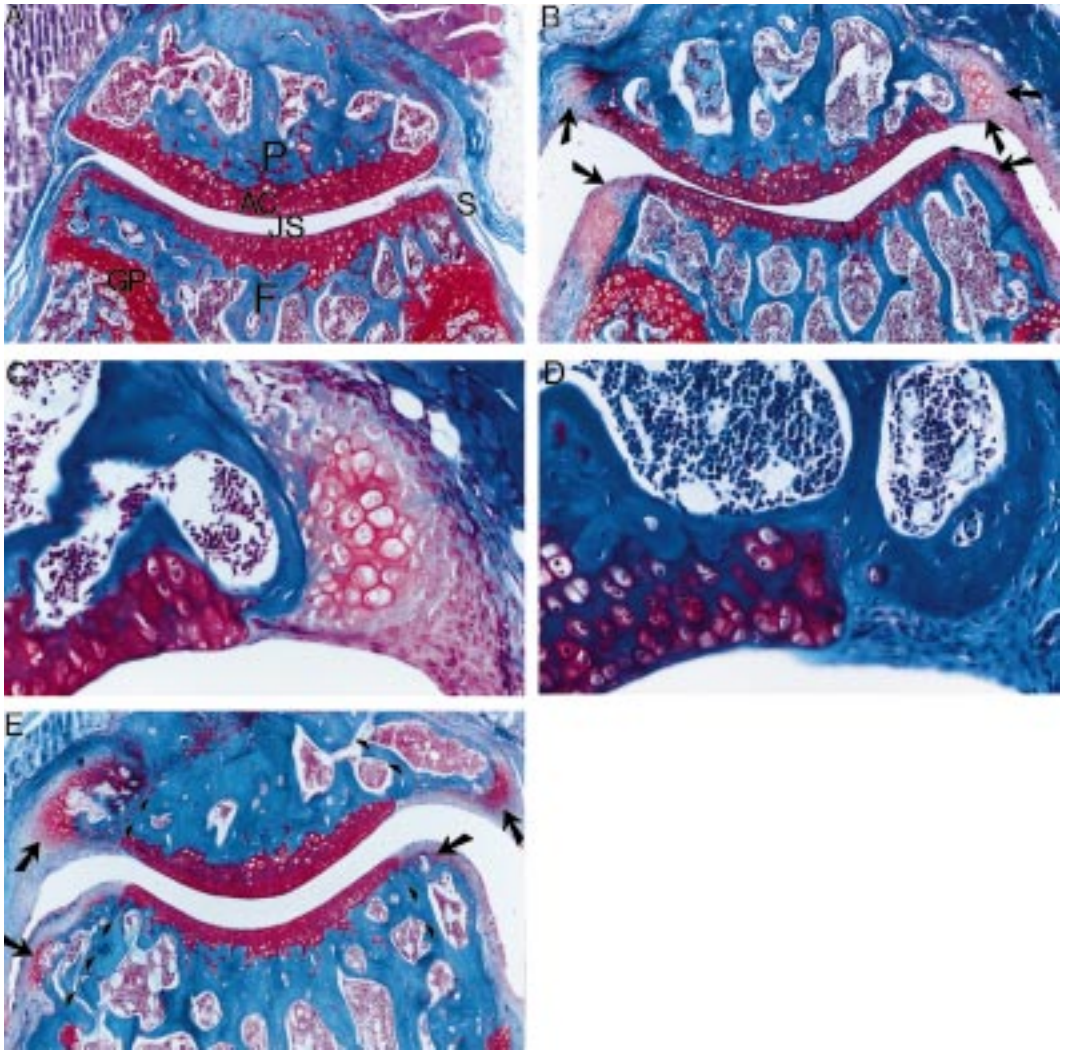

Fig. 3. Osteophyte induction by TGF-β. (A) patella of vehicle-injected animal (B) early phase cartilage formation (arrows) at the margins of articular cartilage of patella and femur, one week after third intra-articular injection of 200 ng TGF-β (C) larger manification of chondrophyte in 3B. (D) one month after the third injection of TGF-β, at the same location osteophytes have developed, which do not bind safranin O any more and contain bone marrow spaces (E) extra TGF-β injections restimulate periosteum (large arrows) of mature osteophytes; adjusted protocol: two extra injections were given 2 weeks after the last of six injections, performed in the first 10 days, and tissue was dissected one week later; small arrows indicate original bone margins. P=patella, F=femur, S=synovium, AC=articular cartilage, GP=growth plate, JS=joint space. Stained with safranin O/fast green. Original magnification: A, B, and E ×100; C and D ×400.

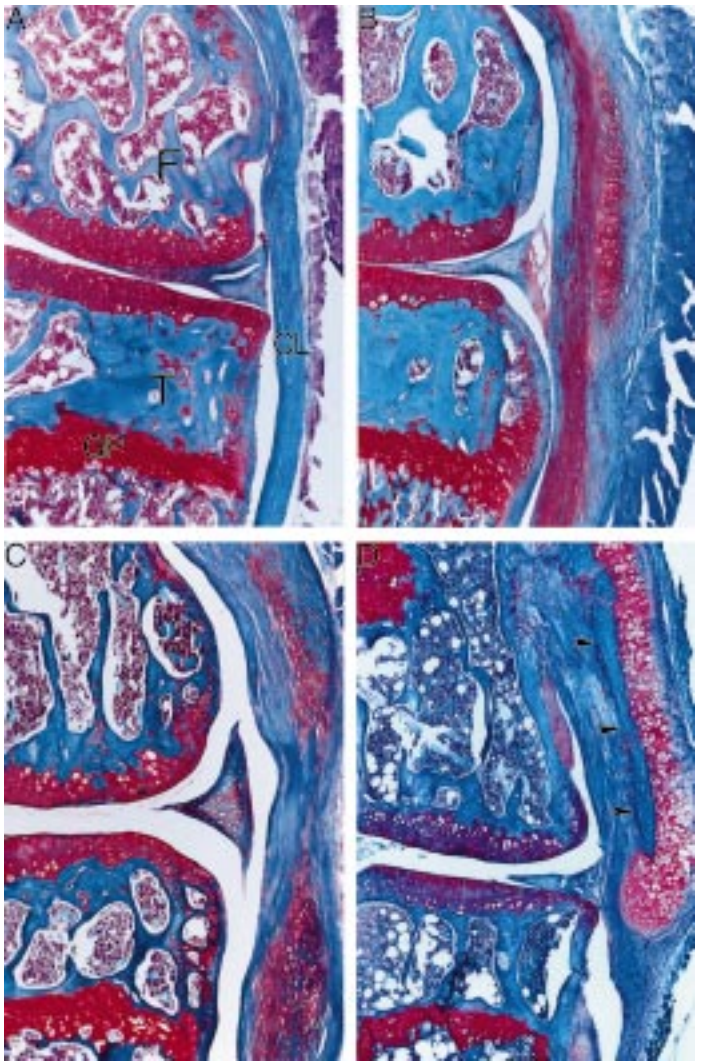

Fig. 4. Effects of TGF-β injections on medial collateral ligament. (A) vehicle-injected joint (B) cartilaginous tissue present in the ligament one month after six injections of TGF-β (C) cartilaginous tissue present in medial collateral ligament of mouse at day 42 after intra-articular injection of collagenase. (D) TGF-β-induced activation of the surface cells of bony structure (ossicle marked by arrows) in medial collateral ligament in 18-months-old mouse with spontaneously occurring osteoarthritis; 1 week after third injection F=femur, T=tibia, CL=collateral ligament, GP=growth plate. Stained with safranin O/fast green. Original magnification: ×100.

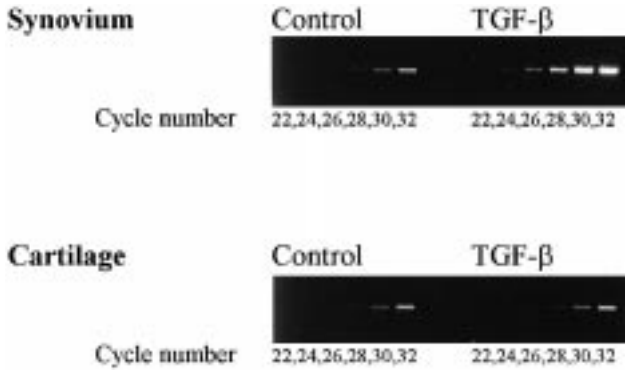

Fig. 5. Semiquantitative TGF-β1 mRNA analysis in synovium and patellar cartilage isolated from murine knee joints. Tissue was taken 8 days after the last of three injections with 200 ng TGF-β1. As a control, the vehicle-injected contralateral joints of the same animals were used. Samples from the PCR reaction mixture where taken with intervals of two cycli and run on an ethidium-stained agarose gel. TGF-β1 mRNA levels were corrected for GAPDH mRNA expression.

marrow spaces (Fig. 3D). Even after prolonged exposure to TGF-β, the periosteum remained responsive to TGF-β. This was shown in a group of mice in which two weeks after the last of six injections, two additional injections caused renewed development of cartilage-like tissue on the recently formed osteophytes (Fig. 3E).

CHONDROGENESIS IN LIGAMENTS

Two striking consequences of triple TGF-β injections were synovial hyperplasia and thickening of ligaments and tendons. Interestingly, another phenomenon also seen in early osteoarthritis was found at day 28 in mice which received six or eight TGF-β injections. Chondrogenesis was observed in the collateral ligaments of these animals. Large rounded cells with lacunae and pericellular PGs, presumably fibrochondrocytes, were found in these sites (Fig. 4B). For comparison, similar chondrogenesis (preceding partial ossification) was also found in collateral ligaments of mice with collagenase-induced ligament disruption and subsequent instability-induced osteoarthritis (Fig. 4C). In time, these chondroid structures also might develop into bony structures, akin to ossicles present in collateral ligaments of aged mice with spontaneous osteoarthritis.

TGF-β EFFECTS IN OLD MICE

In 18-month-old mice, TGF-β injections induced stimulation of PG synthesis and development of osteophytes and these effects were comparable to what was seen in young adults (not shown). This indicates that in old mice, the chondrocytes and the cells of the periosteum still respond to TGF-β. Moreover, TGF-β injections appeared to induce development of cartilage-like tissue on already existing bony structures (ossicles) in ligaments of 18-month-old mice with spontaneous osteoarthritis (Fig. 4D). This suggests that TGF-β could be involved not only in induction, but also in outgrowth of such structures.

TGF-β AUTOINDUCTION

Since TGF-β appeared to have long-lasting effects on articular cartilage, we estimated TGF-β autoinduction in articular cartilage and neighbouring soft tissue. In patellar cartilage, mRNA levels of TGF-β1, -β2, and -β3 were not elevated at day 2 and day 8 after three intra-articular injections with 200 ng TGF-β1. In contrast, in standardized punches from neighbouring joint capsule, GAPDH-corrected mRNA levels of all three isotypes of TGF-β appeared to be elevated at these time points. In joint capsule, intra-articularly injected TGF-β1 increased mRNA of TGF-β1, -β2, and -β3 to levels that were approximately 16 times higher compared to normal joints, at day 2 and 8. Figure 5 shows a characteristic result of RT-PCR, illustrating TGF-β1 mRNA upregulation in synovial tissue.

Discussion

We examined the effects of prolonged TGF-β exposure on articular cartilage and other joint structures *in vivo*, as a way of investigating involvement of TGF-β in osteoarthritis pathology. After multiple intra-articular injections of TGF-β, we observed prolonged disturbances of articular cartilage PG homeostasis. Both PG synthesis and content were enhanced in all articular cartilage surfaces of the knee. In patellar cartilage this was normalized in about one month, but in tibial cartilage the PG content of the surface layer was still enhanced at 2 months after the last injection. Deeper in the tibial cartilage areas with PG loss were found and in several animals cracks were observed at these sites. Furthermore, we observed TGF-β-induced fibrosis, osteophyte formation at specific sites, and development of chondroid tissue in collateral ligaments. All TGF-β-induced changes are very similar to changes that have been reported in experimental OA in dogs,[6,7] and changes we observed in experimental OA in mice and in spontaneous OA in old mice.

Already, one single injection of 200 ng TGF-β induced long-lasting stimulation of patellar cartilage PG synthesis, which started after a lagtime of about 2 days. This suggests the involvement of changes in chondrocyte phenotype, induction of second mediators, and/or TGF-β autoinduction.[18,19] TGF-β autoinduction at the mRNA level was not detected in patellar cartilage, but strong upregulation of all three TGF-β isotypes was observed in adjacent joint capsule specimens during a period of at least 8 days after the last of three intra-articular injections of TGF-β1. This observation suggests that the effects of TGF-β1 injections on articular cartilage can be increased and prolonged by local TGF-β induction in neighbouring soft connective tissue.

In addition to enhanced PG synthesis, the PG content in the superficial layer of articular cartilage was also significantly increased after TGF-β injection. PG content of patellar cartilage was enhanced during 3 weeks after the last of three TGF-β injections, and 1 week later, the extracellular cartilage had normalized and also the chondrocytes had a normal appearance. This is in contrast to results from a study of Hulth and colleagues,[20] who

found in TGF-β-treated rat patellar cartilage an area of necrosis and decreased safranin O staining in the deeper part of the cartilage. However, in tibial cartilage of TGF-β-injected mice we noticed that in the superficial cartilage the PG content was still enhanced even at two months. Interestingly, in deeper cartilage layers, especially of the lateral tibia, focal PG loss occurred in an area just above the tide-mark, in the posterior part of the joint. In control knees we sometimes observed areas with PG loss in the same region, but these were much smaller. Therefore, we postulate that TGF-β accelerates the pre-osteoarthritic changes in murine articular cartilage. For development of fullblown murine OA pathology, either more time is needed, or additional factors are involved, like mechanical overload, joint instability or local generation of other cytokines/growth factors.

TGF-β-induced focal PG depletion was an unexpected finding. TGF-β is known as a stimulator of extracellular matrix production, an inhibitor of protease release and an upregulator of TIMP expression, all fitting with a controling role in further tissue damage. However, TGF-β has recently been shown to be a potent inducer of MMP-13,[21] an enzyme involved in the pathophysiology of human OA.[21–23] Intriguingly TGF-β-induced expression of MMP-13 was mainly found in deeper layers of cartilage, fully in line with our finding of characteristic localization of lesions in the tibial plateau after TGF-β overexposure. It is therefore tempting to speculate that TGF-β induces excessive protease activation at defined, vulnerable sites in the cartilage, and we are in the process of identifying local enzyme levels with in-situ hybridization and cartilage neoepitope immunohistochemistry.

Cracks at the level of the tide-mark were found in several TGF-β-injected joints. Interestingly, spontaneous development of clefts at the level of the lateral tibial tide-mark in C57 black mice, genetically predisposed to develop OA, has been reported.[24] In late stages of both natural and experimental murine OA, we noticed that tibial noncalcified cartilage was either still present or had disappeared completely, suggesting that tearing off at the level of the tidemark is a characteristic feature of murine OA. Probably focal cartilage degeneration plays a role also in the mouse OA process. In the literature, areas of PG loss in early stages of experimental OA models in mice, leading to fissuring at later time points have been described.[11,25,26]

TGF-β injections into murine knee joints not only affected articular cartilage, but also stimulated periosteum to form new cartilaginous structures, socalled chondrophytes. Previous studies have demonstrated that TGF-β can induce cartilage and bone formation when it is applied to periosteal cells in vitro and *in vivo*.[5,27,28] In the underlying study we showed that these early cartilage-like structures develop into osteophytes and fuse with the original bone later on. Like in experimental arthritis and spontaneous and experimental osteoarthritis,[5,11] these osteophytes were found mainly along the margins of articular cartilage and at insertion sites of ligaments.

BMP-2 is also a major inducer of cartilaginous outgrowth after injection into the murine knee joint.[29,30] However, the characteristics of these outgrowths are quite different from the ones induced with TGF-β, and the latter ones are much more close to events found in spontaneous or experimentally induced murine OA. It is clear from our recent studies that another anabolic factor, IGF-1, is not capable of induction of osteophytes after repeated intraarticular injections (unpublished observations).

Furthermore, we found effects of TGF-β on soft connective tissues. Besides the thickening of synovium and ligaments that was found after triple injections, development of chondroid tissue in ligaments was seen after six or eight injections of TGF-β. This is in line with studies describing TGF-β-induced proliferation and matrix synthesis by ligament fibroblasts.[31,32] Also, TGF-β involvement in ossification of ligaments in humans has been suggested.[33]

There is ample evidence from experimental OA that the early stage is characterized by enhanced chondrocyte synthetic activity and hypertrophy. The latter would fit with excessive growth factor stimulation and it is apparent from our studies that TGF-β may be a likely candidate. OA can develop after cartilage injury or ligament disruption and both of these conditions give rise to TGF-β activation, probably in an attempt to reduce further destruction and to promote repair. However, when conditions develop where there is failure in attempted repair, which is the case with ruptured ligaments or unrepairable cartilage lesions, prolonged TGF-β activation is a likely event. As illustrated by our study, prolonged exposure of the joint to TGF-β eventually leads to OA-like events. Apart from excessive local growth factor activation, it is recently demonstrated that a failure in TGF-β receptor activation may also give rise to OA-like lesions.[34] Although this may seem contradictory to our findings, it is reasonable to suggest that both a lack of an essential repair factor as well as its prolonged overexpression may cause joint pathology.

We conclude that multiple injections of TGF-β into the murine knee joint induce OA-like changes in articular cartilage and surrounding tissues, suggesting a pathogenic role for excessive local TGF-β production in osteoarthritis.

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