EXTENDED REPORT

Expression of transforming growth factor-β (TGFβ) and the TGFβ signalling molecule SMAD-2P in spontaneous and instability-induced osteoarthritis: role in cartilage degradation, chondrogenesis and osteophyte formation

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Background: The primary feature of osteoarthritis is cartilage loss. In addition, osteophytes can frequently be observed. Transforming growth factor-β (TGFβ) has been suggested to be associated with protection against cartilage damage and new cartilage formation as seen in osteophytes.
Objective: To study TGFβ and TGFβ signalling in experimental osteoarthritis to gain insight into the role of TGFβ in cartilage degradation and osteophyte formation during osteoarthritis progression.
Methods: Histological sections of murine knee joints were stained immunohistochemically for TGFβ3 and phosphorylated SMAD-2 (SMAD-2P). Expression patterns were studied in two murine osteoarthritis models, representing spontaneous (STR/ort model) and instability-associated osteoarthritis (collagenase-induced instability model).
Results: TGFβ3 and SMAD-2P staining was increasingly reduced in cartilage during osteoarthritis progression in both models. Severely damaged cartilage was negative for TGFβ3. In contrast, bone morphogenetic protein-2 (BMP-2) expression was increased. In chondrocyte clusters, preceding osteophyte formation, TGFβ3 and SMAD-2P were strongly expressed. In early osteophytes, TGFβ3 was found in the outer fibrous layer, in the peripheral chondroblasts and in the core. Late osteophytes expressed TGFβ3 only in the fibrous layer. SMAD-2P was found throughout the osteophyte at all stages. In the late-stage osteophytes, BMP-2 was strongly expressed.
Conclusion: Data show that lack of TGFβ3 is associated with cartilage damage, suggesting loss of the protective effect of TGFβ3 during osteoarthritis progression. Additionally, our results indicate that TGFβ3 is involved in early osteophyte development, whereas BMP might be involved in late osteophyte development.

Osteoarthritis is a degenerative joint disease with a high prevalence in the elderly population. Although osteoarthritis is a complex disease, the primary feature in all osteoarthritis-affected joints is focal cartilage destruction. This can vary from cartilage fibrillation to fissures, or total loss of cartilage, exposing the bone underneath. Within certain limits, chondrocytes are capable of repairing cartilage.[1] In contrast with degradation, excess tissue production is often observed elsewhere in the joint. This might be a remodelling process initiated as a response to injury, resulting in osteophytes at the joint margins. Transforming growth factor-β (TGFβ) is involved in protection against cartilage destruction and in formation of new cartilage, as can be found during osteophyte formation.[2–6] TGFβ stimulates production of extracellular matrix (ECM) components by chondrocytes and has the ability to counteract catabolic cytokines like interleukin 1 (IL-1).[5 7–10] Blocking TGFβ during papain-induced experimental osteoarthritis increases articular cartilage proteoglycan loss.[4] TGFβ injection or adenoviral overexpression induces osteophyte formation in naive knee joints,[3 5 11] whereas inhibition of endogenous TGFβ prevents osteophyte formation during experimental osteoarthritis.[4] In addition, blocking osteophyte formation with alendronate in the rat anterior cruciate ligament transection model is associated with a reduced release of active TGFβ.[12] TGFβ expression has also been found in human femoral head osteophytes, but in variable amounts.[13] These studies indicate a role for TGFβ in protection against cartilage destruction and in osteophyte formation.

In osteoarthritis-affected joints, both cartilage degradation and cartilage formation occur simultaneously in the same joint. To elucidate the role of TGFβ in these processes during osteoarthritis, we investigated TGFβ expression and signalling during cartilage degradation and osteophyte development in two murine models of osteoarthritis with different aetiologies: a spontaneous osteoarthritis model and an instability-induced osteoarthritis model. We examined the expression of the most abundant TGFβ isoform, TGFβ3,[4] and the expression of its downstream signalling molecule SMAD-2 in its phosphorylated state, indicating active TGFβ signalling.

MATERIALS AND METHODS

Animals

Male BALB/c mice aged 10 weeks (n = 12) and male STR/ort mice aged 8 weeks (n = 6), 6 months (n = 5) and 1 year (n = 6) were used. Mice were kept in filter-top cages with woodchip bedding under standard pathogen-free conditions. They were fed standard diet and tap water ad libitum. This

Abbreviations: BMP-2, bone morphogenetic protein-2; ECM, extracellular matrix; TGF-β, transforming growth factor-β

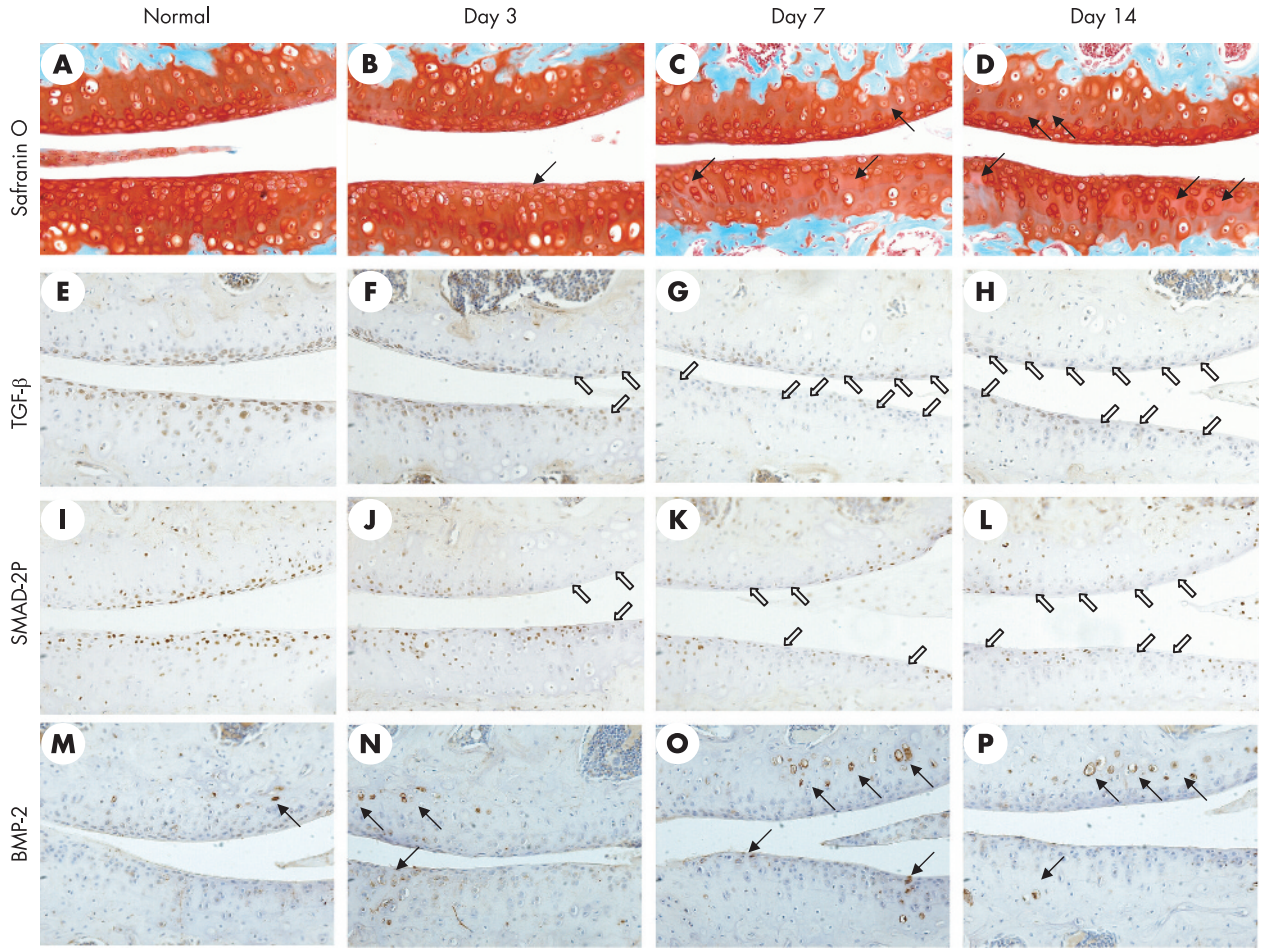

Figure 1 (A) Cartilage: instability model. Original magnification: ×200. Sections were stained with Safranin O and Fast Green (A–D), and were stained immunohistochemically for the transforming growth factor-β3 (TGFβ3) (E–H), SMAD-2P (I–L) and bone morphogenetic protein-2 (BMP-2) (M–P). In Safranin O-stained sections, arrows indicate lighter stained cartilage representing proteoglycan depletion. TGFβ3 and SMAD-2P-stained sections show a clear reduction in immunopositive cells over time. BMP-2 staining increases over time. For Safranin O and Fast Green-stained sections, black arrows indicate loss of red staining in (B–D). In immunohistochemically stained sections, the black arrows indicate positive staining and the open arrows indicate loss of staining.

study was approved by the local animal experimentation committee, Nijmegen, The Netherlands.

Experimental induction of osteoarthritis in BALB/c mice

Six µl solution of bacterial collagenase (5 µ/mg, 5 U/knee) was injected intra-articularly into the right knee joint as described previously.[14] Mice were killed 1, 3, 7 and 14 days after collagenase injection. Knee joints were dissected for histological examination. Non-injected mice served as controls.

The injection of bacterial collagenase leads to joint laxity and osteoarthritic lesions resembling those occurring naturally in old mice: cartilage destruction, synovial fibrosis and osteophytes.[14] This model might be seen as an equivalent to human secondary osteoarthritis resulting from joint instability.

STR/ort mice

STR/ort mice of ages 8 weeks, 6 months and 1 year were killed to compare their pathology. STR/ort mice are genetically predisposed to spontaneously develop osteoarthritis-like lesions. In all, 80% of male STR/ort mice show degenerative cartilage lesions by 6 months of age, starting with lesions at the interface of the cruciate ligament and the medial tibial plateau. The lesions range from mild erosion of the cartilage surface to loss of cartilage exposing the subchondral bone. Additionally, the mice develop osteophytes at the joint margins. The histological lesions seen in this model resemble those seen in humans.[15–17] Therefore, STR/ort mice might be seen as a model for human primary osteoarthritis.

Histological examination

Knee joints of mice were dissected and fixed in phosphate-buffered formalin for 7 days. Thereafter, they were decalcified in 10% formic acid for 1 week. Knee joints were dehydrated with an automated tissue-processing apparatus (VIP) and embedded in paraffin wax. Frontal whole sections of 7 µm were made. Sections were stained with Safranin O and Fast Green (BDH Laboratory Supplies, Poole, UK).

Immunohistochemical analysis

Sections were deparaffinised and washed with phosphate-buffered saline. They were incubated in citrate buffer (0.1 M sodium citrate and 0.1 M citric acid) for 2 h for antigen unmasking. Endogenous peroxidase was blocked with 1% hydrogen peroxidase in methanol for 30 min. Sections were blocked with 5% normal serum of the species in which the secondary antibody was produced. Specific primary antibodies against TGFβ3 (2 µg/ml; Santa Cruz Biotechnology, Santa Cruz, California, USA), SMAD-2P (1:100) (Cell Signalling Technology, Danvers, Massachusetts, USA) or BMP-2 (2 µg/ml) (Santa Cruz Biotechnology) were incubated

overnight at 4°C. After extensive washing with phosphate-buffered saline, the appropriate biotin-labelled secondary antibody was used (Dako, Glostrup, Denmark) for 30 min at room temperature followed by a biotin–streptavidin detection system used according to the manufacturers' protocol (Vector Laboratories, Baiklin Game, California, USA). Bound complexes were visualised using dimethylaminoazobenzene reagent, counterstained with haematoxylin, dehydrated and mounted with Permount (Fisher Chemicals, New Jersey, USA).

Scores

A blinded observer scored sections stained for either TGFβ3 or SMAD-2P. The number of TGFβ3 and SMAD-2P immunopositive cells and the staining intensity in immunohistochemically stained sections were examined in tibial cartilage, femoral cartilage, and osteophytes developing on the femur above the collateral ligament. For the spontaneous model, both left and right knee joints were included in the study. The number of cells staining positive for either TGFβ3 or SMAD-2P was determined visually and stratified into five catagories: negative, no cells staining positive (−); weakly positive, a low number of cells staining positive (+/−); positive, <50% of the cells staining positive (+); strongly positive, between >50% and close to all cells staining positive (++), or all cells stained positive with high staining intensity (+++). We scored four different cartilage surfaces: medial femoral cartilage, lateral femoral cartilage, medial tibial cartilage and the lateral tibial cartilage. At least four sections were examined and averaged per joint. The mean of all the averages determined the outcome of the score. Osteophytes were scored in a similar manner, estimating the number of cells expressing TGFβ3 or SMAD-2P on the femur just below the collateral ligament on the medial side of the joint.

To validate our visual scores, we also determined the number of cells staining positive for SMAD-2P on the medial tibial cartilage with a computerised imaging system (Qwin, Leica Imaging Systems, Wetzcar, Germany). A blinded observer selected the cartilage surface in at least three sections per knee joint. The computerised imaging system subsequently determined the amount of positive cells in the selected area. The obtained values were averaged per knee joint.

Statistical analysis

Statistical significance was calculated using a Mann–Whitney U test. Values of $p<0.05$ were considered to be significant.

RESULTS

Cartilage: instability model

Healthy cartilage was smooth, and stained evenly red with Safranin O. Almost every chondrocyte appeared to be TGFβ3 positive. When osteoarthritis is induced by injecting collagenase, the joint becomes unstable, eventually resulting in cartilage damage. The cartilage of the osteoarthritis joint showed focal depletion of proteoglycans, indicated by reduced red staining in the non-calcified cartilage. This was first observed on day 3 on the medial side of the joint, accompanied by an overall decrease in TGFβ3 expression. The amount of proteoglycans in cartilage diminished over time, with pronounced reduction on day 14, mainly on the medial side of the joint (fig 1). Only 10% of the cells in the medial cartilage expressed TGFβ3, located near the surface of the cartilage. Proteoglycan loss and reduction in TGFβ3 expression on the lateral side of the joint were observed only on day 14 and were less pronounced (table 1).

In naive joints, 80% of cartilage cells were immunopositive for SMAD-2P on the lateral side of the joint. On the medial side this was only 50%. The slightly proteoglycan-depleted cartilage of day 3 already showed a reduced number of SMAD-2P-expressing cells. On day 7, changes in SMAD-2P were minimal. By day 14, the depleted cartilage was almost completely negative for SMAD-2P on the medial side. The few cells that remained positive were predominantly located in the lateral cartilage and stained intensely (table 1, fig 1).

Cartilage: spontaneous model

The cartilage of 8-week-old STR/ort mice displayed proteoglycan loss, which was more pronounced on the medial side of the joint. Both TGFβ3 and SMAD-2P expression varied at this age. The cartilage of the lateral side contained almost 90% TGFβ3 immunopositive cells, whereas the cartilage of the medial side displayed only 10–20% cells immunopositive for TGFβ3. SMAD-2P expression varied from 50% to 75% of the cells in the lateral cartilage, and from non-existent to 20% positive cells on the medial side.

After 6 months the Safranin O staining had diminished further, and cartilage contained clefts or was eroded. Occasionally, the calcified cartilage layer was devoid of proteoglycans. The cartilage was negative for both TGFβ3 and SMAD-2P, except for some positive cells remaining in the lateral tibial cartilage (<5% positive cells for both TGFβ3 and SMAD-2P). After 1 year large portions of the cartilage were gone. The remaining cartilage was completely negative for both TGFβ3 and SMAD-2P (table 1, fig 2).

To validate our arbitrary scoring system, we additionally scored SMAD-2P-positive cells with a computerised imaging system. This showed that after induction of instability there were considerably fewer cells staining positive for SMAD-2P. Additionally, it showed that the number of positive cells in the STR/ort mice was considerably less than in normal mice with almost no expression at ages 6 months and 1 year (fig 3).

BMP-2 in osteoarthritis cartilage

TGFβ3 and SMAD-2P were found to be decreased in early osteoarthritis-affected cartilage and even absent in severe osteoarthritis-affected cartilage. To be sure that this was not caused by overall reduced cell viability resulting in reduced protein synthesis, we examined the expression of another growth factor of the TGFβ family: BMP-2. In contrast with TGFβ3, BMP-2 expression was increased with progression of osteoarthritis pathology. BMP-2 was found in only a small number of chondrocytes in the deeper layer of normal cartilage. In instability-induced osteoarthritis, we observed a small increase in the number of positive cells on day 3. BMP-2 was also found in more superficial, non-calcified layers of cartilage. On days 7 and 14 the number of positive cells had increased further, mainly in the deeper layers. The number of positive cells in the non-calcified layers had increased as well, but still remained <20% of the total number of cells (fig 1).

In the spontaneous osteoarthritis model, we observed intense staining for BMP-2 in degenerating cartilage. At 8 weeks of age, staining was mainly seen in cells of the deeper cartilage layers. In 6-month-old animals cells in the superficial, non-calcified layers also stained strongly. In 1-year-old mice, almost every cell neighbouring severely damaged cartilage stained intensely for BMP-2 (fig 2).

Osteophytes: instability model

Osteophytes are newly formed bone outgrowths that develop at the edges of the joint. The first observations in osteophyte formation are cell clusters in the periosteum resembling chondrocytes. These cells are surrounded by ECM that stains red with Safranin O, similar to cartilage, and was observed as early as 3 days after induction of osteoarthritis. The chondrogenic structure had enlarged by day 7. Some of the cells disappeared, and round, empty lacunae remained. On

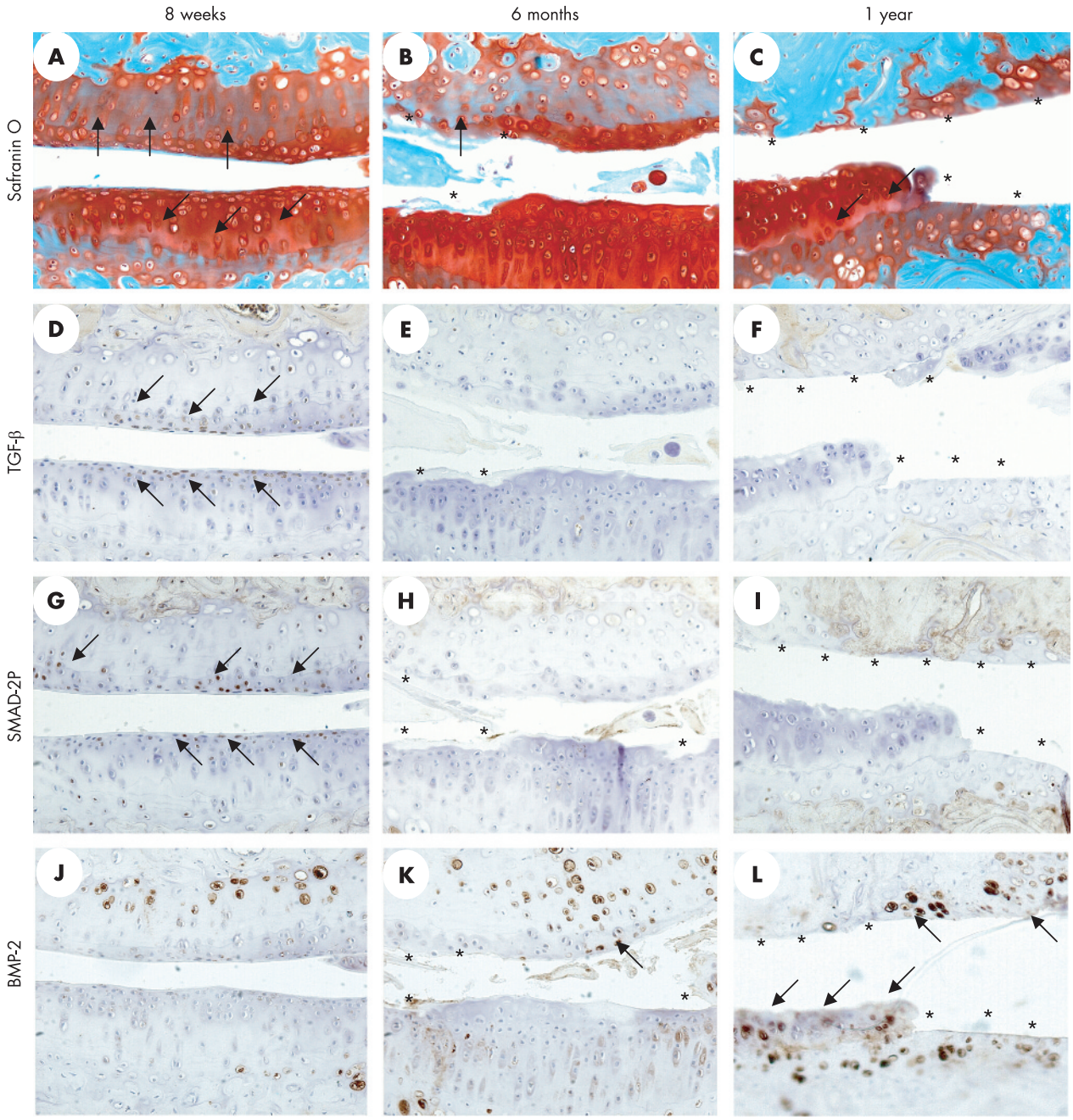

Figure 2 Cartilage: spontaneous model. Original magnification: ×200. Sections were stained with Safranin O and Fast Green (A–C), and were stained immunohistochemically for transforming growth factor-β3 (TGFβ3) (D–F), SMAD-2P (G–I) and bone morphogenetic protein-2 (BMP-2) (J–L). In these sections the cartilage is clearly degrading over time. After 1 year (C,F,I,L) there is hardly any cartilage left. At 8 weeks there is still TGFβ3 and SMAD-2P staining (D,G), but at 6 months and 1 year the remaining cartilage is negative for both TGFβ3 and SMAD-2P (E,F,H,I). BMP-2 expression is strongly expressed in severely damaged cartilage. (L) For Safranin O and Fast Green-stained sections, black arrows indicate loss of red staining (A,B). In immunohistochemically stained sections, the black arrows indicate positive staining. Asterisks indicate the location where cartilage is lost.

day 14, lacunae in the core of the osteophyte had joined together, forming new bonemarrow spaces, filled with new cells. Deposition of bone had begun in the core of the osteophyte around the larger rough-edged lacunae. The developing osteophyte was covered with a layer of fibroblast-like cells surrounded by a small amount of ECM with a different organisation from cartilage. This layer was thicker on day 14 than on day 7.

All the initial cell clusters on day 3 expressed TGFβ3. Even when the chondrogenic structure enlarged, TGFβ3 was expressed in every cell. The round lacunae on day 7 and 14 were negative for TGFβ3. The new bone-marrow spaces contained small cells that expressed TGFβ3. The outer layer of the osteophyte was covered with TGFβ3 immunopositive, fibroblast-like cells. When the core of the osteophyte started to turn into bone (day 14), TGFβ3 resided at the margins (fig 4).

The cell clusters of day 3 and 7 were all positive for SMAD-2P. On day 14, when there was ossification of the core of the osteophyte, the SMAD-2P staining was intense and, in contrast with TGFβ3, observed throughout the entire structure (fig 4). Not all cells had similar staining intensity. In general, cells near the edge usually stained more intensely than those in the core (fig 4).

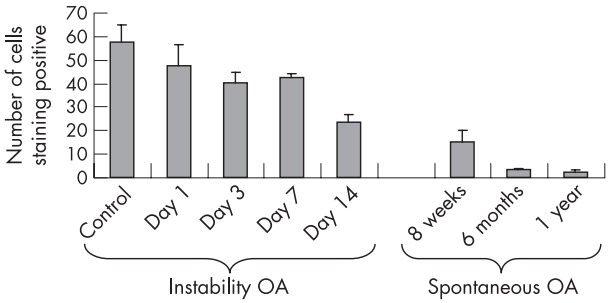

Figure 3 Score of SMAD-2P in medial tibial cartilage. Sections were stained immunohistochemically for SMAD-2P. The number of cells staining positive for SMAD-2P was measured with a computerised imaging system as a control for the visual score. The number of cells after induction of instability was considerably lower than controls. STR/ort mice showed a decreased expression with age. OA, osteoarthritis.

Osteophytes: spontaneous model

One of the most apparent locations of osteophyte formation in STR/ort mice is above the root of the collateral ligament at the medial side of the joint, but osteophytes also develop at other locations. At 8 weeks of age, STR/ort mice displayed primary stages of osteophyte development. The chondrogenic core of the osteophytes was located along the original bone margin. In contrast with osteophytes in the instability model, no red staining was observed in the outer layer, indicating a lack of proteoglycans. The chondrogenic core stained far less red compared with osteophytes in the instability-induced osteoarthritis model. The direct surrounding of the chondrocytes stained red, but the spaces in between were pink or even green. After 6 months the osteophytes had turned into bone. After 1 year the osteophytes that started development around 8 weeks of age were hard to distinguish from the original bone, as margins had become unclear. In some of the 6-month-old mice but most of the 1-year-old STR/ort mice, new osteophytes were developing in ligaments, extending from the menisci or growing on top of old osteophytes. Some of these new osteophytes were already ossified, whereas others were still in developmental stages, displaying chondrogenesis.

The chondrogenic part of the osteophytes in 8-week-old mice was, in contrast with the osteophytes in the instability model, negative for TGFβ3. Only the fibroblasts in the outer layer expressed TGFβ3. Half of the cells of the chondrogenic part were immunopositive for SMAD-2P, and all of the cells in the outer layer stained positive for SMAD-2P. The ossified osteophytes of 6-month-old mice displayed approximately 10% cells that were immunopositive for TGFβ3, and approximately 50% of the cells were positive for SMAD-2P. This remained the same after 1 year (table 1; fig 5).

Early osteophytes expressed only low levels of BMP-2, whereas the bone-like structures that have developed after 1 year displayed intense BMP-2 staining, indicating a role for BMP in late osteophyte development (fig 5).

DISCUSSION

We investigated the role of TGFβ in two osteoarthritis models reflecting aspects of human osteoarthritis. TGFβ3 and TGFβ signalling, assessed by SMAD-2P staining, were studied. TGFβ induces SMAD-2 phosphorylation in a time-dependant and dose-dependent manner, giving an insight into active TGFβ signalling.[4 18 19]

One of the main features of osteoarthritis is destruction of cartilage, which is preceded by loss of proteoglycans. In both osteoarthritis models proteoglycan loss is accompanied by reduced TGFβ3 and SMAD-2P staining. Osteoarthritis lesions in the spontaneous model were predominantly observed on the medial side of the joint corresponding with previous findings of Dunham *et al*,[20 21] describing progressive disorganisation of proteoglycans in the medial cartilage plateau of STR/ort mice younger than 30 weeks of age.[3] We observed that TGFβ3 and SMAD-2P staining also diminished fastest on the medial side in both osteoarthritis models. The STR/ort mice were studied over a longer time period, enabling us to study more progressive cartilage damage. The severely damaged articular cartilage was totally negative for TGFβ3 and SMAD-2P, whereas intact cartilage, even in older STR/ort mice, contained TGFβ3 and SMAD-2P immunopositive cells. Supportive of our findings, Verdier *et al*[22] showed decreased TGFβ expression in degraded human osteoarthritis cartilage and diminished TGFβ II receptor expression in fibrillated cartilage. Furthermore, Wang *et al*[23] found a correlation between expression of TGF-β II receptor/TGFβ1 and intracellular levels of tissue inhibitor of metalloproteinase in human cartilage chondrocytes. Tissue inhibitors of metalloproteinase inhibit matrix metalloproteinases, thereby facilitating accumulation of ECM products, indicating a role for the TGFβ pathway in ECM homeostasis of the cartilage. Kizawa *et al*[24] show an asporin polymorphism in which asporin D14 has a greater inhibitory effect on TGFβ-mediated expression of cartilage matrix gene than does the common form D13. The D14 variant was over-represented in people with arthritis, indicating the lack of TGFβ responsiveness and its association with osteoarthritis progression.[24]

Taken together, these data strongly suggest that loss of TGFβ signalling is associated with cartilage damage.

In the papain model, TGFβ was found to be increased in damaged cartilage.[25] However, papain breaks down the ECM

Table 1 Score of transforming growth factor-β3 and SMAD-2P expression

	Medial femur		Lateral femur		Medial tibia		Lateral tibia		Osteophyte	
	T-β3	S-2P	T-β3	S-2P	T-β3	S-2P	T-β3	S-2P	T-β3	S-2P
Instability OA										
Naive	−									
Day 1	++	+	++	++	++	+	++	++		
Day 3	+	+	++	++	++	+	++	++		
Day 7	+	+/−	+	+	+	+/−	+	++	++	+
Day 14	+/−	+/−	+/−	+/−	+/−	+/−	+/−	+	+++	++
Spontaneous OA	−	−	+/−	+/−	−	−	+/−	+/−	+++	+++
8 weeks										
6 months	+/−	−	+	−	+	−	++	++	+++	+++
1 year	−	−	−	−	−	−	+	−	+/−	+
	−	−	−	−	−	−	+/−	+	+/−	+

OA, osteoarthritis; TGF, transforming growth factor.
Score of TGFβ3 (T-β3) and SMAD-2P (S-2P) expression in several cartilage surfaces, cruciate ligaments and osteophytes in both a spontaneous and an instability OA model: −, negative; +/−, weakly positive; +, positive; ++, >50% of the cells immunopositive; +++, almost every cell immunopositive and high staining intensity.

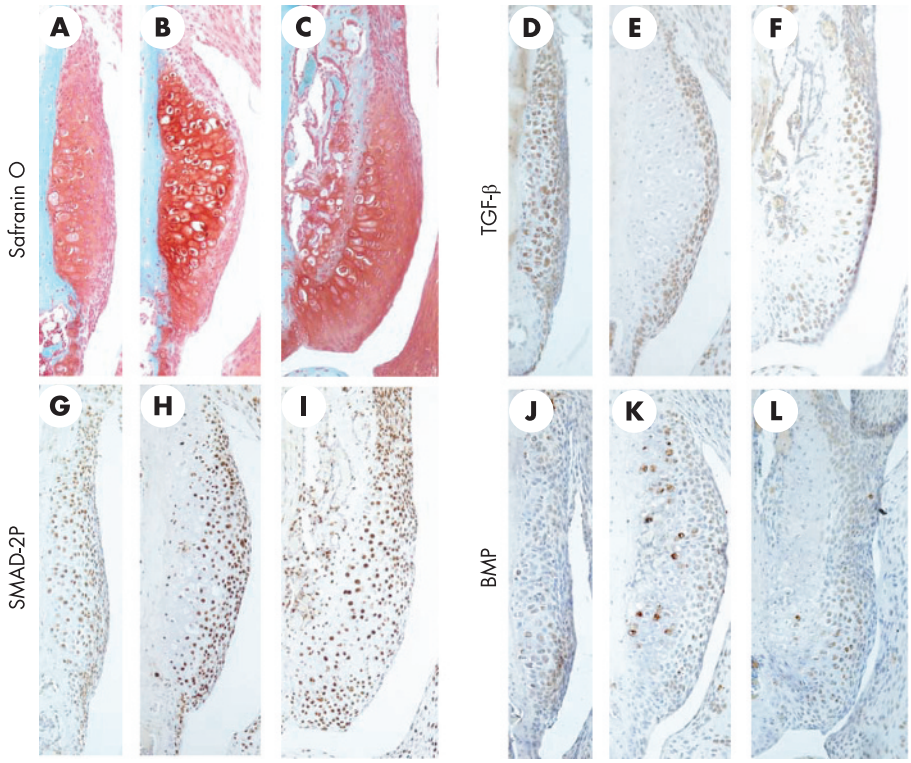

Figure 4 (A) Osteophyte development: instability model. Osteophyte is located on the femur below the attachment of the collateral ligament. Original magnification: ×200. Sections were stained immunohistochemically for Safranin O (A–C), transforming growth factor-β3 (TGFβ3) (D–F), SMAD-2P (G–I) and bone morphogenetic protein-2 (BMP-2) (J–L). Osteophytes had developed 7 days (A,D,G,J) and 14 days (B,C,E,F,H,I,K,L) after collagenase injection. In early development, all of the cells of the newly formed tissue stain positive for both TGFβ3 and SMAD-2P (D,G). When osteophyte formation progresses TGFβ3 resides in the fibrous layer overlaying the cartilage, whereas SMAD-2P is seen in the entire structure (E,H). During ossification of the osteophyte TGFβ3 is observed again in the fibrous layer, but also in the core of the osteophyte. The cartilage-like layer is still negative for TGFβ3 (F). SMAD-2P staining can be observed in all layers of the osteophyte (I). BMP expression is present in all layers, but less prominent than TGFβ (J–L).

of the cartilage, thereby directly affecting cartilage integrity. It can be anticipated that chondrocytes respond differently to this insult when compared with instability-induced cartilage damage, probably involving different roles for TGFβ.[26–29]

To confirm that our findings were not the effect of an overall drop in cell viability in osteoarthritis-affected cartilage, but were TGFβ specific, we studied the expression of another TGFβ family member, BMP-2. In contrast with TGFβ3, BMP-2 was increased with osteoarthritis progression, indicating that the reduced TGF-β3 expression is not simply the result of decreased cell viability.

Another prominent feature of osteoarthritis is osteophyte formation. The chondrocyte-like cell clusters that are observed in early osteophyte development are all positive for TGFβ3 and SMAD-2P. More developed, but not fully ossified, osteophytes have a core of bone-like tissue covered with a layer of cartilage-like tissue and an outer layer with fibroblast-like cells. In this stage, TGFβ3 is observed in the bone marrow of the osteophyte. The cartilage-like tissue is negative, whereas the fibroblast-like cells are all positive for TGFβ3. In contrast, SMAD-2P is found in every layer of the osteophyte. TGFβ3 expression is low to absent in this phase of development or obscured by matrix development. The first seems more plausible, in which case the TGFβ might diffuse from the fibrous layer to the cartilage, inducing TGFβ signalling. Either way, there is SMAD-2P staining indicating active TGFβ signalling.

Overexpression of TGFβ in murine knee joints has been shown to induce osteophytes, whereas blocking endogenous TGFβ in an osteoarthritis model reduces osteophyte formation.[3 5 6 11 25] These findings suggest an important role for TGFβ in osteophyte formation. However, in older STR/ort mice the osteophytes no longer express TGF-β3 or SMAD-2P. The osteophytes in the instability model have been monitored over a shorter time period, showing only the early phases of development. TGFβ seems to be particularly important in these phases. In later phases of osteophyte development, as seen in older STR/ort mice, TGFβ function is supposedly substituted by factors such as BMP. BMP is associated with osteophyte maturation[30 31] and was found to be strongly expressed in late osteophytes in our experiment. Uchino *et al*[13] described TGFβ expression in human osteophytes. They were not able to discriminate between different stages of osteophyte formation, which might explain why they found various amounts of TGFβ in osteophytes. However, the location of TGFβ expression mainly in the fibrous, superficial layer of the human osteophytes corresponds to our findings in mice. This confirms that the process of osteophyte formation observed in our murine osteoarthritis models closely resembles that in human osteoarthritis.

In the spontaneous osteoarthritis model, we observed ectopic bone formation in the collateral ligament. Collins *et al*[32] and Walton[33] also found these chondro-osseous structures in synovia and ligaments in STR/ort mice. This ectopic bone formation is observed initially by red staining in the ligament in Safranin O-stained sections, indicating proteoglycan deposition. This particular area contains cells positive for TGFβ3 and SMAD-2P, whereas the rest of the ligament, which looks normal, is negative for both. Further changes in ligaments resemble those observed in developing osteophytes with respect to TGFβ3 and SMAD-2P expression. In the early process of chondrogenesis TGFβ3 and SMAD-2P are

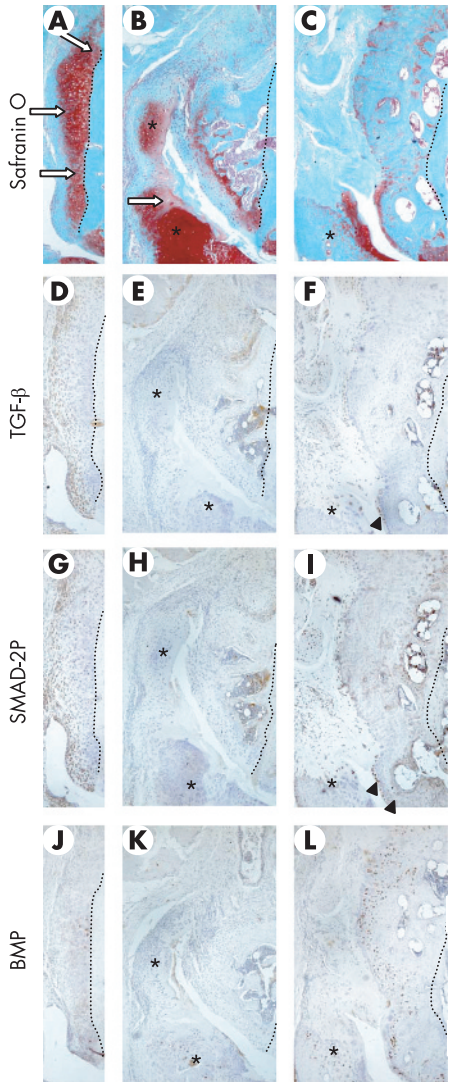

Figure 5 Osteophyte development: spontaneous model. Osteophyte is located on the femur below the attachment of the collateral ligament. Original magnification: ×200. Sections were stained immunohistochemically for Safranin O (A–C), transforming growth factor-β3 (TGFβ3) (D–F), SMAD-2P (G–I) and bone morphogenetic protein-2 (BMP-2) (J–L). Osteophytes at the age of 8 weeks (A,D,G,J), 6 months (B,E,H,K) and 1 year (C,F,I,L). In early development all of the cells of the newly formed tissue stain positive for both TGFβ3 and SMAD-2P (D,G) and there is weak expression of BMP-2 (J). When osteophyte formation progresses into more ossified structures, TGFβ3 and SMAD-2P expression diminishes (E,H). When the osteophyte has turned into a bone structure some TGFβ3 and SMAD-2P expression is found, but it is unclear whether this is from the original osteophyte or newly synthesised tissue (arrowheads in (F,I)). BMP-2 expression is weak at first, but in mice aged 1 year the expression of BMP-2 is clearly elevated (L). Dotted lines approximate the original bone margin. Asterisks indicate ectopic bone formation.

abundantly expressed, whereas fully developed pseudo joints are negative for TGFβ3 and SMAD-2P, again suggesting a role for TGFβ in the early developmental stages of ectopic bone.

Although TGFβ expression is reduced in damaged cartilage, its expression is strongly increased in other compartments of the joint (osteophytes, but also synovial tissue). Therefore, it can be expected that TGFβ will still be released into the joint cavity and could ultimately reach other tissues. We show that TGFβ signalling in cartilage is down regulated or even absent in osteoarthritis-affected cartilage. TGFβ that is produced outside the cartilage does not reach the chondrocytes, either because of scavenging by the extracellular matrix or because the cells have lost the ability to respond. The loss of ability to respond is in concordance with the loss in TGFβ receptor II expression, as has been shown in rabbits with osteoarthritis.[34] Loss of the ability to respond to TGFβ might be related to high levels of proinflammatory cytokines such as IL1 and tumour necrosis factor present in osteoarthritis-affected cartilage. The absence of TGFβ3 expression could also be attributed to the effects of proinflammatory cytokines. Struder *et al*[35] have shown that proinflammatory cytokines can indirectly, via nitric oxide production, modulate TGFβ production. In contrast, IL1 and tumour necrosis factor have been shown to stimulate BMP-2 expression, which could explain our findings of BMP-2 up-regulation during osteoarthritis progression.[36]

From our observations, we can conclude that TGFβ and SMAD-2P expression is reduced in damaged cartilage and is completely absent in cartilage that has started eroding, suggesting a protective role for TGFβ in cartilage. TGF-β and SMAD-2P are up regulated during chondrogenesis, osteophyte and ectopic bone formation, but mainly in the early stages of osteophyte development. In progressive osteoarthritis, TGFβ expression in newly formed osteophytes is, although still present, reduced compared with that in the newly formed osteophytes in early osteoarthritis. At later stages of osteophyte development TGFβ and SMAD-2P are no longer expressed, and other factors are probably involved in further progression of osteophyte formation.

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