TGF-β in osteoarthritis
Age-related loss of protective function in cartilage and player in osteophyte formation and synovial fibrosis

Esmeralda Blaney Davidson
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Age-related loss of protective function in cartilage
and player in osteophyte formation and synovial fibrosis

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<td>BMD</td>
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<td>BMP Responsive Element</td>
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<td>Ct</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>Proliferating Cell Nuclear Antigen</td>
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Chapter 1

General introduction: TGF-β and osteoarthritis

& Outline of the thesis


E.N. Blaney Davidson
P.M. van der Kraan
W.B. van den Berg
Abstract

Osteoarthritis (OA) is a multifactorial disease involving different tissues that form an articular joint. As cartilage destruction is one of the major problems in OA, cartilage is the main target for therapeutic approach. Growth factors have been proposed as a tool to stimulate chondrocytes to repair the damaged cartilage matrix. One growth factor that has great potential in cartilage repair is Transforming Growth Factor-β (TGF-β). In this review, we will focus on the role of TGF-β in OA, potential therapeutic intervention in OA with TGF-β, application of the growth factor TGF-β in cartilage repair and on the side-effects of TGF-β treatment that can occur.

Osteoarthritis

OA is the most common form of arthritis, involving cartilage, synovium and bone. (Figure 1) The main characteristics are cartilage damage, synovial fibrosis, sclerosis of the subchondral bone and osteophyte formation at the joint margins (1). Clinically OA is characterized by joint pain, tenderness, occasional effusions and eventually loss of joint function. The etiology of OA is still unknown although certain risk factors have been established. Systemic risk factors include genetic susceptibility, race, gender, age and nutrition. Besides systemic factors, local joint environment is important. Previous damage to the joint as well as muscle weakness and joint deformation that can cause alteration in joint loading heighten the risk of OA development. In addition, elevated loading caused by obesity or injurious physical activity increases the probability of OA development (2). Although several risk factors are known, in general the cause of OA remains to be discovered. Traditionally OA was assessed with articular cartilage damage as the main pathological event, but lately it has become clear that OA is a whole organ disease in which the role of bone and synovial tissue have come up in relation to OA onset and progression. It is highly feasible that there is not one main initiating event of OA, but that several different triggers can lead to a common disease pathway eventually leading to the same disease. Regardless of the initiating trigger cartilage damage is the main event in OA. Cartilage has a very limited intrinsic reparative capacity. As a consequence, cartilage damage results in progressive disease. This makes it crucial to target cartilage damage at an early stage to prevent further progression.

Cartilage

Cartilage is non-vascular and nutrients are provided by the synovial fluid. On a weight base it is mainly composed of collagens and proteoglycans. Collagens provide tensile strength and proteoglycans retain water molecules in the matrix. In humans, cartilage is composed of three zones: superficial, middle and deep zone, each with a distinct composition. (Figure 2) The superficial zone includes disc-shaped chondrocytes and the collagen fibers are aligned along the surface. The middle zone has a higher proteoglycan content than the superficial zone, cells are more spherical and the collagen fibers are orientated isotropically. The deep zone contains spherical cells and collagens have a perpendicular orientation (1).

Cartilage damage in OA has several hallmarks. Initially, in contrast to what is expected during damage, an increased synthesis of matrix molecules is observed. However, in time cartilage matrix degradation exceeds matrix deposition resulting in net matrix loss. In early OA the cartilage surface is still intact, but shows some focal oedema or even minor fibrillations. The chondrocytes then start to proliferate and form cell clusters. In addition, chondrocyte hypertrophy can be observed.

Figure 1

Schematic representation of osteoarthritis in a synovial joint. The left part of the figure displays a normal joint whereas in the right part of the figure, an osteoarthritic joint is depicted. The latter shows cartilage degradation and loss, synovial fibrosis, osteophyte formation and a change in angle of the opposing bones.
Subsequently, the superficial zone shows fibrillations and loss of chondrocytes. The fibrillations then progress into fissures that extend into the mid zone, followed by cartilage erosion, denudation of bone and finally deformation (3).

Chondrocytes can be stimulated by catabolic cytokines to release cartilage degradation products, ultimately leading to damage. In the 1980’s catabolin, now termed IL-1 (Interleukin-1), was suggested to play a role in OA. Several groups described its capacity to induce metalloproteinases in cartilage and its ability to stimulate chondrocytes to degrade both proteoglycan and collagen (4;5). The exact nature of the major mediator of chondrocyte activation in OA is not identified yet, but Interleukin-1β (IL-1β) is considered to be a principle mediator of joint damage in OA (6). It causes destruction of cartilage by increasing enzyme activity and inflammation while inhibiting synthesis of enzyme-inhibitors (7). IL-1 and TNFα can stimulate chondrocytes to produce nitric oxide (NO) (8), matrix metalloproteinases (MMPs) (9), aggrecanases (ADAMTS) (10) and suppress the synthesis of aggrecan and collagen type II (11-15).

In normal cartilage there is constant degradation as well as synthesis of cartilage matrix molecules, controlled by the chondrocytes. A high degradation rate does not necessarily implicate OA as long as there is enough compensation by synthesis. Besides catabolic factors OA chondrocytes also express anabolic factors, like insulin-like growth factor-1 (IGF-1) and TGF-β that stimulate extracellular matrix (ECM) production (16-19). Increased synthetic activity in early OA has been found accompanied with an up regulation of TGF-β expression (20;21). The initial increase in production of ECM that is found in OA, shows that OA is more than just accelerated cartilage degradation. Unfortunately the increased anabolic impulse is only a temporary state characteristic for early OA and therefore cannot compensate for the overall catabolic insult to cartilage.

Chondrocytes in OA cartilage are different from normal cartilage in their cytokine and growth factor expression patterns. For instance, chondrocytes from fibrillated OA cartilage display higher levels of intracellular IL-1α and β and up regulated plasma membrane-bound IL-1RI, whereas the decoy receptor IL-1RII is down regulated in OA chondrocytes (22;23). Thus, not only are there higher levels of IL-1 present in OA joints, but OA-chondrocytes are also more sensitive to IL-1, thereby increasing susceptibility to IL-1 induced cartilage damage. Not only fibrillated areas show different expression patterns, also cartilage proximal to macroscopic OA lesions show a higher binding of TNF-α and IL-1β compared to chondrocytes from morphologically normal cartilage from the same joint (24). In normal cartilage chondrocytes are able to counteract IL-1 with TGF-β, however, with age, this ability is lost (25).

Synovium

The role of the synovium in OA pathology is becoming more important than initially anticipated. Commonly, hyperplasia and extensive fibrosis are observed in OA joints (26). In addition, inflammation of the synovium, which was thought to be primarily a feature of rheumatoid arthritis, has been found in OA. Mainly in early OA mononuclear infiltration, proinflammatory cytokines and mediators of joint damage are found (27;28). There is a correlation between severity of inflammation and severity of chondropathy, indicating that inflammation may be a predictive factor in OA (29). In patients with early OA, the inflamed synovium releases catabolic cytokines like IL-1α, IL-1β and TNF-α (28;29). The correlation of synovitis with chondropathy as well as the release of catabolic cytokines suggests that synovitis contributes to the pathology seen in OA, including cartilage damage.

Osteophytes

Besides cartilage damage and synovial fibrosis, OA often displays osteophytes. They develop at the joint margins starting with chondrogenesis and eventually developing into bone. Osteophytes are thought to result from mesenchymal cells in the periosteum activated to undergo chondrogenesis by mechanical or biochemical stimuli. Gelse et al have divided osteophyte development into four stages. Stage I starts with condensation of mesenchymal cells, collagen type II and aggrecan expression. Stage II is characterized by addition of fibrous tissue and collagen type I.
expression. In stage III a zonal organization is observed and chondrocyte hypertrophy is seen, in which bone is formed leading to stage IV: the mature osteophyte with largely hyaline articular cartilage and mainly collagen type II, aggrecan and collagen type XI expression (30). Whether osteophyte formation is a good or a bad thing is still under discussion. Osteophytes are associated with malalignment of the joint, limit movement and increased risk of structural progression of OA (2). On the other hand, patients with varus limbs have larger medial osteophytes. Pottenger et al demonstrated that removal of osteophytes in patients with osteoarthritis increased varus-valgus motion, indicating that marginal osteophytes help to stabilize the joint (31). However, development of osteophytes result in severe joint-deformation, sometimes joint fixation. Overall it is unclear whether the osteophytes are formed as a compensatory mechanism for stabilization or a side-effect of joint pathology. Another possibility is a combination of both options: they start out as a means of stabilization but the process eventually runs amok.

TGF-β

The TGF-β family consists of over 35 members and includes, besides TGF-βs, activins and bone morphogenetic proteins (BMPs) (32). They play vital roles in development and homeostasis of various tissues. They regulate cell proliferation, differentiation, apoptosis and migration, as well as control ECM synthesis and degradation. Moreover, these factors mediate cell and tissue responses to injury and modulate immune functions (33). In mammals there are three isotypes of TGF-β, called β1, β2 and β3. All isoforms show a high degree of homology of 84-92%. The expression of the three isoforms is differently regulated at the transcriptional level due to different promoter sequences (34-36). TGF-β is secreted as an inactive complex comprised of a TGF-β dimer, its propeptide LAP (Latency Associated Peptide) and LTBP (Latent TGF-β Binding Proteins) (37;38). Therefore, the secreted TGF-β requires activation before it can bind to its receptor. Activated TGF-β binds to the TGF-β type II receptor to form a complex that recruits the TGF-β type I receptor, which is activated by phosphorylating the serine/threonine residues. There is an additional third TGF-β receptor, also known as betaglycan, which allows high-affinity binding of mainly TGF-β 2 to the TGF-β receptor type II (33).

Upon receptor phosphorylation, R-Smads (Smad2 or 3) are presented to the receptor by SARA (Smad-Anchored for Receptor Activation) and phosphorylated. Then the phosphorylated R-Smads form a complex with the Co-Smad (Smad4), and translocate to the nucleus where they can act either as, or in orchestrate with, transcription factors. (33;39;40) I-Smads (Smad6 and 7) can inhibit TGF-β signaling by interfering with R-Smad phosphorylation, thereby functioning as a negative feedback system. (Figure 3)
TGF-β can also activate Erk, JNK and p38 MAPK pathways (41). Cross talk between the Smad pathway and other TGF-β signaling pathways have also been reported (40,41). They can interact by Smad phosphorylation by ERK or JNK, by controlling Smad expression and by nuclear interaction between Smad complexes and MAPK-activated transcription factors. The latter depends on the structure of the target promoters (42). MAPK activation is not TGF-β signaling specific and can be triggered by various extracellular stimuli, such as IL-1 and TNF-α. Therefore, the Smad-MAPK interactions are not solely the result of multifaceted TGF-β signaling downstream of the receptors, but are a result of interacting cytokines that together modulate the Smad/MAPK signals (42). The mechanism of alternative TGF-β signaling pathways and their biological consequences are poorly understood since there are many factors that can activate the MAPK pathways at various levels, most of which are able to interact.

Genetic aspects of osteoarthritis and TGF-β

Family studies can indicate a relation between genetically determined factors and the development of osteoarthritis. In humans a relationship between TGF-β and osteoarthritis symptoms has been shown in Japanese women. A polymorphism of TGF-β1 on position 29 (T to C, amino acid 10) positioned in the signal sequence region of TGF-β1 is related to an elevated prevalence of spinal osteoporosis and ossification of the posterior longitudinal ligament (43,44). This TGF-β1 polymorphism was associated with a lower frequency of D-13 repeat, which results in a stronger inhibitory effect on TGF-β than the common D-14 repeat. This indicates that in OA there is a higher frequency D-14, resulting in strong TGF-β inhibition, which can result in reduction of ECM of cartilage. This suggests that reduced TGF-β action might be correlated with increased susceptibility to OA. However, the study performed by Kizawa et al. included only Japanese patients. When repeated in a Spanish Caucasian population, by Rodriguez-Lopez et al., the higher susceptibility to OA in patients with the D-14 polymorphism was no longer found (53). In UK Caucasians a trend was seen towards a higher degree of D-14 polymorphism in OA patients, but this was only significant in a specific subset of patients (54). However, in a different ethnic group, Han Chinese, the OA susceptibility was found again (55). The susceptibility was not limited to OA, Torres et al. also found that patients with rheumatoid arthritis (RA) that carried the D-14 polymorphism more frequently produced rheumatoid factor and had an earlier onset of the disease. Although the repeat might not be the major influence in RA, it was concluded to influence the outcome of the disease (56). The studies mentioned above show that TGF-β inhibition can aggravate OA and RA.

Mice deficient for TGF-β1 show 50% embryonic lethality and animals that are born alive develop severe inflammatory disorders and die within 1 month (57,58). Mice with a knockout gene for TGF-β2 and TGF-β3 show numerous developmental defects and perinatal death. Mice lacking TGF-β2 have numerous structural defects in the skeletal elements and show joint laxity (59). This indicates that TGF-β2 is involved in skeletal development. Animals with a non-functional gene for the type I receptors ALK1 or ALK5 or the Smad proteins 2 and 4 are embryonic lethal (60-63).

Null mice for Smad3 developed degenerative joint disease resembling human osteoarthritis, as characterized by progressive loss of articular cartilage, formation of osteophytes and increased expression of type X collagen. These data indicate that Smad3 signaling is essential for repressing chondrocyte terminal differentiation, a hallmark of human OA (64). This observation is supported by studies in mice that overexpress a dominant negative TGF-β type II receptor in skeletal tissues (65). These mice developed progressive skeletal degeneration that strongly resembles human osteoarthritis.

In addition, mice that lack the latent TGF-β binding protein (LTBP-3) also show altered chondrocyte differentiation and early osteoarthritis development (64,66,67). These observations show that interference with TGF-β signaling in chondrocytes results in abnormalities in chondrocyte differentiation and the development of osteoarthritis.

TGF-β and cartilage

Lack of TGF-β or an abnormality in TGF-β signaling apparently results in a cartilage phenotype that resembles cartilage pathology in OA. TGF-β has been shown to be very beneficial for cartilage as it stimulates chondrocytes in vitro to elevate proteoglycan and collagen type II production (16-18,64,68). Also in vivo TGF-β proved to have beneficial effects on cartilage such as stimulation of proteoglycan synthesis in cartilage (69).
Not only does TGF-β stimulate ECM production, it also counteracts the main catabolic players in OA. Our group has shown that TGF-β counteracts 38% of the genes that are regulated by IL-1. For example, TGF-β counteracts IL-1 up regulation of MMP-13 and −14, which have been found important mediators of cartilage damage. In addition, IL-1 down regulation of collagen and ECM-related genes are counteracted by TGF-β (10). Hui et al. show that TNF-α promotes MMP dependent collagen breakdown, which can be prevented by TGF-β1 in bovine cartilage explant cultures. In addition, TGF-β1 reduced expression and secretion of collagens and induced TIMP production (70). In addition, IL-1β has been shown to inhibit proteoglycan biosynthesis in a dose-dependent manner in porcine articular cartilage and increase the rate of degradation in proteoglycans. TGF-β was able to recover the IL-1 induced proteoglycan reduction (71;72). TGF-β is not only able to counteract the effects of IL-1, but can also reduce IL-1 signaling by down regulation of its receptors and increasing the expression of the decoy receptor IL-1Ra, thereby counteracting at several levels (73-77).

Overall, IL-1 and TNF-α produce matrix proteases and suppress the synthesis of collagen and proteoglycan. TGF-β is able to counteract the net effect of catabolic cytokines by stimulating the synthesis of matrix components, of protease inhibitors and down regulating the expression of cytokine receptors and cartilage degrading enzymes (78). These studies show that TGF-β can potently counteract catabolic effects in cartilage and stimulate ECM production and can be used as a potential treatment for cartilage destruction in OA.

TGF-β supplementation

Lack of TGF-β signaling results in susceptibility to cartilage damage, therefore TGF-β supplementation should aid in cartilage maintenance or repair. For years many researchers have focused on this TGF-β quality. We have shown that multiple injections of TGF-β induce strong and long-lasting stimulation of proteoglycan synthesis and increase the GAG content in patellar cartilage in mice under arthritic conditions (69;74). TGF-β stimulation of proteoglycans is a long-lasting effect. A single injection of 200 ng TGF-β into a murine knee joint stimulated proteoglycan synthesis for 3 weeks and elevated proteoglycan content for 2 weeks. Triple injections prolonged the increase in proteoglycan content for 3 weeks (79). In a survey, Grimaud et al. concluded that TGF-β expression and the use of gene transfer might provide an approach for treatment of OA lesions in cartilage (80).

Unfortunately, the effects of injecting TGF-β into a joint are not limited to cartilage. Chondrocytes are embedded in the cartilage without direct contact with other cells. The non-vascular properties make chondrocytes dependent on their direct environment for signals. This makes it very hard to target cartilage without involvement of other tissues. This implies that TGF-β supplementation in a joint also results in responses of other tissues that are in contact with the synovial fluid. TGF-β is implicated in fibrosis in many organs like eye, lung, heart, liver, kidney, pancreas and skin (81). The synovial tissue in articular joints is susceptible to TGF-β induced fibroplasias and fibrosis. As a consequence multiple injections of TGF-β induce synovial fibrosis in murine knee joints (69). In contrast to the lack of sufficient TGF-β expression in cartilage in progressive OA, it is abundantly present in synovial tissue. Therefore, TGF-β might also be involved in the synovial hyperplasia that is observed in OA. Besides being a potent inducer of synovial fibrosis, TGF-β is also able to induce osteophytes similar to those found in OA (69;82). TGF-β expression as well as active TGF-β signaling is found highly expressed in osteophytes in OA, suggesting a role for TGF-β in OA-induced osteophytes. Because of the role of TGF-β in fibrosis and osteophyte formation, the use of TGF-β as a therapeutic agent for cartilage repair should be evaluated thoroughly as side effects will likely occur or aggravate already existing pathology if TGF-β exposure is not confined to the articular cartilage.

TGF-β inhibition

The experiments discussed above are all circumstantial evidence of TGF-β involvement in the OA-changes like fibrosis and osteophyte formation. The only real proof of a protective role for TGF-β in OA and a role in induction of fibrosis and osteophyte can be obtained by blocking endogenous TGF-β during OA to see whether cartilage damage is aggravated by the lack of TGF-β and if fibrosis and osteophyte formation can be prevented.

Our group has shown that inhibition of TGF-β with a soluble receptor enhanced proteoglycan loss and reduced cartilage thickness (83). Serra et al. overexpressed a dominant negative TGF-β receptor resulting in terminal chondrocyte differentiation and OA (65). This proves that endogenous TGF-β is important for maintaining cartilage integrity.

Inhibition of endogenous TGF-β in a murine model for OA revealed that indeed TGF-β plays a role in OA-induced synovial fibrosis and osteophyte formation as both were reduced by blocking TGF-β by adenoval overexpression of different TGF-β inhibitors: Smad6, Smad7 or LAP (84). As TGF-β seems to be important for cartilage, blocking of TGF-β has to be compartmentalized to the locations where it is needed, excluding sites where side-effects occur. Although utilization of Smad6, -7 and LAP works in mice, for human transfection of the synovial lining would mean introducing modifications that are not directly usable as a treatment. Therefore, it would be very beneficial if we could find secondary mediators for the unwanted side-effects of TGF-β.
Potential secondary pathways

A potential solution to abolishing TGF-β side-effects is application of local inhibitors like Smad7. But this is not the only possibility to overcome the problem of having to compartmentalize TGF-β effects in a single tissue of the joint. Another potentially fruitful approach of abolishing TGF-β side-effects, while preserving the beneficial effects of TGF-β, is to evaluate the secondary pathways that might be involved in the side-effects. To enhance cartilage ECM formation with TGF-β while preventing synovial fibrosis, the putative TGF-β-induced secondary mediator that induces fibrosis should be identified. Connective Tissue Growth Factor (CTGF/CCN2) is a good candidate as it is directly induced by TGF-β through a TGF-β response element in the CTGF-promotor. Moreover, CTGF is an established player in various fibrotic disorders including nephropathy, Crohn’s disease, liver fibrosis, scleroderma, systemic sclerosis, lung fibrosis and heart fibrosis (85-92). Wahab et al established that CTGF augments TGF-β signaling by reducing the negative feedback through Smad7 and enhancing Smad2 phosphorylation (93). These data suggest that CTGF might be a candidate secondary mediator of TGF-β induced synovial fibrosis and therefore a potential therapeutic target.

In addition to fibrosis, TGF-β induces osteophytes. Secondary mediators might play essential roles in TGF-β induced osteophyte formation. A potent inducer of osteophytes is Bone Morphogenetic Protein 2 (BMP-2). BMP belongs to the TGF-β superfamily and shares some of the TGF-β functions. BMP has been shown to compensate for TGF-β in Smad3 deficiency. Chondrocytes that are Smad4 deficient, therefore partially lacking TGF-β signaling, have a high up regulation of BMP-signaling indicating a compensatory mechanism (94). Therefore, it is possible that BMPs share some of the TGF-β functions in osteophyte formation, making it a potential candidate for mediating TGF-β induced osteophyte formation.

Outline of the thesis

Osteoarthritis is a common joint disease affecting mainly the elderly in the population, but can also be a result of joint trauma. Although risk factors have been established, the actual cause of OA is still unknown. OA is characterized by cartilage damage, synovial fibrosis and osteophyte formation.

TGF-β is a growth factor that is an important inducer of cartilage extracellular matrix production and is therefore suggested as a potential tool to enhance cartilage repair upon damage. Deficiencies in the TGF-β signal lead to OA-like phenotypes. (64-67) Thus TGF-β is potentially not only a reductive factor, but might also be important for cartilage integrity. This led to the hypothesis that during OA TGF-β function might be compromised. Therefore, we investigated whether the presence and function of TGF-β was deficient during OA. We studied expression of TGF-β and the TGF-β signaling molecules in cartilage during experimental OA (Chapter 2). We found that indeed during OA there is a decrease in expression of the TGF-β isoforms 2 and 3 (not 1), the TGF-β receptors and the phosphorylated form of Smad2. The reduction in TGF-β was not a common reduction of growth factor expression as we found elevated expression of BMP-2. In addition, the reduction of the TGF-β signal was confined to the cartilage as the osteophytes that had developed during OA showed very high expression of TGF-β and Smad3P. The decreased TGF-β signaling was more pronounced with more progressive OA, suggesting an association between decreased TGF-β signaling and cartilage damage.

The potential importance of TGF-β for cartilage maintenance or repair is its strong ability to counteract the major cartilage degrader IL-1 (95). However, with age, which is a risk factor for OA, this counteracting ability is lost (96). This could be due to an age related reduced expression of TGF-β signaling or to an age related altered response to IL-1. Therefore, we evaluated the expression of various TGF-β signaling molecules in aged mice versus young mice as well as the potential influence of IL-1 on their expression (Chapter 3). We found that with age, similar to our findings in OA, the TGF-β signaling cascade was down regulated. As age is a risk factor for OA, this might be preceding the damage that we see during OA. To investigate what the implications were of a reduced TGF-β signal, we blocked endogenous TGF-β while inflicting cartilage damage with IL-1 (Chapter 3). Lack of TGF-β resulted in a more severe damage in response to IL-1. This showed that TGF-β presence is important for preserving cartilage.

Our data showed that lack of TGF-β in cartilage induced a condition that favors damage, but does supplementation of TGF-β compensate for this flaw? We set out to investigate whether TGF-β supplementation had an anabolic effect on damaged cartilage (Chapter 4). But since we know that TGF-β supplementation results in side-effects like synovial fibrosis, we investigated...
whether it was possible to compartmentalize the positive and negative effects by locally blocking TGF-β signaling in the synovium (Chapter 4). We found that indeed it was possible to stimulate cartilage repair with TGF-β while preventing synovial fibrosis by local inhibition of TGF-β in synovial tissue with Smad7. Moreover, we transferred this system to our murine models for OA and found that in this setting we were also able to boost proteoglycan synthesis in OA cartilage, while preventing synovial fibrosis (Chapter 4).

Although this was a adequate proof of principle, it is not directly applicable in the clinic. It would be very beneficial if we could identify specific secondary mediators for the negative side effects induced by TGF-β. As CTGF is strongly induced by TGF-β and seen as a potential target for fibrosis, we evaluated its effects in murine knee joints (Chapter 5). This showed that CTGF was able to induce fibrosis, but only transient. In addition, CTGF induced cartilage damage, which was in contrast to findings in literature. This might be due to the indirect effects via the CTGF-induced fibrosis. As CTGF-induced fibrosis was only transient in contrast to the more persistent fibrosis that was induced by TGF-β, we wanted to find out what caused the differences in fibrosis induced by either TGF-β or CTGF (Chapter 6). We found that TGF-β induced expression of lysyl hydroxylase 2 splice variant B (LH2B). Expression of LH2B will result in collagen cross-linking which is harder to degrade than LH2A, thereby contributing to persistence of fibrosis (97).

In addition, TGF-β induced high levels of TIMP1, which has been found associated with matrix accumulation in fibrosis (98-101).

CTGF is strongly up regulated by TGF-β, but despite the fact that TGF-β is drastically reduced in OA cartilage, CTGF is one of the most highly up regulated factors in OA cartilage. Besides CTGF we found that BMP2 was expressed strongly in areas surrounding OA lesions, whereas in normal cartilage it was hardly present (Chapter 2). The functional consequence of its presence during OA was unknown. Hence, we investigated what the role for BMP-2 was in cartilage (Chapter 7). We found that BMP-2 induced both anabolic and catabolic effects, but overall led to an increase in proteoglycan content. This suggested that BMP-2 might be important for cartilage repair. Therefore we investigated the role of BMP during damage repair (Chapter 7). Upon IL-1 induced cartilage damage BMP-2 boosted the proteoglycan synthesis, whereas blocking BMP during this process aggravated cartilage damage. This implies that the presence of BMP during OA is functional and probably serves as a cartilage-remodeling factor.

BMP-2 itself is able to induce de novo osteophyte formation. Therefore BMP-2 could serve as a secondary mediator of TGF-β induced osteophyte formation. To investigate whether it played a role during TGF-β-induced osteophyte formation or during OA-induced osteophyte formation we compared the patterns of osteophytes in all these conditions (Chapter 8). We found that BMP-2-induced osteophytes initially developed at different locations when compared to the osteophytes induced by TGF-β and experimental OA. TGF-β induced osteophytes developed at similar locations as during OA, indicating that perhaps TGF-β is more important during OA-induced osteophyte development when compared to BMP-2. By blocking BMP during the onset of TGF-β-induced or OA-induced osteophyte formation we could show that BMP is not important for the onset of osteophyte formation in these processes.

Overall, we show that TGF-β is an important factor to maintain cartilage integrity, but also to repair cartilage. The unwanted TGF-β induced fibrosis can be abrogated by local inhibition of TGF-β activity. The secondary mediators that we identified did not dominate the side-effects enough to serve as targets for therapeutic intervention. However, they have contributed to better understanding of the processes underlying TGF-β side-effects.

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Figure 4
Graphical representation of the general scope of this thesis. The arrows show the chapters in which each aspect is discussed.


Chapter 2

Expression of TGF-β and the TGF-β signaling molecule Smad-2P in spontaneous and instability-induced osteoarthritis. Role in cartilage degradation, chondrogenesis and osteophyte formation.


E.N. Blaney Davidson
E.L. Vitters
P.M. van der Kraan
W.B. van den Berg
Introduction

Osteoarthritis (OA) is a degenerative joint disease with a high prevalence in the elderly population. Although OA is a complex disease, the primary feature in all OA 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 to degradation, frequently excess tissue production is observed elsewhere in the joint. This might be a remodeling process initiated as a response to injury, resulting in osteophytes at the joint margins. TGF-β is involved in protection against cartilage destruction and in formation of new cartilage as can be found during osteophyte formation (2-6). TGF-β stimulates production of ECM components by chondrocytes and has the ability to counteract catabolic cytokines like Interleukin-1 (IL-1) (5,7-10). Blocking TGF-β during papain-induced experimental OA enhances articular cartilage proteoglycan loss (4). TGF-β injection or adenoviral overexpression induces osteophyte formation in naive knee joints (3,5,11), while inhibition of endogenous TGF-β prevented osteophyte formation during experimental OA (4). In addition, blocking osteophyte formation in the rat anterior cruciate ligament transection model with alendronate was associated with a reduced release of active TGF-β (12). TGF-β expression has also been found in human femoral head osteophytes, but in very variable amounts (13). These studies indicate a role for TGF-β in protection against cartilage destruction and in osteophyte formation. In OA joints both cartilage degradation and cartilage formation occur simultaneously in the same joint. To elucidate the role for TGF-β in these processes during OA, we investigated TGF-β expression and signaling during cartilage degradation and osteophyte development in two murine models of OA with a different etiology: a spontaneous OA model and an instability-induced OA model. We examined the expression of the most abundant TGF-β isoform, TGF-β3 (4) and the expression of its downstream signaling molecule Smad2 in its phosphorylated state, indicating active TGF-β signaling.

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 old (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 study has been approved by the Local Animal Experimentation Committee, Nijmegen, The Netherlands.
Experimental induction of osteoarthritis in BalbC mice
Six μl solution of bacterial collagenase (5 u/mg, 5 units/knee) was injected intra-articularly into
the right knee joint as previously described (14). Mice were sacrificed 1, 3, 7 and 14 days after
collagenase injection. Knee joints were dissected for histology. 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 OA resulting from joint
instability.

STR/ort mice
The STR/ort mice of ages 8 weeks, 6 months and 1 year old were sacrificed to compare pathology.
STR/ort mice are genetically predisposed to spontaneously develop OA-like lesions. Eighty per-
cent of the 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. In addition, the mice develop osteophytes at the joint margins. The histological lesions
seen in this model resemble those seen in humans (15-17). Therefore, the STR/ort mice might be
seen as a model for human primary OA.

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

Immunohistochemistry
Sections were deparaffinized and washed with PBS. Sections were incubated in citrate buffer
(0.1 M sodium citrate + 0.1 M citric acid) for two hours for antigen unmasking. Endogenous
peroxidase was blocked with 1% hydrogen peroxide in methanol for 30 minutes. Sections were
blocked with 5% normal serum of the species in which the secondary antibody was produced.
Specific primary antibodies against TGF-β3 (2 μg/ml) (Santa Cruz Biotechnology Inc.), Smad-2P
(1:100) (Cell Signaling Technology) or BMP-2 (2 μg/ml) (Santa Cruz Biotechnology Inc.) were incu-
bated overnight at 4°C. After extensive washing with PBS, the appropriate biotin labeled secondary
antibody was used (DAKO) for 30 minutes at room temperature followed by a biotin-streptavidin
detection system according to manufacturers protocol (Vector Laboratories). Bound complexes
were visualized using DAB reagent, counterstained with haematoxylin, dehydrated and mounted
with Permount.

Scores
A blinded observer scored sections stained for either TGF-β3 or Smad-2P. The number of TGF-β3
and Smad-2P immunopositive cells and 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 TGF-β3 or Smad-2P
was determined visually and stratified into 5 categories: Negative (-), meaning no cells staining
positive; weakly positive (+/-), meaning a low number of cells staining positive; positive (+),
meaning less than 50% of the cells staining positive; highly positive, meaning between more
than 50% and close to all cells staining positive (++), or all cells stained positive with high staining
intensity (+++). We scored 4 different cartilage surfaces: medial femoral cartilage, lateral femoral
cartilage, medial tibial cartilage and the lateral tibial cartilage. Per joint at least 4 sections were
examined and averaged. The mean of all the averages determined the outcome of the score.
Osteophytes were scored in a similar manner, estimating the amount of cells expressing TGF β3
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 computerized imaging system (Qwin, Leica Imaging Systems
Ltd.). A blinded observer selected the cartilage surface in at least 3 sections per knee joint. The
computerized 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 with a Mann-Whitney test. Values were considered
significant if the P-value was less than 0.05.

Results
Cartilage: instability model
Healthy cartilage was smooth and stained evenly red with Safranin O. Almost every chondrocyte
appeared to be TGF-β3 positive. When OA is induced by injection of collagenase, the joint
becomes unstable eventually resulting in cartilage damage. The cartilage of the OA joint showed
focal depletion of proteoglycans, indicated by reduced red staining in the non-calcified cartilage.
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 amount 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 very intense (Table 1, Figure 1A).

This was first observed on day 3 on the medial side of the joint, accompanied with an overall decrease in TGF-β3 expression. The amount of proteoglycans in cartilage diminished in time, with pronounced reduction on day 14, mainly on the medial side of the joint (Figure 1A). Only 10% of the cells in the medial cartilage expressed TGF-β3, located near the surface of the cartilage. Proteoglycan loss and reduction in TGF-β3 expression on the lateral side of the joint were only observed on day 14 and were less pronounced (Table 1).

---

**Figure 1A**

Cartilage: instability model. Original magnification: 200x. Sections were stained with Safranin O and Fast Green (A, B, C, D), sections were stained immunohistochemically for TGF-β3 (E, F, G, H), SMAD-2P (I, J, K, L) and BMP-2 (M, N, O, P) in Safranin O stained sections arrows indicate lighter stained cartilage representing proteoglycan depletion. TGF-β3 and SMAD-2P stained sections show a clear reduction in immunopositive cells in time. BMP-2 staining increases in time. For Safranin O and Fast Green stained sections arrows indicate loss of red staining in B, C and D. In immunohistochemically stained sections the black arrows indicate positive staining and the open arrows indicate loss of staining.

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**Figure 1B**

Cartilage: spontaneous model. Original magnification: 200x. Sections were stained with Safranin O and Fast Green (A, B, C), sections were stained immunohistochemically for TGF-β3 (D, E, F), SMAD-2P (G, H, I) and BMP-2 (J, K, L). In these sections the cartilage is clearly degrading in time. After 1 year (C, F, I, L) there is hardly any cartilage left. At 8 weeks there is still TGF-β3 and SMAD-2P staining (D, G), but at 6 months and 1 year the remaining cartilage is negative for both TGF-β3 and SMAD-2P (E, F, H, I). BMP-2 expression is highly expressed in severely damaged cartilage. (L) For Safranin O and Fast Green stained sections black arrows indicate loss of red staining in A and B. In immunohistochemically stained sections the black arrows indicate positive staining. Asterisks indicate the location where cartilage is lost.
To validate our arbitrary scoring system we additionally scored Smad-2P positive cells with a computerized imaging system. This showed that after induction of instability there were significantly less cells staining positive for Smad-2P. Additionally, it showed that the amount of positive cells in the STR/ort mice was significantly less than in normal mice with almost no expression at ages 6 months and 1 year (Figure 2).

**Cartilage: spontaneous model**

Cartilage of 8-week-old STR/ort mice already displayed proteoglycan loss, which was more pronounced on the medial side of the joint. Both TGF-β3 and Smad-2P expression varied at this age. The cartilage of the lateral side contained almost 90% TGF-β3 immunopositive cells whereas the cartilage of the medial side displayed only 10-20% cells immunopositive for TGF-β3. Smad-2P expression varied from 50-75% of the cells in the lateral cartilage and was 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 TGF-β3 and Smad-2P except for some positive cells remaining in the lateral tibial cartilage (<5% positive cells for both TGF-β3 and Smad-2P). After 1 year large portions of the cartilage were gone. The remaining cartilage was completely negative for both TGF-β3 and Smad-2P (Table 1, Figure 1B).

<table>
<thead>
<tr>
<th>Score of SMAD-2P in medial tibial cartilage</th>
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</thead>
<tbody>
<tr>
<td><img src="image.png" alt="Smad-2P staining in the medial tibial cartilage" /></td>
</tr>
</tbody>
</table>

Table 1

| Score of TGF-β3 (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; ++ more than 50% of the cells immunopositive; +++ almost every cell immunopositive and high staining intensity. |

<table>
<thead>
<tr>
<th>Instability OA</th>
<th>Femur medial</th>
<th>Femur lateral</th>
<th>Tibia medial</th>
<th>Tibia lateral</th>
<th>Osteophyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Day 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Day 3</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+++</td>
</tr>
<tr>
<td>Day 7</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+++</td>
</tr>
<tr>
<td>Day 14</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Spontaneous OA</td>
<td>8 weeks</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>6 months</td>
<td>--</td>
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<td>--</td>
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<td></td>
<td>1 year</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

To validate our arbitrary scoring system we additionally scored Smad-2P positive cells with a computerized imaging system. This showed that after induction of instability there were significantly less cells staining positive for Smad-2P. Additionally, it showed that the amount of positive cells in the STR/ort mice was significantly less than in normal mice with almost no expression at ages 6 months and 1 year (Figure 2).
Figure 3A
Osteophyte development: instability model. Osteophyte is located on the femur below the attachment of the collateral ligament. Original magnification: 200x. Sections were stained with Safranin O (A, B, C) and immunohistochemically for TGF-β3 (D, E, F) SMAD-2P (G, H, I) and BMP-2 (J, K, 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 TGF-β3 and SMAD-2P (D, G). When osteophyte formation progresses TGF-β3 resides in the fibrous layer overlaying the cartilage while SMAD-2P is seen in the entire structure (E, H). During ossification of the osteophyte TGF-β3 is observed again in the fibrous layer, but also in the core of the osteophyte. The cartilage-like layer is still negative for TGF-β3 (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 TGF-β (I, K, L).

Figure 3B
Osteophyte development: spontaneous model. Osteophyte is located on the femur below the attachment of the collateral ligament. Original magnification: 200x. Sections were stained with Safranin O (A, B, C) and immunohistochemically for TGF-β3 (D, E, F) SMAD-2P (G, H, I) and BMP-2 (J, K, 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 TGF-β3 and SMAD-2P (D, G) and there is low expression of BMP-2 (J). When osteophyte formation progresses into more ossified structures TGF-β3 and SMAD-2P expression diminishes (E, H). When the osteophyte has turned into a bone structure some TGF-β3 and SMAD-2P expression is found, but it is unclear whether this is from the original osteophyte or newly synthesized tissue (arrowheads in F, I). BMP-2 expression is low 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.
BMP-2 in OA cartilage

TGF-β3 and Smad-2P were found declined in early OA-cartilage and even absent in severe OA-cartilage. To make 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 TGF-β family: BMP-2. In contrast to TGF-β3, BMP-2 expression was elevated with increasing OA pathology. BMP-2 was found in only a small number of chondrocytes in the deeper layer of normal cartilage. In instability-induced OA we observed a small increase in number of positive cells on day 3. BMP-2 was also found in more superficial, non-calcified layers of cartilage. On day 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 under 20% of the total number of cells (Figure 1A).

In the spontaneous OA model we observed very 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 months old animals also cells in the superficial, non-calcified layers stained very intense. In one-year-old mice almost every cell neighboring severely damaged cartilage stained very intense for BMP-2 (Figure 1B).

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 OA. The chondrogenic structure had enlarged by day 7. Some of the cells disappeared and round, empty lacunae remained. On day 14, lacunae in the core of the osteophyte had joined together forming new bone marrow 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 organization than cartilage. This layer was thicker on day 14 than on day 7.

The initial cell clusters on day 3, all expressed TGF-β3. When the chondrogenic structure enlarged TGF-β3 was still expressed in every cell. The round lacunae on day 7 and 14 were negative for TGF-β3. The new bone marrow spaces contained small cells that expressed TGF-β3. The outer layer of the osteophyte was covered with TGF-β3 immunopositive, fibroblast-like cells. When the core of the osteophyte started to turn into bone (day 14) TGF-β3 resided at the margins (Figure 3A).

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 very intense and, in contrast to TGF-β3, observed throughout the entire structure (Figure 3A). Not all cells had similar staining intensity. In general, cells near the edge usually stained more intense than those in the core (Figure 3A).

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 to 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 to osteophytes in the instability-induced OA 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 grew on top of old osteophytes. Some of these new osteophytes were already ossified while others were still in developmental stages displaying chondrogenesis.

The chondrogenic part of the osteophytes in 8-week-old mice was, in contrast to the osteophytes in the instability model, negative for TGF-β3. Only the fibroblasts in the outer layer expressed TGF-β3. Of the chondrogenic part 50% of the cells 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 TGF-β3 and approximately 50% of the cells were positive for Smad-2P. This remained the same after 1 year (Table 1, Figure 3B). Early osteophytes expressed only low levels of BMP-2, whereas the bone-like structures that had developed after 1 year displayed intense BMP-2 staining indicating a role for BMP in late osteophyte development (Figure 3B).
Discussion

We investigated the role of TGF-β in two OA models reflecting aspects of human OA. TGF-β3 and TGF-β signaling, assessed by Smad-2P staining, were studied. TGF-β induces Smad-2 phosphorylation in a time- and dose-dependent manner, giving insight in active TGF-β signaling (4,18,19). One of the main features of OA is destruction of cartilage, which is preceded by loss of proteoglycans. In both OA models proteoglycan loss is accompanied by reduced TGF-β3 and Smad-2P staining. OA lesions in the spontaneous model were predominantly observed on the medial side of the joint corresponding with previous findings of Dunham et al., describing progressive disorganization of proteoglycans in the medial cartilage plateau of STR/ort mice younger than 30 weeks of age (3,20,21). We observed that TGF-β3 and Smad-2P staining also diminished fastest on the medial side in both OA models. The STR/ort mice are studied over a longer time period, enabling us to study more progressive cartilage damage. The severely damaged articular cartilage is totally negative for TGF-β3 and Smad-2P, whereas intact cartilage, even in older STR/ort mice, contains TGF-β3 and Smad-2P immunopositive cells. Supportive of our findings, Verdier et al. show decreased TGF-β expression in degraded human OA cartilage and diminished TGF-βRII expression in fibrillated cartilage (22). Furthermore, Wang et al. found a correlation between expression of TGF-βRII/TGF-β1 and intracellular levels of TIMP in human cartilage chondrocytes. TIMPs inhibit MMPs, thereby facilitating accumulation of ECM products indicating a role for the TGF-β pathway in ECM homeostasis of the cartilage (23). Kizawa et al. show an asporin polymorphism in which asporin D14 has a greater inhibitory effect on TGF-β-mediated expression of cartilage matrix gene than the common form D13. The D14 variant was over-represented in individuals with arthritis indicating the lack of TGF-β responsiveness and its association with OA progression (24). Taken together these data strongly suggest that loss TGF-β signaling is associated with cartilage damage.

In the papain model, TGF-β was found increased in damaged cartilage (25). However, papain breaks down the ECM of the cartilage, thereby directly affecting cartilage integrity. It can be anticipated that chondrocytes respond differently to this insult when compared to instability-induced cartilage damage, probably involving different roles for TGF-β (26-29).

To confirm that our findings were not the effect of an overall drop in cell viability in OA cartilage, but TGF-β specific, we studied the expression of another TGF-β family member: BMP-2. In contrast to TGF-β3, BMP-2 is elevated with OA progression, indicating that the reduced TGF-β3 expression is not simply the result of decreased cell viability.

Another prominent feature of OA is osteophyte formation. The chondrocyte-like cell clusters that are observed in early osteophyte development are all positive for TGF-β3 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, TGF-β3 is observed in the bone marrow of the osteophyte. The cartilage-like tissue is negative, whereas the fibroblast-like cells are all positive for TGF-β3. In contrast, Smad-2P is found in every layer of the osteophyte. TGF-β3 expression is low to absent in this phase of development or obscured by matrix development. The first seems more plausible, in which case the TGF-β might diffuse from the fibrous layer to the cartilage inducing TGF-β signaling. Either way, there is Smad-2P staining indicating active TGF-β signaling.

Overexpression of TGF-β in murine knee joints has been shown to induce osteophytes while blocking endogenous TGF-β in an OA model reduces osteophyte formation (3,5,6,11,25). These findings suggest an important role for TGF-β 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. Particularly in these phases TGF-β seems to be very important. In later phases of osteophyte development, as seen in older STR/ort mice, TGF-β function is supposedly substituted by factors such as BMP. BMP is known to be involved in osteophyte maturation (30,31) and was found highly expressed in late osteophytes in our experiment. Uchino et al. describes TGF-β expression in human osteophytes (13). They were not able to discriminate between different stages of osteophyte formation, which might explain why they found various amounts of TGF-β in osteophytes. However, the location of TGF-β 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 OA models closely resembles that in human OA. In the spontaneous OA model we observe ectopic bone formation in the collateral ligament. Collins et al. and Walton also found these chondro-osseous structures in synovia and ligaments in STR/ort mice (32,33). 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 TGF-β3 and Smad-2P while 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 TGF-β3 and Smad-2P expression. In the early process of chondrogenesis TGF-β3 and Smad-2P are abundantly expressed whereas fully developed pseudo joints are negative for TGF-β3 and Smad-2P. Again suggesting a role for TGF-β in the early developmental stages of ectopic bone.

Although TGF-β expression is reduced in damaged cartilage, its expression is highly elevated in other compartments of the joint (osteophytes, but also synovial tissue). Therefore, it can be expected that TGF-β will still be released into the joint cavity and could ultimately reach other
tissue. We show that TGF-β signaling in cartilage is down regulated or even absent in OA cartilage. TGF-β that is produced outside the cartilage does not reach the chondrocytes, either due to scavenging by the extracellular matrix or the cells have lost the ability to respond. The latter is in concordance with the loss in TGF-β receptor II expression as has been shown in rabbits with osteoarthritis (34). Loss of the ability to respond to TGF-β might be related to high levels of proinflammatory cytokines like IL-1 and TNF present in OA cartilage. The absence of TGF-β3 expression could also be attributed to effects of pro-inflammatory cytokines. Struder et al has shown that pro-inflammatory cytokines can indirectly, via nitric oxide production, modulate TGF-β production (35). In contrast, IL-1 and TNF have been shown to stimulate BMP-2 expression, which could explain our findings of BMP-2 up regulation during OA progression (36).

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

Reference List


Chapter 3

Reduced transforming growth factor-β signaling in cartilage of old mice: role in impaired repair capacity


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Introduction

Osteoarthritis (OA) is characterized by cartilage damage, osteophyte formation and thickening of the joint capsule. The etiology of OA is unknown, but OA is strongly correlated with age. OA may be a result of an age-related alteration in responsiveness of cells to anabolic and catabolic stimuli.

IL-1 is a cytokine that plays an important catabolic role in OA. IL-1 is highly expressed by chondrocytes of joints that are affected by OA, both in mice and humans (1,2). Patients with OA have high levels of IL-1 in their synovial fluids as well (3). IL-1 itself can induce cartilage damage (4) by reducing proteoglycan (PG) synthesis, increasing matrix metalloproteinase expression (5), and stimulating nitric oxide production (6).

Transforming growth factor (TGF)-β is an important anabolic factor in OA. It is very beneficial for cartilage as it stimulates PG and collagen type II synthesis and can down regulate cartilage-degrading enzymes (7-13). In addition, TGF-β is able to counteract IL-1 induced suppression of PG synthesis (9,14-16). Through this action TGF-β is able to protect cartilage from damage by IL-1 (9,17,18). In humans, expression of an asp orin variant with a high TGF-β inhibitory effect is significantly correlated with an increased incidence of OA (19).

Old animals show more prolonged suppression of PG synthesis after IL-1 exposure than young mice (4) and display a reduced response to counteraction of IL-1 by TGF-β (20). This indicates a shift in response to catabolic and anabolic stimuli, eventually leading to loss of cartilage homeostasis and OA.

TGF-β signals predominantly through two receptors, TGF-βRI (ALK5) and TGF-βRII. TGF-β binds to the type II receptor, recruits and phosphorylates the type I receptor and subsequently activates its receptor Smad, Smad2, Smad3, or Smad6 and Smad7. The complex is subsequently translocated to the nucleus where TGF-β responsive genes are transcribed (22). Inside the cell there are also inhibitory Smads (Smad6 and Smad7) that can prevent TGF-β signaling (23,24). We postulate that the lack of responsiveness to TGF-β counteraction of IL-1 in old mice is due to an overall lack of responsiveness to TGF-β caused by a down regulation of receptors and/or Smad expression or and increase in inhibitory Smads. Therefore, we investigated the expression of the various TGF-βs (1, 2 and 3) as well as their signaling molecules (TGF-βRI and TGF-βRII, Smad2, Smad-2P, Smad3, Smad4, Smad6 and Smad7) immunohistochemically in the cartilage of knee joints of young and old mice. In addition, we assessed whether these expression levels were altered differently in young and old mice by intra-articular injection of IL-1α.

We show that old mice have a profoundly lower expression of TGF-β receptors (I and II) than...
Immunohistochemistry for TGF-β1, TGF-β2, TGF-β3, TGF-βRI, TGF-βRII and Smad-2P, as well as Safranin O/Fast Green staining, were performed on paraffin sections from total knee joints. Knee joints were fixed in phosphate buffered formalin for 7 days. They were dehydrated using an automated tissue-processing apparatus (Tissue Tek VIP, Sakura, Ramsey, MN, USA) and embedded in paraffin. Tissue sections of 7 μM were prepared.

Immunohistochemistry
Sections were deparaffinized and washed with PBS. For antigen unmasking, sections were incubated in citrate buffer (0.1 M sodiumcitrate, 0.1 M citric acid) for 2 hours. Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 30 minutes. Thereafter, sections were blocked with 5% normal serum of the species in which the secondary antibody was produced. Specific primary antibodies against TGF-β1, TGF-β2, and TGF-β3 (1.0 μg/ml), TGF-βRI and TGF-βRII, Smad2, Smad3 and Smad4 (0.5 μg/ml), Smad6 (1.0 μg/ml), Smad7 (3.3 μg/ml) and Smad-2P (1:100) were incubated overnight at 4°C. (Smad6 antibody was purchased from Invitrogen (Breda, The Netherlands), Smad-2P from Cell Signaling Technology (Beverly, MA, USA), and all other primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA)). As a negative control, the primary antibody was replaced with goat or rabbit IgGs. After extensive washing in PBS, the appropriate biotin labeled secondary antibody was used at a concentration of 2 μg/ml in 1% bovine serum albumin/PBS for 2 hours (Vector Laboratories Inc., Burlingame, CA, USA), followed by a biotin-streptavidine detection system according to the manufacturers protocol (Vector Laboratories Inc.). Bound complexes were visualized via reaction with 3′,3′diaminobenzidine (Sigma Chemicals Co., Zwijndrecht, The Netherlands) and H2O2 resulting in a brown precipitate. Sections were briefly counterstained with hematoxylin and mounted with Permount.

Image analysis: quantification of positively stained articular chondrocytes
For the different antigens, the number of positive articular chondrocytes in the tibia was determined by a blinded observer. The microscopic image was displayed on a computer monitor using the Qwin image analysis system (Leica Imaging Systems, Rijswijk, The Netherlands) and a Leica DC 300F digital camera. The area representing the non-calcified articular cartilage was selected by hand. For each antigen, a threshold was set in such a manner that only chondrocytes that were found to be positive (brown stained cell) as judged by the observer were selected. The computer program determined the number of positive cells in the cartilage for the different antigens. For each knee joint, the expression of the different antigens was measured in at least three tissue sections. The intensity of the staining was not taken into account as no obvious

Materials and Methods

Animals
Male C57BL/6 mice aged 5 months or 2 years were used. Animals were kept in filtertop cages with woodchip bedding under standard pathogen free conditions. They were fed a standard diet with tap water ad libitum. The local animal committee approved this study.

Experimental design
TGF-β counteraction of IL-1 effects is most likely mediated by TGF-βRI, TGF-βRII and the intra-cellular Smad proteins. We investigated if young (n = 14) and old (n = 14) mice differ in expression of these TGF-β signaling mediators. Therefore, knee joints were isolated and prepared for immunohistochemistry. Half of the joints were prepared for paraffin sections, half were prepared for frozen sections. The number of cells staining positive for the various proteins were measured with a computerized imaging system. In addition to the comparison between young and old mice, we checked whether IL-1α injection 24 h prior to knee joint isolation (10 ng) (R&D Systems, Wiesbaden, Germany) influenced the expression patterns. Thus, the right knee joint of every mouse was injected with IL-1α and the left knee served as the non-injected group.

Histology
For the different classes of Smads, knee joints were decalcified for 14 days in EDTA/PVP and subsequently cryosections of total knee joints (7 μM) were prepared and stored at -20°C. Before use, sections were air-dried for 30 minutes and freshly prepared paraformaldehyde (4%, 5 minutes) was used to fix the sections.
Results

Chondrocyte cell number is reduced with age

The percentage of cells expressing the different TGF-β signaling proteins in murine cartilage was calculated by correction for the total number of cells present in the articular cartilage of the tibia. Therefore, the total number of cells in both medial and lateral tibial cartilage was quantified for all experimental groups by computerized quantification of cell number in hematoxylin and eosin (H&E) stained sections. Old mice had a significantly lower number of chondrocytes in the tibial cartilage: the reduction was more pronounced in the medial tibial cartilage, with a reduction in cell number of 34%; the number of cells in lateral tibial cartilage had reduced 17%. (Figure 1). Treatment with IL-1 had no effect on the total number of cells (data not shown).

Reduction of various TGF-β signaling molecules with age

To assess whether the reduced TGF-β responsiveness in old mice was due to a lower amount of TGF-β expression we compared the number of TGF-β positive cells in the tibial cartilage of young (5 months old) and old (2 years old) mice in immunohistochemically stained sections. Old mice had a lower number of chondrocytes in the tibial cartilage: the reduction was more pronounced in the medial tibial cartilage, with a reduction in cell number of 34%; the number of cells in lateral tibial cartilage had reduced 17%. (Figure 1). Treatment with IL-1 had no effect on the total number of cells (data not shown).

Image analysis: proteoglycan content

PG content of articular cartilage was measured in sections stained with Safranin O and Fast Green using a computerized imaging system as previously described (25). Briefly, Safranin O stains PGs in the cartilage red. A blinded observer captured an image on screen and selected the cartilage. The computer then measured the amount of blue light passing through the selected area. The higher the amount of light passing through, the lower the amount of PGs in cartilage. The average of three sections per knee joint was calculated.

Proteoglycan synthesis

PG synthesis was assessed by measurement of 35S-sulfate incorporation. Isolated patellae were immediately placed in Dulbecco’s modified Eagle’s medium with gentamicin (50 mg/ml) and pyruvate. After half an hour, this medium was replaced by medium containing 35S-sulfate 20 μCi/ml in which patellae were incubated for 3 hours at 37°C and 5% CO2. Thereafter, patellae were further prepared for determining the amount of 35S-sulfate incorporation in the articular cartilage as previously described (22).

Statistical analysis

Results were analyzed with the Student’s t-test and considered significant if the p-value was smaller than 0.05.
the joint and from 32% to 2% on the lateral tibial cartilage (Figure 2F,H). TGF-β3 showed a similar pattern in medial tibial cartilage, where the number of positive cells was 31% in young mice compared to 1% in old mice. On the lateral side of the joint, ageing also resulted in a lower number of TGF-β3 positive cells, but this was not significant (Figure 3).

We also examined the effect of aging on the number of cells staining positive for the TGF-βRs. TGF-βRI was expressed by a significantly lower number of cells in the medial tibial cartilage in old mice compared to young mice, 2% compared to 21%, respectively. On the lateral side, the number of TGF-βRI positive cells was also lower in old mice, but this was not significant. The amount of cells expressing TGF-βRII was significantly lower in old mice, both on the medial and on the lateral side of the joint. On the medial side, the number of immunopositive cells was reduced with age from 27% in young mice to 4% in old mice; in the lateral tibial cartilage the reduction was from 26% to 6% (Figs 4 and 2E,G).

In contrast to the receptors, the number of cells positive for the several Smad molecules had hardly changed with age. The percentage of cells positive for receptor-Smad Smad2 was equal in young and old mice (Figure 2A,C). The expression of receptor-Smad Smad3 had increased in old

<table>
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<th>A: TGF-β expression in medial tibial cartilage</th>
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In the medial tibial cartilage, TGF-β1 and TGF-β2 expression were significantly reduced with age. In lateral cartilage, TGF-β2 was significantly reduced. Error bars display the standard error. For statistical analysis, a Student’s t-test was used. (* = p < 0.05).

<table>
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<th>A: TGF-β receptor expression in medial tibial cartilage</th>
<th>B: TGF-β receptor expression in lateral tibial cartilage</th>
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In the medial tibial cartilage, TGF-βRI and TGF-βRII expression were significantly reduced with age. In lateral cartilage, TGF-βRII was significant only in lateral tibial cartilage. Error bars display the standard error. For statistical analysis, a Student’s t-test was used. (* = p < 0.05; ** = p < 0.005; *** = p < 0.0005).
amount of immunopositive cells from 85% to 30% (Figs 6 and 2B,D). This indicates a decrease in active TGF-β signaling in old mice, possibly related to the decreased number of TGF-βRs in old mice.

To assess whether IL-1 itself altered TGF-β signaling in old mice, thereby reducing the counteractive abilities of TGF-β to IL-1, we examined the effect of IL-1 injection on the expression of TGF-β signaling components in the articular cartilage. Injection of IL-1 24 hours prior to knee joint isolation resulted in an increased expression of TGF-β1 and TGF-β2 in lateral tibial cartilage in old mice and a higher number of Smad2 positive cells in the medial tibial cartilage. IL-1 treatment did not influence TGF-β receptor, Smad or Smad2P expression in old mice (Figure 7). IL-1 did not alter the expression of the TGF-β signaling components in young mice.

Effect of blocking TGF-β on proteoglycan synthesis and proteoglycan content

To assess the functional consequence of depressed TGF-β signaling, we blocked TGF-β by adenoviral overexpression of the TGF-β inhibitor LAP two days after IL-1 insult. Four days after primary insult, knee joints were isolated for assessment of PG synthesis and PG content. PG synthesis was measured by 35S-sulfate incorporation into cartilage ex vivo. A normal response to IL-1 insult is an initial drop in PG synthesis the first 2 days after IL-1 injection, followed by a rapid increase in synthesis within the next 2 days (4). The increased synthesis levels are above normal turnover levels. LAP overexpression after IL-1 injection was able to completely block this
intrinsic increase in PG synthesis as shown by the 35S-sulfate incorporation, which was lower than after IL-1 insult alone (Figure 8A).

In addition, PG content was measured by quantification of Safranin O staining intensity of the cartilage. The block of endogenous TGF-β resulted in an aggravation of cartilage damage as the PG content of the cartilage was significantly reduced beyond IL-1 induced PG depletion (Figure 8B). These data show that deprivation of TGF-β resulted in a reduced repair capacity of the cartilage.

Figure 8
Effect of TGF-β deprivation on intrinsic cartilage repair capacity. Murine knee joints of young mice were injected with IL-1. After two days an adenovirus expressing the TGF-β inhibitor latency associated peptide (LAP) was injected intra-articularly. Four days after the initial injections with IL-1, patellae were isolated for 35S-sulfate incorporation and whole knee joints were isolated for histology. (a) 35S-sulfate incorporation into isolated patellar cartilage after treatment with IL-1 and Ad-LAP. IL-1 treatment induces an initial decrease in 35S-sulfate incorporation, but by day 4 the 35S-sulfate incorporation increased above normal levels, indicating an overshoot in proteoglycan synthesis. By blocking endogenous TGF-β with LAP, this overshoot is completely abolished.

(b) Proteoglycan content of patellar cartilage after treatment with IL-1 and Ad-LAP. IL-1 injection results in depletion of proteoglycans in cartilage. Blocking endogenous TGF-β with LAP results in an aggravation of this depletion beyond IL-1 induced damage alone.

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Figure 7
Effect of IL-1 expression of TGF-β signaling proteins in cartilage. Knee joints of (a) young (5 months old) and (b) old (2 years old) mice were injected with IL-1 24 hours prior to isolation of the knee joints. Paraffin sections of knee joints were stained immunohistochemically for TGF-β1, TGF-β2, TGF-β3, TGF-βRI, TGF-βRII, Smad2, Smad3, Smad4, Smad6, Smad7 and Smad2P. Subsequently, the number of cells staining positive were scored with a computerized imaging system and corrected for the total number of cells. After IL-1 injection, Smad2 expression increased only in the medial tibial cartilage and TGF-β1 and TGF-β2 expression increased only in the lateral tibial cartilage. Error bars display the standard error. For statistical analysis, a Student’s t-test was used. (* = p < 0.05).
Discussion

OA is characterized by cartilage damage with an increasing incidence with age. The etiology of OA is unknown, but an imbalance between catabolic and anabolic factors appears to be involved. Whereas chondrocytes of young mice respond well to TGF-β counteraction of IL-1, those of old mice show less efficient counteracting of IL-1 by TGF-β (20). In addition, they display prolonged suppression of PG synthesis. This might be due to a decreased response to TGF-β in cartilage of old mice. We compared, therefore, the expression of TGF-β and the TGF-β signaling components in cartilage of young and old mice. The cartilage of old mice contained a lower number of cells than young mice. We thus corrected our findings for the total number of cells in the examined cartilage. In this study, only the tibial cartilage is discussed, but similar changes occurred in the femoral cartilage. The reduced cell number we found in old mice corresponds to the decreased number of chondrocytes that was found in cartilage of human donors older than 40 (26). A decrease in chondrocyte cell number could be due to an age-related decline in (TGF-β-induced) chondrocyte proliferation rate (27,28).

Our results show that old mice have significantly lower numbers of cells expressing TGF-β2 and TGF-β3 than young mice. In addition, old animals had a significantly lower number of chondrocytes expressing TGF-βR. The lack of responsiveness to TGF-β counteraction in old mice is not likely a result of alterations in Smad expression, as they are unaffected or even elevated by aging. Smad3 was elevated in tibial cartilage, and in the medial tibial cartilage we found an elevation of Smad4 with age. The basal material for signaling inside the cell is present, only the action is lacking. This lack of action might be due to the reduced receptor expression combined with a drop in TGF-β2 and TGF-β3 in old mice. This could also explain the lower Smad2 phosphorylation in old mice. Smad2 itself is not a problem as it is present in equal numbers in both young and old mice, but if there are less receptors and less ligands, Smads are unlikely to be phosphorylated in high amounts.

In lateral tibial cartilage we found an elevation of Smad6 expression with age, while in medial tibial cartilage Smad7 was elevated with age; these changes were restricted to one cartilage surface only instead of both. Although it might contribute, it is unlikely that this elevation is the cause of the overall unresponsiveness to TGF-β.

We wanted to make sure that IL-1 itself did not alter TGF-β signaling and cause the reduced counteraction. Therefore, mice were exposed to IL-1 prior to knee joint isolation. IL-1 treatment had only little effect on TGF-β signaling. In old mice, we found an up-regulation of TGF-β1 and TGF-β2 in lateral tibial cartilage. In the medial tibial cartilage, we observed an IL-1-induced increase in Smad2. Although there was elevation of these factors, it had no effect on Smad2P, indicating that IL-1 treatment did not alter the outcome of TGF-β signaling.

Iqbal et al found a decrease in the expression of mRNA for TGF-β1, TGF-β2 and TGF-β3 with age in equine cartilage, supporting our findings (29). It is not clear why the TGF-β isoforms show a different pattern but it is known that all three isoforms are differentially regulated and have a different promoter region. Also, during embryogenesis all three isoforms show a different, developmental stage related expression pattern (30). Gómez-Camarillo et al also showed a progressive decrease of TGF-βRI with age (31). Matsunaga et al found similar expression patterns in cervical intervertebral discs in mice (32). They showed a decrease in expression of TGF-β1, TGF-β2 and TGF-β3 as well as TGF-βRI and TGF-βRII with age. In myogenic progenitor cells in mice, Beggs et al described similar observations (33). They found that TGF-βRI and TGF-βRII were down regulated and Smad2, Smad3, Smad4 and Smad7 remained unchanged (33). These data indicate that our findings are similar to those found in other species and cell types and that the phenomenon of reduced TGF-βRs and reduced TGF-β expression it is not limited to cartilage of murine knee joints.

IL-1 treatment increased the expression of TGF-β1 and TGF-β2 in tibial cartilage. Andriamanalijaona et al have also shown the ability of IL-1 to increase TGF-β1 of articular chondrocytes (34). Kaiser et al showed that IL-1 treatment resulted in elevated expression of Smad7 mRNA in vitro after 3 days (35). In our in vivo experiment, however, no significant alterations in inhibitory Smad expression were found. In contrast to Kaiser et al (35), we measured the percentage of cells expressing Smad7 1 day after IL-1 injection in vivo. The discrepancies in time, measurement and system probably explain why different results were found.

We previously examined TGF-β expression in OA. In severe OA in STR/ort mice, we did not find any TGF-β expression or Smad2-P at all, whereas younger STR/ort mice with only mild damage still expressed both factors (data not shown). In addition, others have also found discrepancies between OA cartilage and healthy cartilage with respect to TGF-β expression. Gomez-Camarillo and Kouri showed that TGF-β1 receptors were very scarce in experimental OA (31). The drop in expression levels of TGF-β and their signaling molecules that we found in old mice might precede OA.

The expression patterns in the cartilage suggest that a lack of TGF-β signaling plays a potential role in the reduced repair capacity in old mice and possibly in OA. To further investigate whether the disturbed TGF-β signaling could cause a reduction in repair, we inhibited endogenous TGF-β after IL-1 insult. This resulted in a total block of the increased PG synthesis, thereby reducing the intrinsic repair capacity of the cartilage. The reduced PG synthesis resulted in an aggravation of the IL-1-induced PG loss in cartilage. These results show that not only do old mice have a reduced TGF-β signaling capacity, but also that disrupted TGF-β signaling can indeed induce a distorted repair capacity of cartilage.
It has been hypothesized that TGF-β treatment can be used as a factor for cartilage repair. However, old mice respond poorly to TGF-β, so the use of TGF-β for repair might be more difficult than expected. It has already been shown that human articular chondrocytes stimulated with TGF-β1, fibroblast growth factor-2 and platelet derived growth factor-BB, contained more glycosaminoglycans than non-stimulated controls, but only if donors were younger than 40 (26). In addition, stimulation of equine articular cartilage with TGF-β resulted in lower $[35S]Na_2SO_4$ incorporation in horses of 20 years old compared to 9 month old horses (26,29). Although the response to TGF-β is reduced with age, it does not mean that the cartilage does not respond at all. There was still an increase in incorporation of $[35S]Na_2SO_4$ after TGF-β stimulation found by Livne et al in mice (36), but it has to be considered that this response in old animals cannot be compared to the massive stimulation in young animals. However, finding ways to stimulate cartilage repair bypassing the TGF-β receptor pathway appears to be an attractive option to boost repair of aged cartilage.

Conclusions

Our data show that there are less chondrocytes expressing TGF-βRs in cartilage in old mice. Smad expression is unchanged, but Smad phosphorylation is reduced with age. These data suggest that the reduced TGF-β counteraction of IL-1 induced cartilage damage of old mice is due to an overall lack in TGF-β signaling capacity. Blocking endogenous TGF-β in young mice induced a distorted repair capacity in cartilage. The reduced ability of chondrocytes to respond to anabolic factors during aging might play a role in the development of the age-related disease OA.


Chapter 4

TGF β-induced cartilage repair is maintained but fibrosis is blocked in the presence of Smad7


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E.L. Vitters
W.B. van den Berg
P.M. van der Kraan
Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by cartilage breakdown, synovial fibrosis, and bone spurs. An imbalance between catabolic and anabolic factors favoring the catabolic side is very likely involved in the pathological features of OA. Currently, many attempts are being made to repair the cartilage that has been damaged in OA. One approach focuses on shifting the metabolic imbalance back by stimulating the anabolic side. Transforming growth factor-β (TGF-β) is one of the anabolic factors involved in cartilage maintenance and appears to be a good candidate for cartilage repair. TGF-β is a stimulator of extracellular matrix production, like collagen type II and proteoglycan (PG), in chondrocytes and it down regulates matrix-degrading enzymes (1). High amounts of TGF-β are stored in healthy cartilage (2-6), whereas in OA cartilage the expression of TGF-β is reduced (7). Injection of TGF-β into naive murine knee joints results in an increase in PG content of the articular cartilage (8). Moreover, in murine experimental rheumatoid arthritis, injection of TGF-β protected cartilage from PG loss (9). In addition, TGF-β counteracts the anabolic factor interleukin-1 (IL-1), which is a very potent inducer of cartilage degradation (10;11) both in vivo and in vitro (1;12-16). These data indicate that TGF-β has great potential as a tool for stimulating cartilage repair.

To obtain sufficient amounts of TGF-β in the joint for a prolonged period of time, an adenovirus can be used as a vehicle. In vitro, chondrocytes that are transfected with an adenovirus encoding TGF-β responded by elevation of PG and collagen production (17). We wanted to assess whether adenoviral overexpression of TGF-β in the synovial lining could stimulate repair of damaged cartilage in vivo.

Unfortunately, introducing high amounts of TGF-β into a knee joint has adverse effects. Administration of 20 ng TGF-β is already sufficient to result in an increased cellularity of the synovial lining, expansion of fibroblast population in the synovial connective tissue, and continued collagen deposition (18). Injection of high amounts of TGF-β, either as a bolus injection or via adenoviral transfection, results in marked hyperplasia of the synovium and chondro-osteophyte formation (8;18-21). This illustrates that the use of TGF-β for cartilage repair can result in side effects that are deleterious for future therapeutic applications.

The aim of this study was to use TGF-β as a cartilage repair factor but at the same time to prevent the TGF-β-induced fibrotic side effect. Therefore, we examined the effect of adenoviral overexpression of active TGF-β on cartilage repair and additionally studied whether simultaneous Smad7 overexpression could block TGF-β-induced fibrosis. Smad7 is an intracellular molecule that inhibits the TGF-β signaling pathway. TGF-β binds to its type II receptor, which then forms a complex with the type I TGF-β receptor.
Subsequently, the intracellular signaling molecule Smad2 or Smad3 gets phosphorylated, forms a complex with common Smad, Smad4, and shuttles to the nucleus for transcription (22). Smad7 inhibits Smad2 and Smad3 phosphorylation, thereby preventing further signaling (23;24).

To both stimulate cartilage and block side effects, we took advantage of the fact that adenoviruses, once injected into the murine knee joint, transfect the synovial lining but do not penetrate the cartilage (25). We co-transfected the synovial lining with an adenovirus overexpressing TGF-β and an adenovirus overexpressing Smad7. The transfected synovial lining cells will produce TGF-β but due to an intracellular signaling block caused by Smad7, will no longer respond to this factor.

We show that adenoviral overexpression of TGF-β results in increased PG content of the cartilage both after IL-1-induced damage and in a spontaneous model of experimental OA. In both cases, the TGF-β-induced fibrosis can be prevented by simultaneous Smad7 overexpression.

Materials and Methods

Animals

C57Bl/6 mice (10 weeks old) and STR/ort mice (4 weeks old) were used. Mice were kept in filter-top cages with woodchip bedding under standard pathogen-free conditions. They were fed a standard diet and tap water ad libitum. The local animal committee had approved this study.

Stimulation of cartilage repair by TGF-β after IL-1 insult

To assess whether adenoviral overexpression of TGF-β could stimulate cartilage repair, we inflicted cartilage damage in 73 C57Bl/6 mice by intra-articular injection of 10 ng IL-1 (R&D Systems, Inc., Minneapolis, MN, USA). Two days after IL-1 injection, PG synthesis will have reached a low point (11). At this time point, an adenovirus overexpressing active TGF-β (Ad-TGF-β223/225) (Gift by Dr. C.D. Richards) was injected intra-articularly (plaque-forming units (pfu) 10^7/6 μl) and compared with a control virus (Ad-del70-3). Four days after the primary insult, 53 mice were used for patellae isolation for PG synthesis measurement by 35SO_4^- incorporation. The other 20 mice were used for isolation of whole knee joints for histology.

Blocking TGF-β-induced fibrosis

To block TGF-β-induced fibrosis, 24 C57Bl/6 mice were injected intra-articularly with adenoviruses in the combinations of Ad-TGF-β223/225 + Ad-luciferase (Ad-luc), Ad-Smad7 + Ad-luc, and Ad-TGF-β223/225 + Ad-Smad7 (at a pfu of 0.5 × 10^7 per adenovirus in 6 μl) or Ad-luc alone (at a total pfu of 10^7) as a control. After 14 days, when synovial fibrosis can be observed histologically, knee joints were isolated for histology.

Simultaneously stimulating cartilage repair and blocking of fibrosis

To make sure that Smad7 did not interfere with TGF-β-stimulated PG synthesis, we assessed whether stimulation of cartilage repair was not blocked by co-transfection with Ad-Smad7, and cartilage damage was again introduced in 48 C57Bl/6 mice by intra-articular injection with 10 ng IL-1. After 2 days, mice were injected with adenoviruses in the combinations of Ad-TGF-β223/225 + Ad-luc, Ad-TGF-β223/225 + Ad-Smad7, or Ad-luc alone. Four days after IL-1 injection, 24 mice were used for isolation of patellae for 35SO_4^- incorporation measurements. After 2 weeks, the other mice were used for isolation of knee joints for histological assessment of fibrosis.

Cartilage repair while blocking fibrosis in spontaneous OA

To test whether we could stimulate cartilage repair in a spontaneous experimental OA model while preventing fibrosis, we extended our experiment to STR/ort mice. STR/ort mice develop OA spontaneously and show pathological changes by 8 weeks of age. We injected adenoviruses intra-articularly into the knee joint of 24 4-week-old STR/ort mice and repeated this injection after 2 weeks. The adenoviruses were injected in the combinations of Ad-TGF-β223/225 + Ad-luc, Ad-Smad7 + Ad-luc, and Ad-TGF-β223/225 + Ad-Smad7 at a pfu of 0.5 × 10^7 per adenovirus or Ad-luc at a pfu of 10^7 alone as a control. Four weeks after the first injection, knee joints were isolated for histological analysis of synovial fibrosis and PG content of the cartilage.

Histology

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) (Tissue Tek VIP, Sakura, Ramsey, MN, USA) and embedded in paraffin. Coronal whole knee joint sections of 7 μm were made. Sections were stained with Safranin O and Fast Green.

Immunohistochemistry

Sections were deparaffinized and washed with phosphate-buffered saline (PBS). For antigen unmasking, sections were incubated in citrate buffer (0.1 M sodium citrate + 0.1 M citric acid) for 2 hours. Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 30 minutes. Thereafter, sections were blocked with 5% normal serum of the species in which the secondary antibody was produced. Specific primary antibodies against procollagen type I (2 μg/ml) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were incubated overnight at 4°C. After extensive washing with PBS, the appropriate biotin-labeled secondary antibody was used (DakoCytomation, Glostrup, Denmark) for 30 minutes at room temperature followed by a biotin-streptavidine detection system according to manufacturer's protocol (Vector Laboratories,
Burlingame, CA, USA). Bound complexes were visualized using DAB (3,3'-diaminobenzidine) reagent, counterstained with haematoxylin, dehydrated, and mounted with Permount.

**PG synthesis**

For measurement of PG synthesis, $^{35}$SO$_4^{2-}$ was incorporated into isolated patellae. Immediately after isolation, the patellae were placed in Dulbecco’s modified Eagle’s medium with gentamicin (50 mg/ml) and pyruvate. After half an hour, medium was replaced by medium containing $^{35}$SO$_4^{2-}$ (20 μCi/ml) and incubated for 3 hours at 37°C in 5% CO$_2$. Patellae were then further prepared for measurement of $^{35}$SO$_4^{2-}$ incorporation in the articular cartilage as previously described (26).

**PG content**

PG content was measured in sections stained with Safranin O and Fast Green, using a computerized imaging system as previously described (27). Briefly, Safranin O stains PGs in the cartilage red. The amount of PGs is determined by a computerized calculation of the amount of blue light passing through the red-stained cartilage. An increase in PGs leads to more intense red staining and reduced blue light passing through. The PG content of the tibia was calculated by the average of three sections per joint.

**Measurement of fibrosis**

Sections were stained immunohistochemically for procollagen type I as a measure of fibrosis. Subsequently, the number of cells that stain positive in the synovial tissue was determined. A blinded observer selected the synovial tissue in three sections per knee joint. A computerized imaging system subsequently determined the number of positive cells in the selected area. The obtained values were averaged per knee joint.

In addition, synovial hyperplasia was assessed by measurement of synovial thickness. This was determined in sections stained with Safranin O and Fast Green. The thickness of the synovial tissue was measured with a computerized imaging system again in three sections per knee joint and averaged per joint as previously described (27) (Qwin; Leica Imaging Systems Ltd., Cambridge, UK). In short, the width of the joint from bone edge to joint capsule, minus the width of the joint space itself, was determined.

**Statistical analysis**

Results were analyzed with a Student’s t test and stated significant if the p value was lower than 0.05.

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**Results**

**Overexpression of TGF-β stimulates cartilage repair**

Knee joints that are injected with IL-1 initially show a reduced incorporation of $^{35}$SO$_4^{2-}$ into their patellar cartilage. After 2-3 days, this has reached a low point, and thereafter the incorporation rapidly increases above normal incorporation levels. By day 4, the incorporation of $^{35}$SO$_4^{2-}$ had significantly increased to 165% of the normal value ($p < 0.005$). On day 2, an adenovirus encoding active TGF-β was injected into the knee joint. The TGF-β overexpression boosted the incorporation of $^{35}$SO$_4^{2-}$ beyond the already elevated incorporation to 200% of the normal value (Figure 1A). As a result, the overexpression of TGF-β after IL-1 injection almost completely restored the significantly reduced PG content of cartilage (96% of normal control, $p < 0.0005$) (Figure 1B). These data show that adenoviral overexpression of TGF-β can stimulate cartilage repair.

**Figure 1**

Effect of adenoviral TGF-β expression on PG synthesis and content in cartilage. (a) PG synthesis was measured by $^{35}$SO$_4^{2-}$ incorporation into patellar cartilage 4 days after IL-1 injection. (CPM = counts per minute) PG synthesis increased after IL-1 injection ($p < 0.005$), and this increase was boosted by TGF-β ($p < 0.0005$ compared with IL-1). (b) PG content of cartilage was measured by Safranin O staining intensity of the cartilage in Safranin O/Fast Green stained sections. The mean PG content of non-treated knee joints was set at 100%. After IL-1 exposure, a clear reduction in PG content was observed ($p < 0.05$). By adenoviral expression of TGF-β after IL-1-induced damage, the PG content of the cartilage was almost normal. Error bars display standard error.
TGF-β induces synovial fibrosis that can be blocked with Smad7

Intra-articular injection of Ad-TGF-β resulted in a significant increase of synovial thickness (2.5-fold the width of controls) 2 weeks after injection. The percentage of cells expressing procollagen I increased accordingly (2.45-fold the amount of positive cells in controls) (Figure 2). By simultaneous overexpression of Ad-TGF-β and Ad-Smad7, the TGF-β-induced synovial thickness had been significantly reduced (p < 0.05). Sixty-five percent of the increased thickness and 65% of the elevated number of procollagen I-positive cells had been blocked by co-expression of Smad7 (Figure 2). Smad7 itself had no effect on synovial thickness or procollagen I expression. This illustrates that the TGF-β-induced fibrosis can be significantly blocked by co-transfection with Ad-Smad7.

Figure 2

Synovial fibrosis was assessed in knee joints 2 weeks after intra-articular injection of Ad-TGF-β combined with Ad-luc or Ad-Smad7. As a measure of fibrosis, the synovial width opposite the growth plates in the femur was measured (a). In addition, the amount of cells staining positive in immunohistochemically stained sections for procollagen type I was calculated with a computerized imaging system (b). The data are represented as an increase of the viral control. Histological representations of the measurements give an indication of actual thickness (c) and procollagen positive cells (d). There were no differences between viral and non-injected controls. TGF-β overexpression resulted in an increase in synovial thickness and number of procollagen type I-positive cells (p < 0.05). By co-expression with Smad7, most of the TGF-β-induced fibrosis was prevented (p < 0.05). Error bars display standard error.
TGF-β overexpression repairs cartilage while fibrosis is blocked with Smad7

To use the combination of TGF-β and Smad7 as a potential therapeutic intervention, we had to make sure that Smad7 overexpression did not interfere with the beneficial effect of TGF-β on cartilage repair. Therefore, TGF-β and Smad7 adenoviruses were injected 2 days after IL-1 injection. This combination turned out to be beneficial for PG synthesis and could still induce a significant increase in PG synthesis (80% increase of PG synthesis compared with IL-1 alone, p < 0.005). Simultaneously overexpressing Smad7 and TGF-β did not result in blocking of the repair-stimulating effects of TGF-β on cartilage (Figure 3).

Adenoviral overexpression of TGF-β resulted in a significant increase of 3.5 times as many cells expressing procollagen type I as the IL-1 control alone (p < 0.005). By co-expression of Smad7, 38% of the increase was blocked (p < 0.005). The synovial tissue had expanded significantly to almost four times in width after TGF-β overexpression compared with IL-1 + control virus (p < 0.0005). Almost half of the increase was significantly blocked by simultaneous exposure of the synovial cells to TGF-β and Smad7 (p < 0.005) (Figure 4).

These data show that after IL-1-induced cartilage damage, the TGF-β-induced fibrosis could still be blocked by Smad7 overexpression without interfering with the effect TGF-β elicits on cartilage.
In experimental OA, simultaneous overexpression of TGF-β and Smad7 increased PG content of cartilage and prevented synovial fibrosis

The experiments conducted so far had been done in a relatively simple model introducing cartilage damage by injection of IL-1. However, in OA we are dealing with a more complex situation. We introduced combined overexpression of TGF-β and Smad7 in STR/ort mice. These mice develop OA spontaneously; therefore, they can be used as a model of primary OA. In STR/ort mice, OA progresses relatively slowly. Therefore, we examined the final result of TGF-β and Smad7 overexpression 2 weeks after the second of (in total) two viral injections. STR/ort mice that had been injected with the adenovirus for TGF-β (combined with a control virus) alone displayed a significantly higher PG content in cartilage than did controls without TGF-β overexpression \( (p < 0.05) \) (Figure 5). However, these mice had massive synovial hyperplasia. The synovial thickness had increased significantly to 4.4 times the width of non-injected controls, and almost every cell expressed procollagen type I \( (p < 0.005) \) (Figure 6).

Combining overexpression of TGF-β and Smad7 managed to maintain a significantly higher PG content in cartilage than controls \( (p < 0.005) \) (Figure 5). More than half of the increased synovial thickening that had been caused by TGF-β was inhibited significantly by overexpression of Smad7 \( (p < 0.005) \). The amount of procollagen type I-positive cells had been reduced accordingly (Figure 6).

These data clearly indicate that PG synthesis can be stimulated while inhibiting an increase of synovial fibrosis in an experimental model of OA.

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**Figure 5**
Proteoglycan content of osteoarthritits cartilage in STR/ort mice. STR/ort mice (4 weeks old) were injected intra-articularly with Ad-TGF-β combined with Ad-Smad7 or Ad-luciferase and injected again 2 weeks later. Four weeks after the first injection, knee joints were isolated and the effect of TGF-β overexpression on PG content was assessed. TGF-β exposure resulted in a significantly higher PG content of cartilage than Ad-luc-injected controls \( (p < 0.05) \). Co-expression with Smad7 had no effect on the PG content. Error bars display standard error.

**Figure 6**
Blocking of TGF-β-induced synovial fibrosis in osteoarthritis. STR/ort mice (4 weeks old) were injected intra-articularly with Ad-TGF-β combined with Ad-Smad7 or Ad-luciferase (Ad-luc) and injected again 2 weeks after the last injection. Knee joints were isolated for histology and synovial fibrosis was assessed. Synovial thickness was determined by measuring the width of the synovium opposite the growth plates in the femur. (a) Synovial thickness had increased significantly after adenoviral TGF-β overexpression \( (p < 0.005) \). More than 50% of this increase in width was prevented by simultaneous expression of Smad7 \( (p < 0.005) \). The number of procollagen type I-expressing cells (b) had increased as well after exposure to TGF-β \( (p < 0.0005) \). This was also inhibited by more than 50% by co-expression of Smad7. Histological representations of the measurements give an indication of actual thickness (c) and procollagen positive cells (d). Error bars display standard error.
The cartilage damage in OA is thought to be a consequence of a misbalance between anabolic and catabolic factors, favoring the catabolic side. In this study, we used TGF-β as the anabolic factor for cartilage repair. TGF-β has been reported to enhance periosteal chondrogenesis in explants in a dose-dependent manner (28). Morales et al demonstrated that TGF-β increased PG synthesis and suppressed its degradation in articular cartilage organ cultures (4;5). In addition, van Beuningen et al showed that in vivo TGF-β injections result in prolonged elevation of PG synthesis and PG content of cartilage in mice (8). These studies indicate that TGF-β has good potential for repairing cartilage. We showed that adenoviral overexpression of TGF-β was indeed able to boost cartilage repair in vivo. In vitro, it had already been shown that chondrocytes exposed simultaneously to IL-1 and TGF-β could reverse the IL-1-induced suppression of PG incorporation in their extracellular matrix (15). Supportive of our findings, van Beuningen et al demonstrated that, in vivo, TGF-β also counteracted deleterious effects of IL-1 on cartilage PG synthesis and PG content (13). In the current study, we first damaged cartilage by IL-1 injection and subsequently overexpressed TGF-β. In this way, we could assess whether TGF-β was able to restore, instead of prevent cartilage damage. We introduced TGF-β via adenoviral overexpression, thereby gaining prolonged high expression of TGF-β, instead of via a bolus injection that results in short TGF-β exposure. This way, we were able to demonstrate increased PG synthesis and higher PG content in cartilage not only in a clean setting introducing cartilage damage with IL-1 but also in a spontaneous OA model.

The drawback of using TGF-β is that it can have adverse effects in joints. TGF-β is a known inducer of fibrosis in various tissues, and synovial tissue is no exception (8;21). We took advantage of the fact that adenoviruses transflect only the synovial lining. In addition, we profited from the fact that Smad7 is an intracellular inhibitor of TGF-β. Smad7 stays inside the cell that is transfected with the adenovirus encoding Smad7. Because the synovial lining is where TGF-β induces synovial fibrosis, by co-transfection with Smad7, the lining appeared to be less sensitive to TGF-β-induced fibrosis. The reduction of TGF-β-induced fibrosis was not optimal and resulted in only a partial block of the fibrosis. This is likely due to the fact that not all cells in the synovial lining will be targeted. By optimizing this, we might be able to target every single one of the synovial lining cells and thereby fully block the TGF-β-induced fibrosis.

We have previously demonstrated that blocking TGF-β with Ad-Smad7 in OA resulted in reduction of the synovial fibrosis that was induced by the OA process itself (27). Now we combined the Smad7 adenovirus with Ad-TGF-β to block the TGF-β-induced fibrosis. We showed that the Ad-TGF-β transfection was still functional in combination with Smad7. Moreover, we demon-
strated for the first time that adenoviral overexpression of TGF-β could stimulate repair of damaged cartilage and that co-expression with Smad7 could prevent a great deal of the TGF-β-induced synovial fibrosis. Combining Smad7 and TGF-β resulted in a higher PG synthesis after IL-1 insult than did TGF-β alone. This is likely due to the reduced synovial fibrosis when combined with Smad7.

Unfortunately, synovial fibrosis is not the only side effect of TGF-β overexpression in knee joints. TGF-β can induce osteophyte formation (8;19;20;29-31). In the case of OA, TGF-β can aggravate the osteophyte formation that already occurs. We show that it is possible to target synovial cells to prevent fibrosis. In a similar fashion, we could potentially target the mesenchymal stem cells that eventually form the osteophytes after TGF-β exposure. This could be an option when key players of osteophyte formation are identified and can be blocked selectively.

**Conclusion**

We demonstrated that adenoviral overexpression of TGF-β increases PG synthesis and PG content in cartilage, even in experimental OA. In addition, co-transfecting the synovial lining with Ad-Smad7 can block the fibrosis that is induced by TGF-β overexpression.

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**Reference List**


Chapter 5

Connective Tissue Growth Factor/CCN2 overexpression in mouse synovial lining results in transient fibrosis and cartilage damage

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Introduction

Osteoarthritis (OA) is a joint disease affecting most of the elderly population. The disease is characterized by cartilage damage, fibrosis and osteophyte formation. However, the etiology of OA is still unknown. Currently, many efforts are made to find cures for OA (1-3). To find targets for OA therapy it is important to know the factors that are involved in the etiology of this disease. CTGF (=CCN2) is the most abundantly expressed growth factor in chondrocytes in severe human OA (4). In addition, CTGF mRNA was found to be up regulated next to damaged areas of cartilage surfaces and present in chondro-osteophytes (5). Therefore, CTGF is a likely candidate to contribute to the pathogenesis of OA and is likely to play a role in synovial fibrosis and osteophyte formation. However, it is unclear whether CTGF protects against cartilage damage. From many reports focussed on distinct disorders that involve fibrosis it can be concluded that CTGF has a major role in fibrotic diseases, including disorders like Crohn’s disease, liver fibrosis, scleroderma, systemic sclerosis lung fibrosis and heart fibrosis (6-11). This gave rise to the hypothesis that CTGF is likely involved in synovial fibrosis as seen in OA.

Furthermore, CTGF is expressed in hypertrophic chondrocytes, proliferating chondrocytes and proliferating periostal cells. It induces chondrocyte maturation and differentiation (12;13). Additionally, CTGF has angiogenic activities and promotes proliferation and differentiation of osteoblasts. Therefore, CTGF is considered a major factor for promoting enchondral ossification (14-17). These are all processes that are involved in initiation or progression of osteophyte formation similar to that observed in OA, suggesting a potential role for CTGF in OA osteophyte formation.

Nishida et al introduced CTGF in hydrogel into the joint cavity of rats in an OA model and showed repair of damaged cartilage (18). They also demonstrated that CTGF is capable of stimulating proliferation and differentiation in cultured articular chondrocytes. The cells that were transfected with CTGF also produced higher levels of proteoglycans in a dose dependent manner (19). These data indicate that CTGF can have protective effects on cartilage. Until now it is unknown whether CTGF is even capable of inducing fibrosis in diarthrodal joints. The presence of CTGF in OA joints and the potential of CTGF to induce fibrosis in many tissues make a role for CTGF in OA synovial fibrosis probable. From literature it seems that CTGF has beneficial effects on cartilage and it is likely to play a role in osteophyte development. Therefore, we investigated the overall effect overexpression of CTGF in the synovial lining of normal knee joints. We assessed effects of adenoviral CTGF overexpression in murine knee joints histologically, and subsequently focussed on the most prominently changed tissues: synovial tissue and cartilage. In addition, we examined alterations in gene expression patterns in synovia and cartilage.

Abstract

Objective

The characteristics of osteoarthritis (OA) are cartilage damage, fibrosis and osteophyte formation. To find cures for OA, we have to elucidate the factors causing OA pathology. Connective Tissue Growth Factor (CTGF, also known as CCN2 (for CYR61, CTGF, NOV), is found in high levels in OA chondrocytes and is frequently involved in fibrosis, bone formation and cartilage repair. Therefore, we investigated the potential role of CTGF in OA pathophysiology.

Methods

We transfected the synovial lining of murine knee joints with a recombinant adenovirus expressing human CTGF and measured synovial fibrosis and proteoglycan content in cartilage on days 1, 3, 7, 14 and 28. We measured mRNA expression in synovium and cartilage at days 3, 7 and 21.

Results

CTGF induced synovial fibrosis, as indicated by accumulation of extracellular matrix and increase in procollagen-I positive cells. The fibrosis reached a maximum at day 7 and had reversed by day 28. Matrix Metalloproteinases (MMPs) 3 and 13, A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS) 4 and 5, Tissue Inhibitor of Metalloproteases 1 (TIMP1) and Transforming Growth Factor-β (TGF-β) mRNA were elevated in the fibrotic tissue. TIMP-1 expression was elevated on day 3 while expression of other genes did not increase before day 7. CTGF induced proteoglycan depletion in cartilage already on day 1. Maximal depletion was observed at days 3-7. Cartilage damage was reduced by day 28. In cartilage, a high MMP3 mRNA expression was found. CTGF overexpression did not induce osteophytes.

Conclusion

CTGF induces transient fibrosis that is reversible within 28 days. Overexpression of CTGF in knee joints results in reversible cartilage damage, either induced by the high CTGF levels or via factors produced by the CTGF-induced fibrotic tissue.
after adenoviral CTGF overexpression. We found that CTGF is capable of inducing transient synovial fibrosis. Moreover, we found unexpected deleterious effects on proteoglycan content in cartilage. No osteophyte formation was found.

Materials and Methods

Transfection with Ad-CTGF
We injected murine knee joints intra-articularly with \(1 \times 10^7\) pfu virus in the right knee joint, thereby transfecting the synovial lining with an adenovirus overexpressing human CTGF (Ad-CTGF) (provided by FibroGen, Inc., South San Francisco, CA, USA) (\(n=65\)). As a viral control Ad5.CMV-Null was used, which has the same backbone as the CTGF virus (Qbiogene, Irvine, CA, USA) (\(n=40\)). To gain insight into the effect of CTGF we investigated a time course of 1, 3, 7, 14 and 28 days after viral injection. The mice were sacrificed on these days and knee joints were isolated for histology. For RNA isolation of synovial tissue or cartilage knee joints were isolated 3, 7 and 21 days after viral injection.

Animals
Male C57Bl/6 mice aged 12 weeks were used. Animals were kept in filtertop cages with woodchip bedding. They were fed a standard diet with tap water ad libitum. The local animal committee has approved this study.

Histology
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. The knee joints were then dehydrated with an automated tissue processing apparatus (Miles Scientific Tissue-Tek VIP tissue processor) and embedded in paraffin. Frontal whole sections of 7 μm were made. Sections were stained with Safranin O and Fast Green.

Immunohistochemistry
Sections were deparaffinized and washed with PBS. For antigen unmasking, sections were incubated in citrate buffer (0.1 M sodium citrate + 0.1 M citric acid) for two hours. Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 30 minutes. Subsequently, sections were blocked with 5% normal serum of the species in which the secondary antibody was produced. Specific primary antibodies against CTGF or procollagen type I (both 2 μg/ml) (Santa Cruz Biotechnology Inc.) were incubated overnight at 4°C. After extensive washing with PBS, the appropriate biotin labelled secondary antibody was used (DAKO) for 30 minutes at room temperature followed by a biotin-streptavidine detection according to the manufacturer's protocol (Vector Laboratories). Bound complexes were visualised using DAB reagent, counterstained with haematoxylin, dehydrated and mounted with Permount.

Quantification of synovial fibrosis
Sections were stained immunohistochemically for procollagen type I as a measure of fibrosis. Subsequently, the amount of cells that stain positive in the synovial tissue was determined. A blinded observer selected the synovial tissue in 3 sections per knee joint. A computerized imaging system (Qwin, Leica Imaging Systems Ltd.) subsequently determined the amount of positive cells in the selected area. The obtained values were averaged per knee joint. In addition, the amount of extracellular matrix present in synovial tissue was measured in a similar manner. This was determined in Safranin O and Fast Green stained sections: Fast Green stains the collagen green. The area of the green stained extracellular matrix in the synovial tissue was measured with a computerized imaging system, again in 3 sections per knee joint and averaged per joint.

Quantification of proteoglycan content of cartilage
The proteoglycan (PG) content of the articular cartilage was measured on the tibia. The cartilage was selected in 3 sections per knee. The amount of blue light passing through the red, Safranin O-stained, cartilage was measured with a computerized imaging system as described previously (20). Control joints were measured and considered to represent 100% PG content.

Quantitative PCR
Tibial cartilage was stripped off the joint and biopsy punches were taken from synovium as previously described (time points 3, 7, and 21 after injection of the adenovirus, \(n=5\) per group per time point). RNA was isolated from the tissue with an RNeasy Mini Kit (Qiagen) after which an RT-PCR was performed. Individual samples of each group were pooled and a Q-PCR was run in duplicate. A Q-PCR was prepared as follows: a primer mix of 1.5 μl forward primer (5 μM), 1.5 μl reverse primer (5 μM) and 4.5 μl \(d\text{H}_2\text{O}_2\), was added to 12.5 μl Sybr Green. Then 5 μl cDNA was added and the Q-PCR was performed by a “ABI/PRISM 7000 sequence detection system” (Applied Biosystems) according to manufacturers protocol. PCR conditions were as follows: 2 minutes at 50°C and 10 min at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with data collection in the last 30 seconds. In addition, for each PCR melting curves were run. The genes that were measured and the corresponding primer sets are displayed in Table 1. Efficiencies
CTGF that is expressed by the adenovirus is of human origin. We designed primers specific for either human or murine CTGF mRNA. This made it possible to discriminate between human CTGF mRNA, which is encoded by the Ad-CTGF adenovirus, and the endogenous murine CTGF mRNA.

Nucleotide sequences of the human CTGF primers were:

5'- AGGCAGTTGGCTCTAATCATAGTTG - 3'
5'- GCCCTCGCGGCTTACC - 3'

Statistics

Results were analyzed with the student T-test and considered significant if the p-value was smaller than 0.05.

Results

CTGF expression after synovial transfection

Injection of Ad-CTGF in murine knee joints resulted in transfection of the synovial lining with the adenovirus. Immunohistochemical staining with an antibody against CTGF showed clear upregulation of CTGF expression compared to controls (Figure 1A). Three days after injection with the adenovirus the synovial tissue showed high expression of human CTGF, which diminished in time (Figure 1B). Human CTGF was not detected in control samples.

CTGF overexpression induces fibrosis

Three different parameters were used to assess synovial fibrosis: synovial thickening, procollagen type I expression and ECM area.

The synovium of knee joints that had been transfected with Ad-CTGF had increasingly thickened on day 1, 3, and 7. The synovial enlargement was most prominent 7 days after viral transfection. On day 14 and 28, the synovial thickness had still increased compared to controls, but was less thick than on day 7 (Figure 2 and 3A).

Knee joints that had been injected with Ad-CTGF also had significantly increased content of procollagen type I positive cells in the synovial tissue on day 7 and 14. This increase was most prominent on day 7 (138% compared to controls). On day 28, the amount of procollagen type I positive cells was restored to normal values (Figure 3B).

The area of synovial ECM had increased on day 1, 3, 7 and 14 in knee joints injected with Ad-CTGF compared to controls. This increase was also significant on day 1, when the increase was most prominent. On day 28 the amount of ECM present in the synovium was comparable to controls (Figure 3C).

(E) for all primer sets were determined (Table 1) using a standard curve of 5 serial cDNA dilutions in water in duplicate. Primers were accepted if the deviation from slope of the standard curve was less than 0.3 compared to the slope of GAPDH standard curve and if the melting curve showed only one product. For each primer pair non-template controls were run in duplicate. The cycle threshold value (Ct) of the genes of interest were corrected for the Ct of the reference gene GAPDH. Relative mRNA expression was calculated by 2 to the power of delta Ct. Gene expression levels after transfection with CTGF were compared to the control virus group. The

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Table 1

Murine primers used for Q-PCR. Primers were designed using the ABI/PRISM Primer Express software (Applied Biosystems). Efficiencies (E) of all primers were validated with a standard curve of 5 cDNA dilutions in water. E is expressed as fold increase in fluorescence per PCR cycle. R² is the correlation coefficient for the PCR dilution series.

Statistics

Results were analyzed with the student T-test and considered significant if the p-value was smaller than 0.05.

Results

CTGF expression after synovial transfection

Injection of Ad-CTGF in murine knee joints resulted in transfection of the synovial lining with the adenovirus. Immunohistochemical staining with an antibody against CTGF showed clear up regulation of CTGF expression compared to controls (Figure 1A). Three days after injection with the adenovirus the synovial tissue showed high expression of human CTGF, which diminished in time (Figure 1B). Human CTGF was not detected in control samples.

CTGF overexpression induces fibrosis

Three different parameters were used to assess synovial fibrosis: synovial thickening, procollagen type I expression and ECM area.

The synovium of knee joints that had been transfected with Ad-CTGF had increasingly thickened on day 1, 3, and 7. The synovial enlargement was most prominent 7 days after viral transfection. On day 14 and 28, the synovial thickness had still increased compared to controls, but was less thick than on day 7 (Figure 2 and 3A).

Knee joints that had been injected with Ad-CTGF also had significantly increased content of procollagen type I positive cells in the synovial tissue on day 7 and 14. This increase was most prominent on day 7 (138% compared to controls). On day 28, the amount of procollagen type I positive cells was restored to normal values (Figure 3B).

The area of synovial ECM had increased on day 1, 3, 7 and 14 in knee joints injected with Ad-CTGF compared to controls. This increase was only significant on day 1, when the increase was most prominent. On day 28 the amount of ECM present in the synovium was comparable to controls (Figure 3C).
Figure 2
Increase in synovial fibrosis after transfection with Ad-CTGF. Paraffin sections of murine knee joints were stained with Safranin O and Fast Green. Left figure displays a normal joint indicating the area displayed in right figure. Synovial tissue stained with Safranin O and Fast Green. Synovial fibrosis had increased after CTGF overexpression compared to naïve knee joints. Maximum fibrosis was reached on day 7 and thereafter returned to almost normal on day 28. (Original magnification 100x)

Besides histological assessment of fibrosis, biopsies of the synovium were taken for gene expression analysis by determination of mRNA levels. CTGF overexpression in the synovial lining resulted in an increase in mRNA of MMP3, MMP13, ADAMTS4, ADAMTS5, TIMP-1 and TGF-β1 in the synovium. These factors were up regulated on day 3 and even more on day 7. By day 21, expression levels of genes that were elevated on day 3 and 7 were restored to normal levels, except for ADAMTS4. No auto induction of murine CTGF and only minimal up-regulation of collagen type I mRNA were found (Figure 4A).

Effects of CTGF overexpression on cartilage
CTGF overexpression in the synovial lining induced a reduction in PG content in articular cartilage. A maximum reduction in PG content was observed 7 days after injection. Reduction was still present 28 days after transfection with CTGF (Figure 5). Although the PG content was reduced there was no sign of cartilage erosion.

Overall these data show that CTGF is able to induce transient synovial fibrosis. The maximum fibrosis was found on day 7, thereafter thickening slightly decreased on day 14, and normal levels were reached by day 28.
overexpression of CTGF. MMP-3 was influenced the most by CTGF overexpression in the synovial lining: it was up regulated 11 fold compared to controls on day 7. MMP3 expression was normal on day 21. TIMP1 was already up regulated on day 3, displayed normal expression levels on day 7, and was also down regulated by day 21. In contrast to synovial tissue, the cartilage did show an up regulation of murine CTGF mRNA. The response of cartilage to adenoviral CTGF overexpression in the synovium was mainly up regulation of degrading enzymes (Figure 4B).
This study shows the ability of CTGF to induce synovial fibrosis and cartilage damage, both of which are also OA characteristics. No osteophyte formation was seen after CTGF overexpression. The fibrosis that was induced by CTGF overexpression had reversed by day 28. Although CTGF expression in the synovial lining was high at day 3, the synovial thickness and procollagen I expression were highest at day 7. All factors that were examined by Q-PCR showed maximum effect on day 7 as well. The synovial thickness had reached a maximum at day 7, was slightly reduced at day 14 and had resolved by day 28. Bonniaud et al have found similar fibrotic patterns after adenoviral CTGF overexpression in the lung. They show a moderate fibrosis with a maximum around day 14, which had reversed by day 28 (10).

Strikingly, we found high numbers of cells that stained intensely positive for procollagen type I after CTGF expression, but hardly any up regulation of collagen type I mRNA. This implies that a post-translational alteration is more likely than an up regulation of mRNA. CTGF is known to interact directly with other growth factors, thereby stimulating or inhibiting their actions (21;22). There is a possibility that CTGF elicits effects through these interactions rather than direct up regulation of genes.

We did find an up regulation of TIMP-1, which was up regulated earlier than other genes (day 3). McLennan et al studied the expression of TIMP-1, -2 and –3 in mesangial cells after treatment with rhCTGF and also found an up regulation of TIMP-1 (23). They also observed an up regulation of TIMP-3 and no effect on TIMP-2 expression. The increase in TIMP-3 gene expression, however, was reflected in the media at the protein level, nor did they find TIMP-2, whereas TIMP-1 was clearly detectable. It has been suggested that CTGF requires TIMP-1 to cause fibrosis (10). Bonniaud et al show a correlation between TIMP-1 expression and the sensitivity for fibrosis development. Strains of mice that were prone to develop fibrosis showed higher levels of TIMP-1 than fibrosis-resistant strains (24). Moreover, resistant strains could develop pulmonary fibrosis when transfected with Ad-CTGF combined with a bleomycin. Also in the joint TIMP-1 up regulation and fibrosis appears to be related.

Of all the factors examined mainly the MMPs were up regulated in the synovial tissue. Although these are ECM-degrading enzymes, there is still an increase in ECM in the synovial tissue at first. After day 7 the synovial fibrosis resolves, but the MMP expression decreases to normal levels as well. MMPs are excreted in an inactive pro-form and have to be activated outside the cell, so the high levels of MMP mRNA are not necessarily correlated with high levels of active proteases. During accumulation of the fibrotic tissue MMP activity might be low, while there are high levels of the MMP inhibitor TIMP-1. Beyond day 7 the TIMP-1 expression drops and newly synthesized...
MMPs might become activated. It can be speculated that increased MMP activity in combination with decreased TIMP-1 expression could be a cause for the resolving of fibrosis by day 28.

The synovial fibrosis that was found in our CTGF experiments was milder than the TGF-β induced synovial fibrosis in earlier experiments we performed (25;26). However, TGF-β is the main inducer of CTGF (9;27-29), therefore overexpression of TGF-β induces a strong CTGF up regulation presumably enhancing the TGF-β induced fibrosis. We cannot exclude the possibility that CTGF has a key role in the more persistent TGF-β induced fibrosis. In OA-fibrosis, TGF-β is also found (30). TGF-β and CTGF might work together or in turn to maintain the fibrosis. Moussad et al have proposed that for persistent fibrosis TGF-β is needed as an induction factor followed by CTGF as a maintenance factor (31). Chuo et al have also demonstrated that serial injections of CTGF after TGF-β induced more persistent skin fibrosis than TGF-β alone (32). In line with their conclusions, our data show that a single injection of Ad-CTGF alone is not enough to induce persistent fibrosis. Adenoviral overexpression of CTGF is only transient. Our data only show that a short exposure to CTGF is not sufficient to maintain prolonged synovial fibrosis. More sustained presence of CTGF might cause more persistent effects.

We found depletion of proteoglycans in cartilage after CTGF overexpression in the synovial tissue. Supportive of our finding, Omoto et al had previously suggested a relation between CTGF expression and degeneration of cartilage in OA. They showed strong immunopositivity for CTGF next to damaged cartilage surfaces (5). In addition, Minato et al demonstrated that 3 different antibodies against CTGF stimulated PG synthesis in chondrocyte cell lines (33) indicating that CTGF is involved in inhibition of PG synthesis.

In contrast, Nishida et al found increased proteoglycan synthesis in a rabbit articular cartilage cell line, as well as an increase in type II collagen and aggrecan, 48 hours after transfection with an adenovirus expressing CTGF in vitro (19). We found only a mild increase in type II collagen expression on day 7 alone and no changes in aggrecan expression. Nishida et al suggested that CTGF might be useful for cartilage repair, but in our experiment we saw a reduction in proteoglycan content suggesting the opposite. Nakanishi et al illustrated that proteoglycan synthesis stimulation could be neutralized by adding anti-CTGF antibodies suggesting that CTGF stimulates PG synthesis (34). Although the general consensus in literature is that CTGF induces chondrocyte production of PG, our data show that adenoviral overexpression of CTGF results in a reduction of PG-content in the articular cartilage.

In our experiment we examined complete knee joints, thereby taking into account the entire cartilage and not ruling out indirect effects of CTGF via other tissue. Our findings of reduced proteoglycan content in cartilage might be explained by the high up regulation of MMPs after CTGF overexpression in the cartilage itself. However, fibrotic tissue also shows mRNA up regulation of degrading enzymes. The cartilage proteoglycans could have been degraded by factors that were excreted by the fibrotic synovial tissue into the synovial fluid and reach the articular cartilage. The high MMP3 expression found in the cartilage, as well as the lack of aggrecan and collagen type II up regulation, could also be a result of factors secreted by the synovial tissue instead of a direct effect of high CTGF levels. The latter might explain the discrepancy between our findings and those of Nishida et al who showed that local administration of recombinant CTGF in cartilage defects could regenerate the articular cartilage (18).

Although we found clear reduction in proteoglycan content in cartilage, this does not mean that the cartilage is irreversibly damaged. As long as the collagen network is still intact, damage can be reversed. Since there is no consensus on whether CTGF is a factor inducing either degradation of cartilage or an anabolic stimulus, one might consider CTGF to be both a degrading and stimulating factor, thereby playing a role in cartilage remodelling.

Much research has shown a role for CTGF in chondrogenesis and endochondral bone formation (14;16;35-38), but we found no osteophyte formation after CTGF overexpression. If CTGF plays a role in osteophyte formation in OA at all, it is clear that CTGF needs another factor to start the process and cannot do it alone.

In conclusion, our data show that CTGF overexpression in murine knee joints induces fibrosis and cartilage damage, both reversible within 28 days, but does not induce osteophytes. CTGF is likely to play a role in osteophyte formation in OA at all, it is clear that CTGF needs another factor to start the process and cannot do it alone.
Chapter 6

Difference in persistence of TGF-β or CTGF induced fibrosis

Manuscript in preparation

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Introduction

Fibrosis is characterized by excess connective tissue formation, which can occur in many organs and can lead to organ failure. One of the main players in fibrotic diseases is TGF-β. It has been shown to play a role in fibrosis in organs like kidney, liver, lung, skin, heart etcetera (1-6). TGF-β promotes extracellular matrix deposition, decreases expression of matrix metalloproteinases and increases expression of tissue inhibitors of metalloproteinases (7-9).

In murine models of osteoarthritis (OA) we have found that fibrosis is an unwanted characteristic of OA. We have shown that the growth factor TGF-β plays an important role in synovial fibrosis during experimental OA as blocking TGF-β could prevent synovial fibrosis (10). However, blocking TGF-β during OA is not an option as it is crucial for cartilage maintenance and repair (11;12). As overall blocking of TGF-β is not an option, we wanted to find another way to block the fibrosis and searched for a secondary pathway that induced fibrosis, which we could block specifically.

In search for a potential mediator of synovial fibrosis, we found that besides TGF-β, CTGF has been proposed as a major player in fibrotic diseases.

CTGF belongs to the CCN family (for CYR61, CTGF and nov) and is also known as CCN2 and fisp-12. It has been discovered in 1991 by Bradham et al as a molecule with PDGF-like biological activity (13). During development it is important for skeletal development (14). Under non-pathological conditions it is expressed during adulthood in endothelia and neurons in the cerebral cortex where it promotes angiogenesis and tissue integrity and in the female reproductive tract (13;15;16). When it is expressed in other tissues during adulthood, it is mostly due to pathology such as wound healing, vascular diseases and fibrosis (17). During repair of skin wounds and after partial hepatectomy an up regulation of TGF-β mRNA preceded elevation of CTGF, suggesting that CTGF was induced by TGF-β (18;19). CTGF can indeed be induced by TGF-β through a TGF-β response element, but can also work independent of TGF-β (20-22). CTGF can also be induced by other factors like PDGF, EGF and FGF, but this induction is very weak compared to TGF-β-dependent up regulation (18).

To investigate whether there was a role for CTGF in inducing synovial fibrosis, we previously injected an adenovirus overexpressing CTGF and found that it did induce fibrosis (23). However, in contrast to TGF-β induced fibrosis, which is persistent, CTGF overexpression resulted in fibrosis that had been resorbed by day 28. Others have also found that adenoviral expression of TGF-β induced marked fibrosis, while adenoviral expression of CTGF induced only transient fibrosis (4;24). This raised the question what was different between TGF-β and CTGF-induced fibrosis. Therefore, we assessed the effect of either TGF-β or CTGF overexpression on mRNA expression levels of various ECM components, ECM degraders, growth factors and differences in cross-linking.
We found that TGF-β induced more pronounced changes in expression of the analysed factors when compared to CTGF. TGF-β up regulated collagen type I, aggrecan, MMP3, -9, -13, ADAMTS4 and -5, TIMP1 and -2, TGF-β1 and CTGF. In addition, we found that TGF-β induced lysyl hydroxylase expression, especially LH2b, which eventually induce cross-links that are hard to degrade. These data suggest that the induction of LH2b and TIMP1 by TGF-β might contribute to the persistent nature of TGF-β induced fibrosis.

Materials and Methods

Animals
Male C57Bl/6 mice aged 12 weeks were used. Animals were kept in filtertop cages with woodchip bedding. They were fed a standard diet with tap water ad libitum. The local animal committee has approved this study.

Experimental setting
We injected murine knee joints intra-articularly with 1x10^7 pfu virus in the right knee joint, thereby transfecting the synovial lining with an adeno virus overexpressing active TGF-β (Ad-TGF-β223/225) (gift from Dr. C.D. Richards) (n=30) human CTGF (Ad-CTGF) (provided by FibroGen, Inc., South San Fransisco, CA, USA) (n=30). As a viral control Ad-del70-4 was used (n=30). The mice were sacrificed on day 3, 7 and 21 and synovial biopsies were taken for RNA isolation of synovial tissue.

Quantitative PCR
Biopsy punches were taken from synovium as previously described (time points 3, 7, and 21 after injection of the adeno virus). RNA was isolated from the tissue with an RNeasy Mini Kit (Qiagen) after which an RT-PCR was performed. Individual samples of each group were pooled and a Q-PCR was run in duplicate. A Q-PCR was run as follows: a primer mix of 1.5 μl forward primer (5 μM), 1.5 μl reverse primer (5 μM) and 4.5 μl dH2O2, was added to 12.5 μl Sybr Green. Then 5 μl cDNA was added and the Q-PCR was performed by a “7000 sequence detection system” (ABI Prism) according to manufacturers protocol. The genes that were measured and the corresponding primer sets are displayed in Table 1. Efficiencies (E) for all primer sets were determined (Table 1) using a standard curve of 5 serial cDNA dilutions in water in duplicate. Primers were accepted if the deviation from slope of the standard curve was less than 0.3 compared to the slope of GAPDH standard curve and if the melting curve showed only one product. For each primer pair non-template controls were run in duplicate. The cycle threshold value (Ct) of the genes of interest were corrected for the Ct of the reference gene GAPDH. Relative mRNA expres-

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Table 1
Primer that were used for Q-PCR.
Results

We have previously found that TGF-β-induced fibrosis was more pronounced and more persistent than CTGF-induced fibrosis. CTGF-induced fibrosis was reversed by day 28 after viral injection (23). In contrast, TGF-β induced fibrosis is still pronounced 3 months after the last of triple injections with 200 ng TGF-β and also 28 days after intra-articular injection of Ad-TGF-β (data not shown). To gain insight in the differences between the two types of fibrosis we analyzed the synovial tissue of mice injected with either Ad-CTGF or Ad-TGF-β on mRNA level. To address the question which factors are expressed differently in the synovial tissue upon exposure to high levels of TGF-β or CTGF, we evaluated the expression of several extracellular matrix components, matrix proteases, as well as growth factor expression and modulators of collagen cross linking.

First we checked whether the adenoviruses induced expression of human CTGF and porcine TGF-β. This showed that the expression of both factors was still elevated at day 21. (Table 2)

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<th>Porcine TGF-β</th>
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Table 2
Ct values of human CTGF and procine TGF-β. Mice were injected intra-articularly with an adenovirus encoding either TGF-β or CTGF or a control virus. Three, 7 and 21 days after injection of the adenovirus synovial biopsy punches were taken of which RNA was isolated. Primers detecting only viral CTGF (human, not murine) and only viral TGF-β (porcine, not murine) were used to evaluate whether expression was sustained over a period of time. Ct values were corrected for GAPDH. Higher Ct values thus represent a lower expression. Nd = not detectable. Clearly, viral CTGF was only found in synovial biopsy punches of mice injected with Ad-CTGF and viral TGF-β was only detected in synovial biopsy punches of mice injected with Ad-TGF-β. The expression levels declined in time, but were still clearly detectable by day 21.
Relative RNA expression levels of various enzymes in the synovial tissue. Mice were injected intra-articularly with an adenovirus encoding either TGF-β or CTGF. Three, 7 and 21 days after injection of the adenovirus synovial biopsy punches were taken of which RNA was isolated. Relative mRNA levels were calculated by correction for GAPDH and for control samples. TGF-β induced the highest changes on day 7, with most marked changes in expression of MMP3, MMP13 and ADAMTS4.

CTGF overexpression resulted in elevated levels of MMP3, MMP13, ADAMTS4 and ADAMTS5 after 7 days. The level of elevation does not compare to the highly elevated levels of RNA expression seen after TGF-β overexpression. TGF-β induced the highest changes on day 7, with most marked changes in expression of MMP3, MMP13 and ADAMTS4.

Oddly enough, although CTGF is known as a major player in fibrosis, and did induce increased extracellular matrix and procollagen I in synovial tissue, it did not induce major changes in relative RNA expression levels of the expected extracellular matrix components.

Figure 1
Relative RNA expression levels of various ECM components in the synovial tissue. Mice were injected intra-articularly with an adenovirus encoding either TGF-β or CTGF. Three, 7 and 21 days after injection of the adenovirus synovial biopsy punches were taken of which RNA was isolated. Relative mRNA levels were calculated by correction for GAPDH and for control samples. TGF-β elevated the expression of all of the measured ECM components. The most marked changes were observed in collagen type I expression and the drastic increase in aggrecan expression. In contrast, CTGF decreased aggrecan expression on day 21.

Extracellular Matrix: Collagen and aggrecan
To evaluate the effect of adenoviral overexpression of both growth factors on the extracellular matrix, we measured the relative RNA levels of collagen type I, II and III as well as aggrecan. Adenoviral overexpression of TGF-β resulted in up regulation of collagen type I mRNA on all days measured, with a maximum of 37.9 fold compared to controls at day 7. Collagen type II and III showed only very mild changes, whereas aggrecan expression displayed a striking increase after injection with the TGF-β adenovirus, which reached a maximum around 7 days after injection to almost 700 fold (Figure 1A). In contrast to TGF-β, and rather unexpected, CTGF did not influence the expression of collagen type I mRNA, nor did it induce any changes in collagen type III expression. It decreased the expression of collagen type II on all days with a maximum of almost 9 fold on day 7. Aggrecan expression had decreased drastically on day 21 (55 fold) (Figure 1B). Oddly enough, although CTGF is known as a major player in fibrosis, and did induce increased extracellular matrix and procollagen I in synovial tissue, it did not induce major changes in relative RNA expression levels of the expected extracellular matrix components.

Degrading enzymes
Besides inducing expression of several ECM components, TGF-β also induced elevated levels of several MMPs and ADAMTS. We measured the expression of MMP3, 9, 13 and ADAMTS 4, and 5.
Figure 4
Relative RNA expression levels of various growth factors in the synovial tissue. Mice were injected intra-articularly with an adenovirus encoding either TGF-β or CTGF. Three, 7 and 21 days after injection of the adenovirus synovial biopsy punches were taken of which RNA was isolated. Relative mRNA levels were calculated by correction for GAPDH and for control samples. Both TGF-β and CTGF induced elevated levels of TGF-β RNA, but the effect was higher after exposure to TGF-β. In addition, TGF-β overexpression resulted in elevation of CTGF RNA expression.

Expression of TIMPs
As TGF-β induced elevated levels of matrix degrading enzymes, but at the same time induced a more persistent fibrosis, we additionally evaluated the levels of RNA expression of various TIMPs. TIMPs expression was highly elevated by TGF-β overexpression with a maximum of 87.7 times normal expression by day 7. TIMPs showed a similar pattern, only in much lower values with a maximum of 10.1 times normal on day 7 (Figure 3A). CTGF also elevated TIMPs expression on day 7, but this was much lower (5.7 fold). In contrast to the other TIMPs, TIMP4 was decreased both by TGF-β and CTGF (Figure 3B).

All of these enzymes had high levels of RNA expression on day 7. Expression of MMP3 elevated to 94.4 times normal expression on day 7 and to 50 times normal expression by day 21. ADAMTS4 had increased expression on all days measured up to 73.3 times relative to controls on day 7 (Figure 2A).

Compared to TGF-β, CTGF hardly changed expression levels of any of the enzymes measured. The biggest change was observed in ADAMTS4 expression that had reached a 5.6 fold increase on day 7, but this did not linger as it had dropped below normal expression levels by day 21 (Figure 2B). Thus, although TGF-β induced a more persistent kind of fibrosis, it also induces much higher RNA levels of matrix-degrading enzymes.
Lysyl hydroxylases

As we found a difference in persistence of the fibrosis between CTGF and TGF-β, we also addressed the possibility of differences in cross-linking of the extracellular matrix between the two growth factors. Hydroxylation of lysine residues is a key step in collagen maturation and determines the nature of cross-linking. Lysyl Hydroxylase is responsible for converting lysine into hydroxylysine, thereby inducing a route leading to crosslinks that are more difficult to degrade (25). The family of lysyl hydroxylases consists of LH1, LH2, and LH3. LH2 has 2 alternative splice-variants: LH2a and LH2b. The latter was found associated with overhydroxylation. CTGF had only minimal effect on LH expression levels, whereas TGF-β induced an elevation of all LHs, with the highest levels by day 21. Strikingly, TGF-β overexpression resulted in very high levels of LH2, especially LH2b by day 21, indicating a high degree of hard-to-degrade cross-links (Figure 5).

TGF-β induced elevation in CTGF levels

When comparing TGF-β induced fibrosis to CTGF-induced fibrosis, we found that TGF-β overexpression also enhanced CTGF mRNA levels. Therefore, we measured whether this indeed translated into elevated levels of CTGF protein. Three, 7, and 14 days after Ad-TGF-β injection, we isolated the patella-synovial area of the knee joints of C57Bl/6 mice and incubated them in DMEM for 24 hours. Protein levels were measured in the 24 hour washouts. Ad-TGF-β exposure clearly induced elevation of CTGF protein levels compared to controls.

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Growth factor expression

Adenoviral overexpression of TGF-β mainly induced high relative RNA levels of endogenous TGF-β1 and endogenous CTGF. TGF-β1 had been elevated already by day 3, but was highest on day 7, where it had reached 18.5 fold compared to normal. In contrast, CTGF was induced highest on day 3, where its expression was 15.8 fold compared to normal CTGF expression. By day 7 it had decreased, but was still 5.5 fold compared to normal and it continued to decrease to 3.9 fold at day 21 (Figure 4A). Adenoviral overexpression of CTGF hardly changed any expression levels of the various growth factors that were assessed. Only a very mild increase of TGF-β1 was found on day 7 (3.9 fold) (Figure 4B).
In this paper we evaluated the differences between TGF-β and CTGF-induced synovial fibrosis on mRNA levels. We found major differences in expression of collagen type I, aggrecan, TIMP1 and LH2b on mRNA levels, which were highly up regulated by TGF-β when compared to controls and to CTGF. These changes might contribute to the more persistent nature of TGF-β induced fibrosis. Although we would have expected collagen type I up regulation by both TGF-β and CTGF, we only found a 1.3 and 1.5 fold up regulation on day 3 and day 7 respectively and 0.73 fold on day 21 after Ad-CTGF exposure. This was unexpected as collagen type I is the major component of fibrosis and has frequently been found up regulated by CTGF (21;24;26;27). In addition, we had previously found an increase in procollagen type I positive cells and an increase in extracellular matrix in the synovium upon Ad-CTGF injection (23). However, Bonniaud et al found approximately 1.5 fold increase in procollagen type I mRNA expression on day 3 and 7 after Ad-CTGF exposure and approximately 0.75 fold at day 21, which perfectly fits our results (24). In addition, they did find a 3 fold up regulation of procollagen type I mRNA at day 14. Thus, we might have missed the window in which this increased expression could have been found. In addition, the elevation in collagen type III was also minimal after CTGF overexpression, whereas TGF-β elevated, collagen type III, but also type II and aggrecan. The expression of aggrecan was elevated already on day 14. This might explain why the elevated MMP13 and ADAMTS4 mRNA levels do not interfere with the persistence of TGF-β induced fibrosis. This also might explain why CTGF-induced fibrosis is transient, whereas TGF-β-induced fibrosis lingers for months. It is still under discussion whether CTGF alone is sufficient to induce fibrotic disorders, or whether it acts as a downstream mediator of TGF-β in fibrosis induction. TGF-β overexpression results in the induction of endogenous TGF-β, thereby providing an amplifying loop, which could contribute to potentiation of the TGF-β induced effects. However, several groups have established that fibrosis induced by TGF-β alone was not persistent and that simultaneous application of CTGF and TGF-β resulted in long-term fibrotic tissue formation in skin of mice (38-40).

One must keep in mind that once TGF-β is overexpressed, CTGF is up regulated resulting in a bias comparison. In the case of TGF-β induced fibrosis as we have shown in this paper, CTGF is induced as well, already on day 3. This implicates that the fibrosis that is observed is no longer dependent fibrosis sensitivity in mice (32). In addition, TIMP1 expression has been found associated with matrix accumulation in other fibrosis disorders (4;33-35). Thus the elevation of TIMP1 might contribute to persistent fibrosis.

Van der Slot et al found an association between expression of the lysyl hydroxylase 2 isofrom LH2b and overhydroxylation of lysine residues within collagen telopeptides in fibrotic skin of patients with Systemic Sclerosis (36). They suggested that LH2b plays an important role in the irreversible accumulation of collagen in fibrosis. They also showed that fibroblasts of several fibrotic disorders had increased levels of LH2b mRNA expression (25). TGF-β was found to be able to induce LH2b in skin fibroblasts (37). Therefore we also evaluated whether there was a difference in lysyl hydroxylase expression in synovial fibrosis induced by TGF-β or CTGF. A prominent observation is that TGF-β induced high expression of LH2, especially 2b, whereas CTGF does not induce any changes in LH mRNA levels. LH2b expression will result in cross-linking of collagens that are hard to degrade. This might explain why the elevated MMP13 and ADAMTS4 RNA levels do not interfere with the persistence of TGF-β induced fibrosis. This also might explain why CTGF-induced fibrosis is transient, whereas TGF-β-induced fibrosis lingers for months. It is still under discussion whether CTGF alone is sufficient to induce fibrotic disorders, or whether it acts as a downstream mediator of TGF-β in fibrosis induction. TGF-β overexpression results in the induction of endogenous TGF-β, thereby providing an amplifying loop, which could contribute to potentiation of the TGF-β induced effects. However, several groups have established that fibrosis induced by TGF-β alone was not persistent and that simultaneous application of CTGF and TGF-β resulted in long-term fibrotic tissue formation in skin of mice (38-40).

One must keep in mind that once TGF-β is overexpressed, CTGF is up regulated resulting in a biased comparison. In the case of TGF-β induced fibrosis as we have shown in this paper, CTGF is induced as well, already on day 3. This implicates that the fibrosis that is observed is no longer induced by TGF-β alone, but also affected by CTGF.

This should not be ignored as CTGF has been shown to potentiate TGF-β signaling. In mesangial cells it has been shown that CTGF can block the negative feedback loop of TGF-β by down regulating Smad7. Smad7 prevents phosphorylation of receptor Smads that is required for TGF-β signaling. CTGF up regulates TIEG which in turn represses Smad7 transcription, thereby allowing continued TGF-β activation (41). In addition, CTGF has the ability to bind to growth factors like TGF-β, BMP and VEGF, thereby modulating their function. For example, CTGF binding to BMP4...
or VEGF prevents receptor-binding, whereas CTGF binding to TGF-β1 enhances receptor-binding (42,43). A specific CTGF-receptor has not been found, but its association with other factors seems to point towards a role in modulating effects induced by other factors rather than a direct effect. Given our previous results showing that CTGF on its own gives only very mild and transient fibrosis, it holds true that CTGF likely needs additional factors like TGF-β (or TIMP1) before having sufficient effects on its own. Moreover, we induced fibrosis with an adenovirus overexpressing CTGF under the control of a CMV-promotor. This will result in a high expression the first couple of days, but thereafter a rapid decline in expression. During “naturally” occurring fibrosis the expression of CTGF will be different. Most likely it will be a more sustained expression over a longer period of time instead of a short high expression. This prolonged expression might contribute to maintaining fibrosis rather than inducing it. This does still leave the potential of a therapeutic effect in blocking CTGF as this might block the persistent nature of the fibrosis.

We previously showed that we can prevent synovial fibrosis during OA by blocking TGF-β signaling by adeno viral overexpression of Smad7 (10). However, this was done in an experimental model of OA in which we can control the onset of fibrosis. In the clinic, patients already have fibrosis. In this case, blocking TGF-β might not be sufficient to reverse the fibrosis that already exists. It remains to be investigated whether the role for CTGF in maintaining persistence of fibrosis is big enough to provide a solid therapeutic target.

Overall, we show that the differences in persistence between TGF-β and CTGF-induced synovial fibrosis might be caused by TGF-β induction of highly elevated levels of TIMP1 and LH2b.


Chapter 7

Elevated Extracellular Matrix Production and degradation upon BMP-2 stimulation point towards a role for BMP-2 in cartilage repair and remodeling.

Submitted for publication

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**Introduction**

Cartilage damage is a major problem in joint diseases like osteoarthritis (OA) and rheumatoid arthritis (RA). As a response to cartilage injury, chondrocytes display a reparative response (1,2). Unfortunately, this response is very limited, resulting in suboptimal repair (3). Until now, reparative responses that have been induced by drilling and microfractures are unable to overcome this problem (4). They yield a new tissue, often fibrocartilage that does not compare to original cartilage in structural, biomechanical and biochemical aspects (5). Currently, in experimental settings, growth factors are utilized to promote chondrogenic differentiation in vitro. This has the potential to eventually produce cartilage that can overcome the current problems.

Bone Morphogenetic Protein-2 (BMP-2) is one of the candidate growth factors with good potential in cartilage tissue engineering as well as cartilage repair. BMP-2 belongs to the Transforming Growth Factor-β (TGF-β) superfamily, consisting of TGF-βs, growth differentiation factors, BMPs, activins, inhibins, and glial cell line-derived neurotrophic factor (6). BMPs have been identified as very potent inducers of bone, but since then it has become evident that its function is not limited to skeletal development (7). BMP-2 expression is found in mesenchymal condensation in embryonic development (8). BMP-2 is able to induce chondrogenesis in human mesenchymal stem cells in culture (9). For cartilage reparative reasons BMP-2 can be used to induce chondrogenesis by coating a scaffold with BMP-2 before implantation (10). Thereby the scaffold itself can be replaced by the original tissue. This can be combined with culturing mesenchymal stem cells or tissue specific cells on the coated scaffold to gain de novo tissue formation in the scaffold (11).

Although BMP-2 is able to induce cartilage formation, we found that the expression of BMP-2 in normal cartilage was low, but that its expression was elevated in area’s surrounding cartilage lesions and in OA cartilage (12). In addition, mechanical injury was found to up regulate BMP-2 as well as BMP-2 signaling in human cartilage explants (13). This could indicate that BMP-2 is up regulated as a reparative response, but could also indicate that BMP-2 is merely up regulated as a pathological side effect, thereby further stimulating injury. Therefore, the effect of elevated BMP-2 on healthy cartilage and in cartilage that has been damaged by exposure to IL-1 was investigated.

**Abstract**

BMP-2 has been proposed as a tool for cartilage repair and as a stimulant of chondrogenesis. In normal cartilage, BMP-2 is hardly present, whereas it is highly expressed during OA. To assess its function in cartilage, BMP-2 was over expressed in normal murine knee joints and the effects on proteoglycan synthesis and degradation were evaluated. Moreover, the contribution of BMP to repair of damage induced by IL-1 was investigated.

Ad-BMP-2 was injected intra-articularly into murine knee joints, which were isolated 3, 7 and 21 days after injection for histology, immunohistochemistry and autoradiography. In addition, patellar and tibial cartilage was isolated for RNA isolation or measurement of proteoglycan (PG) synthesis by means of 35SO4 incorporation.

To investigate the role of BMP-2 in cartilage repair, cartilage damage was induced by i.a. injection of IL-1. After 2 days Ad-BMP-2, Ad-BMP-2 + Ad-gremlin, Ad-gremlin or a control virus were injected. Whole knee joints were isolated for histology at day 4 or patellae were isolated to measure 35SO4 incorporation.

BMP-2 stimulated PG-synthesis in patellar cartilage on all days and in tibial cartilage on day 21. Aggrecan mRNA expression had increased on all days in patellar cartilage with the highest increase on day 7. Collagen type II expression showed a similar expression pattern. In tibial cartilage, collagen type II and aggrecan mRNA expression had increased on day 7 and 21.

BMP-2 overexpression also induced increased aggrecan degradation in cartilage. VDIPEN staining (indicating MMP activity) was elevated on day 3 on tibial cartilage and on day 3 and 7 in patellar cartilage, but no longer by day 21. Increased NITEGE staining (indicating aggrecanase activity) was found on day 7 and 21.

In IL-1 damaged patellar cartilage BMP-2 boosted proteoglycan synthesis. Blocking of BMP activity resulted in a decreased proteoglycan synthesis compared to IL-1 alone. This decreased proteoglycan synthesis was associated with proteoglycan depletion in the cartilage. These data show that BMP-2 boosts matrix turnover in intact and in IL-damaged cartilage. Moreover, BMP contributes to the intrinsic repair capacity of damaged cartilage. Increased matrix turnover might be functional to replace matrix molecules in the repair of a damaged cartilage matrix.
Materials and Methods

Construction of the BMP-2 adenovirus
A PCR was performed on cDNA of synovial fibroblast cells isolated from a human knee joint biopsy samples. As primers (Biolegio, Nijmegen, the Netherlands) 5’-CCCCAGCGTGAAAAGAGAC-GAC-3’ (forward primer) and 5’-AAATCTAGACTAGCGA-3’ (reverse primer) were used, thereby introducing the XbaI restriction site. The PCR-product was ligated blunt into the Srf restriction site of the PCR-Script vector (Statagene, La Jolla, CA, USA). The vector containing the product was introduced into JM109 cells via heat shock and plated on ampicillin resistant agar plates. Several colonies were cultured and the vector was isolated by miniprep (QIAGEN, Venlo, the Netherlands) according to manufacturers protocol, followed by restriction analysis. The miniprep product of one of the colonies that contained the BMP-2 PCR-product was cut with restriction enzymes XbaI and SalI (New England Biolabs, Ipswich, MA, USA). The same restriction was performed on the pShuttle-CMV vector (Statagene, La Jolla, CA, USA). Thereafter, the PCR-product that was isolated from the PCR-Script vector was ligated into the pShuttle-CMV vector using T4 DNA Ligase. (Invitrogen, Grand Island, N.Y., USA) The adenovirus was then produced with the AdEasy Adenoviral Vector System (Statagene, La Jolla, CA, USA) by co-transfection of the vector with the plasmid in N52E6 cells according to manufacturers protocol.

Construction of the gremlin adenovirus
An adenovirus overexpressing the BMP-inhibitor gremlin was constructed. Therefore, a PCR was performed on cDNA of 3T3 cells. The following primers were used: (Biolegio, Nijmegen, the Netherlands) 5’-ACCACCATGAATCGACCCGC -3’ (forward primer) and 5’- GTCAAGCGGGCACA-TTCA-3’ (reverse primer). The PCR-product was ligated blunt into the Srf restriction site of the PCR-Script vector (Statagene, La Jolla, CA, USA). The vector containing the product was introduced into JM109 cells via heat shock and plated on ampicillin resistant agar plates. Several colonies were cultured and the vector was isolated by miniprep (QIAGEN, Venlo, the Netherlands) according to manufacturers protocol, followed by restriction analysis. The miniprep product of one of the colonies that contained the gremlin PCR-product was used for PCR again in order to introduce the XhoI and XbaI restriction sites. This was performed with 5’-CGCTCGAGACCACATGAATCGACC GGCACC-GC -3’ as a forward primer and 5’- GTCTAGATGAAATGTCGCCGT-3’ as the reverse primer. The PCR product was cut with XhoI and XbaI. The same enzymes were used to cut the pShuttle-CMV vector (Statagene, La Jolla, CA, USA). The PCR-product was then ligated into the pShuttle-CMV vector using T4 DNA Ligase. (Invitrogen, Grand Island, N.Y., USA) The adenovirus was produced with the AdEasy Adenoviral Vector System (Statagene, La Jolla, CA, USA) by co-transfection of the vector with the plasmid in N52E6 cells according to manufacturers protocol.

Functional test Ad-BMP-2 and Ad-gremlin: BRE-luciferase stably transfected cell-line
The BRE-luciferase construct was obtained from Dr. ten Dijke (14). It contains a BMP Responsive Element (BRE) that drives a luciferase gene. The BRE-luciferase construct was isolated from its pGL3-Basic vector by cutting with the enzymes MluI and BamHI (New England Biolabs, Ipswich, MA, USA). The pcDNA3.1(-)/Myc-HisB vector (Invitrogen, Grand Island, N.Y., USA) was cut with the same enzymes, thereby also removing the CMV-promoter. Subsequently, the BRE-luciferase construct was cloned into the pcDNA3.1 vector. After restriction analysis confirming the correct product, 3T3 cells were transfected with polyfectamin (Invitrogen, Grand Island, N.Y., USA) and cultured with neomycin (800 μg/ml). By limiting dilution cloning, a cell-line was created. Its responsiveness was effectively tested with serial dilutions of BMP-2 (R&D Systems Inc, Minneapolis, MN, USA) in culture medium as well as a combination of BMP-2 with several concentrations of noggin (R&D Systems Inc, Minneapolis, USA).

To test the functionality of the BMP-2 adenovirus, 911 cells were transfected with Ad-BMP-2 (MOI 10). After 48 hours the supernatant of the cells was incubated with the BRE-luciferase cell line. The luciferase production had reached a maximum, thus production could not be quantified. Therefore, the supernatant of the transfected cells was diluted 25 or 125 times to measure the quantity of BMP-2 that was produced.

To test the functionality of the Ad-gremlin adenovirus 3T3 cells were transfected with Ad-gremlin (MOI 25). After 28 hours the supernatant of the cells was incubated with the BRE-luciferase cell line and a variety of known concentrations of BMP-2 protein.

Animals
Male C57Bl/6N mice (n=272) aged 8 weeks 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 study has been approved by the Local Animal Experimentation Committee, Nijmegen, The Netherlands.

Experimental design
To assess the effect of BMP-2 on normal cartilage, mice were injected intra-articularly with either Ad-BMP-2 (an adenovirus expressing human BMP-2) or Ad-luc as a control virus (an adenovirus expressing the luciferase gene) at a pfu of 2x10^6. After 3, 7 and 21 days knee joints were isolated for histology, autoradiography and immunohistochemistry (n = 30; 5 mice per group per time point); for RNA-isolation of tibial and patellar cartilage (n = 54; 9 mice per group per time point) or for measurement of proteoglycan synthesis by 35S incorporation in tibial and patellar cartilage (n = 72; 12 mice per group per time point).
In addition, the role of BMP in the intrinsic cartilage repair upon damage was investigated. Therefore, mice were injected with 6 μl solution of IL-1β (10 ng/knee) (R&D Systems, Minneapolis, USA) in 0.9% NaCl intra-articularly into the right knee joint (n = 116). Two days after IL-1 injection Ad-BMP-2 (pfu of 2x10^6), an adenovirus expressing the specific BMP-inhibitor gremlin (Ad-gremlin; pfu of 1x10^7) or a combination of both or a control virus (Ad-luc) that has been previously described (15) was injected intra-articularly into the right knee joint (pfu of 1x10^7). Four days after IL-1 injection patellae were isolated for proteoglycan synthesis measurement by 35SO_4^- incorporation (n = 92) or whole knee joints were isolated for histological assessment of proteoglycan content of patellar and tibial cartilage (n = 24). Gremlin inhibits not only BMP-2 signaling, but also that of other BMPs. Therefore, in cases where gremlin was used, BMP instead of BMP-2 was mentioned.

**Histology**
Knee joints of mice were isolated and fixed for 7 days in phosphate buffered formalin. They were decalcified for a week in 10% formic acid. Knee joints were dehydrated with an automated tissue processing apparatus (Miles Scientific Tissue-Tek VIP tissue processor, Elkhart, IN, USA) and embedded in paraffin. Frontal whole sections of 7 μm were made. Sections were used for immunohistochemistry, autoradiography, or stained with Safranin O and counterstained with Fast Green (Brunschwig, Amsterdam, The Netherlands).

**Quantitative PCR**
Patellar and tibial cartilage was stripped off the joint as previously described (16) (time points 3, 7, and 21 after injection of the adenovirus, n = 9 per group per time point). RNA was isolated from the tissue with an RNeasy Mini Kit (QIAGEN, Venlo, the Netherlands) after which an RT-PCR was performed. Individual samples of each group were pooled and a Q-PCR was run in duplicate. A Q-PCR was prepared as follows: a primer mix of 1.5 μl forward primer (5 μM), 1.5 μl reverse primer (5 μM) and 4.5 μl dH_2O, was added to 12.5 μl Sybr Green PCR master mix (Applied Biosystems, Foster City, CA, USA). Then 5 μl cDNA was added and the Q-PCR was performed by an “ABI/PRISM 7000 sequence detection system” (Applied Biosystems, Foster City, CA, USA) according to manufacturers protocol. PCR conditions were: 2 minutes at 50°C and 10 min at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C, with data collection in the last 30 seconds. In addition, for each PCR melting curves were run. The genes that were measured and the corresponding primer sets are displayed in Table 1. Efficiencies (E) for all primer sets were determined (Table 1) using a standard curve of 5 serial cDNA dilutions in water in duplicate. Primers were accepted if the deviation from slope of the standard curve was less than 0.3 compared to the slope of GAPDH standard curve and if the melting curve showed only one product. For each primer pair non-template controls were run in duplicate. The cycle threshold value (Ct) of the genes of interest were corrected for the Ct of the reference gene GAPDH. Relative mRNA expression was calculated by 2 to the power of delta Ct. Gene expression levels after transfection with BMP-2 were compared to the control virus group. If the mRNA expression was higher after BMP-2 expression, the fold change is positive, decreases in expression are negative.

**Quantitative measurement of proteoglycan synthesis**
Proteoglycan synthesis was assessed by measurement of 35SO_4^- incorporation. Isolated patellae and tibia were immediately placed in DMEM medium (Invitrogen, Grand Island, NY, USA) with gentamicin (Centrafarm Services B.V, Etten-Leur, the Netherlands) (50 mg/ml) and pyruvate (Invitrogen, Grand Island, NY, USA). After half an hour medium was replaced by medium containing 35SO_4^- (20 μCi/ml) and incubated for 3 hours at 37°C 5% CO_2. Thereafter, patellae and tibia were further prepared for determining amount of 35SO_4^- incorporation in the articular cartilage as previously described using a liquid scintillation counter (17). Cartilage from the separate surfaces of one tibia were pooled.

**Autoradiography**
For assessment of proteoglycan synthesis the amount of 35SO_4^- incorporation in cartilage was measured histologically. Mice were injected i.p. with 75 μCi radiolabelled 35SO_4^- 4 hours prior to knee joint isolation. After histological processing, sections were dipped in nuclear research emulsion (Ilford, Basildon, Essex, U.K.) and exposed for 4-8 weeks. Slides were developed in Kodak D-19 developer (Kodak, Chalon-sur-Saone, France) and counterstained with H&E.

**Immunohistochemistry: NITEGE**
Sections were deparaffinized and washed with PBS. Sections were incubated in citrate buffer (0.1 M sodium citrate + 0.1 M citric acid) for two hours for antigen unmasking. Endogenous peroxidase was blocked with 1% hydrogen peroxide in methanol for 30 minutes. Sections were blocked with 5% normal serum of the species in which the secondary antibody was produced. Specific primary antibodies were incubated overnight at 4°C. To assess degradation of aggrecan a polyclonal antibody to the aggrecan neoepitope NITEGE (1:1000) (Aggrecan Neo) (Acris, Hiddenhausen, Germany) was used. The antibody recognizes CGGNITEGE, which is an epitope revealed from aggrecan core proteins upon aggrecanase cleavage at the Glu373-Ala374 site. After extensive washing with PBS, the appropriate biotin labeled secondary antibody was used (DAKO, Glostrup, Denmark) for 30 minutes at room temperature followed by a biotin-strepta-
Results

Ad-BMP-2 and Ad-gremlin tested on stably transfected BRE-luciferase cell line

To examine the efficiency of Ad-BMP-2, 911 cells were transfected with Ad-BMP-2 at a MOI of 10. The amount of BMP-2 produced in the diluted samples was 40.16 ng/ml in the sample diluted 25 times and 8.39 ng/ml in the sample diluted 125 times. Thus, transfection with MOI 10 Ad-BMP-2 results in a production of 1 μg/ml BMP-2 biologically active protein after 48 hours.

Immunohistochemistry: VDIPEN staining

After deparaffinization of the sections, they were digested with chondroitinase ABC for 2 hours at 37°C. Then the sections were treated with 1% H2O2 in methanol for 20 minutes and subsequently washed with 0.1% Triton X-100 in PBS for 5 minutes followed by an incubation in 1.5% normal goat serum for 20 minutes. The primary antibody was affinity-purified rabbit anti-VDIPEN IgG, detecting the VDIPEN C-terminal neoepitope of aggrecan generated by MMPs (18-20). The primary antibody was incubated overnight at room temperature. As a secondary antibody biotinylated goat anti-rabbit antibody was used and detected with biotin-streptavidin-peroxidase staining (Elite kit; Vector Laboratories, Burlingame, CA, USA). Peroxidase staining was developed using nickel enhancement and counterstained with orange G (21%).

Histological scores

A blinded observer scored sections stained with Safranin O, VDIPEN, NITEGE and autoradiography. The uncalcified area of the cartilage surfaces was selected in at least 3 sections per knee joint. The computerized imaging system subsequently determined the area that stained positive and the total area that was selected. The percentage of the total area that stained positive was calculated. A computerized imaging system was used for all histological measurements (Qwin, Leica Imaging Systems Ltd., Wetzlar, Germany). The obtained values were averaged per knee joint.

Statistical analysis

Data were analyzed with a student t-test. P values smaller than 0.05 were considered significant. Error bars in all graphs display the Standard Error of the Mean. Bonferroni correction was performed in cases of multiple comparisons.
Co-incubation of several dilutions of BMP-2 protein with the supernatant of cells transfected with Ad-gremlin showed that gremlin was able to effectively block luciferase expression whereas supernatant of control virus transfected cells had no effect. Thus transfection with the Ad-gremlin adenovirus results in efficient blocking of BMP-2 (Figure 5A).

Histological appearance of cartilage
To assess the effect of BMP-2 overexpression on joint cartilage, C57Bl/6N mice were injected with either Ad-BMP-2 or Ad-luc. BMP-2 overexpression resulted in an altered appearance of chondrocytes in the cartilage. The chondrocytes that had been exposed to BMP-2 were larger than normal. In some joints this was already visible by day 3, but all joints displayed altered chondrocyte appearance by day 7 (Figure 1A-D). This was more apparent in the patella than in the tibia.

BMP-2 induces expression of aggrecan and collagen type II
On mRNA levels Ad-BMP-2 transfection induced elevated expression of the extracellular matrix molecules collagen type II and aggrecan. No changes were found in collagen type X expression. (C and D) Effect of BMP-2 overexpression on proteoglycan synthesis. Murine knee joints were injected with either Ad-BMP-2 or a control virus. Cartilage was isolated 3, 7 or 21 days after viral injection and incubated with $^{35}$SO$_4$$^2-$ after which the amount of incorporation was measured (A). In addition, other mice were injected with $^{35}$SO$_4$$^2-$ i.p. prior to knee joint isolation 3, 7 or 21 days after viral injection for autoradiography. (B) These data show that BMP-2 stimulation of cartilage results in increased synthesis proteoglycans. (Statistical analysis with a Student T-test. * = p<0.05; ** = p<0.005; *** = p<0.0005)
BMP-2 induces elevated proteoglycan synthesis

Elevated aggrecan expression was found on mRNA-level. To investigate whether this translated into an actual production of aggrecan, $^{35}$SO$_4$$^{2-}$ incorporation into patellar and tibial cartilage was assessed. The cartilage of patella and the tibia were isolated 3, 7 and 21 days after viral injection and incubated with $^{35}$SO$_4$$^{2-}$ for 3 hours. The proteoglycan synthesis was found elevated in all cartilage surfaces. In tibial cartilage, $^{35}$SO$_4$$^{2-}$ incorporation had increased significantly on day 7 with 2.5 fold compared to Ad-luc controls. In patellar cartilage, the $^{35}$SO$_4$$^{2-}$ incorporation had reached 2.6 fold by day 3, 2.5 fold on day 7 and 1.7 fold by day 21 (Figure 2C).

In addition, to evaluate whether the increase in $^{35}$SO$_4$$^{2-}$ incorporation was evenly distributed in the cartilage or incorporated in a more focal fashion autoradiography was performed. Therefore, mice were injected with $^{35}$SO$_4$$^{2-}$ prior to knee joint isolation. Autoradiography displayed the $^{35}$SO$_4$$^{2-}$ incorporated into the cartilage, which was distributed evenly along the chondrocytes in the non-calcified cartilage (Figure 1E-H). BMP-2 significantly increased proteoglycan synthesis in patellar cartilage on all days, up to almost 3 fold on day 7. The tibia also showed clear elevated proteoglycan synthesis upon stimulation with BMP-2, which had reached statistical significance by day 7. In patellar cartilage the elevated proteoglycan synthesis seemed to have reached a plateau around day 7. In the cartilage of the tibia, $^{35}$SO$_4$$^{2-}$ incorporation increased with time (Figure 2D). The data obtained with autoradiography were not exactly similar with the data obtained with in vitro $^{35}$SO$_4$$^{2-}$ incorporation. However, the data were collected in different experiments and incubation periods and conditions were different (vivo versus vitro) which could have led to a difference in pattern. In both methods a clear increase in proteoglycan synthesis was found.

Proteoglycan content

Although elevated levels of proteoglycan synthesis were found, by mere visual investigation, no differences in Safranin O staining intensity were observed between BMP-2 exposed cartilage and controls. Therefore, the Safranin O staining intensity was scored in patellar and tibial cartilage with a computerized imaging system. There was a significant 30% increase in Safranin O staining intensity in the patella on day 7. When the data of all time points were pooled a significant increase in Safranin O staining was observed. The tibial cartilage however, did not display any alterations in Safranin O staining intensity (Figure 3). This is a discrepancy with the previously found elevated proteoglycan synthesis in the tibia indicating there might be additional degradation as well.

MMP-mediated proteoglycan cleavage

To explore the possibility of elevated aggrecan degradation upon BMP-2 stimulation, paraffin sections of the knee joints isolated on day 3, 7 and 21 after Ad-BMP-2 injection and stained immunohistochemically for VDIPEN (Figure 1I-L). BMP-2 initially induced an increase in VDIPEN staining on day 7 in patellar cartilage. In tibial cartilage elevated levels of VDIPEN staining were found on day 3. A significant decrease in VDIPEN staining was observed on the medial side of the tibia on day 21 (Figure 4A).

ADAMTS-mediated proteoglycan cleavage

In addition to VDIPEN staining, NITEGE was stained as well (Figure 1M-P). NITEGE staining was lower than controls on day 3 in the cartilage on the medial side of the tibia and no differences in the lateral tibial cartilage after BMP-2 stimulation. By day 7 NITEGE staining had increased in BMP-2-treated samples with a significant 2.5 fold increase in the patella. By day 21 NITEGE staining was still significantly increased in the patella, but no differences were found in the tibia (Figure 4B).
adenovirus gave rise to an increase in proteoglycan synthesis to over 300% compared to normal
turnover proteoglycan synthesis. To investigate the role of endogenous BMP in cartilage repair,
BMP activity was inhibited by adenoviral overexpression of the BMP inhibitor gremlin. The
adenovirus overexpressing gremlin was found to efficiently block BMP activity (Figure 5A).
Gremlin expression was not only able to totally abolish the boost in proteoglycan synthesis that
was induced by BMP-2, but restrained the IL-1 related elevation in proteoglycan synthesis (Figure 5B).
To investigate the influence of the various conditions on the total proteoglycan content, the
staining intensity of the Safranin O was measured in the cartilage. The damage that had been
inflicted by IL-1 had been overcome by the natural repair of chondrocytes by day 4 (Figure 5C).
Although BMP-2 induced an increase in proteoglycan synthesis, the outcome in proteoglycan
content was comparable to the natural reparative response. However, when BMP activity was
blocked by gremlin, the natural reparative response was abolished and resulted in proteoglycan
depletion of the cartilage.

These data show that not only is BMP-2 able to boost proteoglycan synthesis in damaged
cartilage, but BMP also plays a role in the natural reparative response of chondrocytes as a
reaction to damage.
Discussion

In literature, BMP-2 is proposed as a stimulant for cartilage (re-)generation. BMP-2 is able to stimulate proteoglycan synthesis in murine cartilage and enhances collagen type II expression in chondrocytes seeded in alginate (22,23). Also in species like rats and most important, humans, BMP-2 is able to stimulate the chondrogenic phenotype on mRNA level and to stimulate cartilage extracellular matrix proteoglycan production (24,25). In this study, BMP-2 induced an increase in mRNA levels of collagen type II and aggrecan and stimulated proteoglycan synthesis up to 3-fold in vivo, both in healthy and in damaged cartilage. All these data, including current data, confirm a strong anabolic effect of BMP-2 on cartilage.

What most studies neglect to investigate is whether there is a catabolic effect of BMP on intact cartilage. Indeed BMP-2 exposure led to degradation of aggrecan as shown by the increase in MMP- and ADAMTS- mediated proteoglycan degradation. This is not necessarily negative for cartilage integrity, especially if the use of BMP-2 is intended as a stimulant of cartilage repair. In that case, it is not unlikely that old tissue has to be removed in order to provide space for the high amounts of newly synthesized extracellular matrix. The catabolic effects that were observed were temporary, as the evidence for MMP-mediated degradation was totally reversed by day 21 to levels lower than in control cartilage. ADAMTS-mediated degradation lingered, but had also been reduced more than 50% compared to day 7. The degradational response might be initial impulse of the chondrocytes to create space in the cartilage for the new tissue that will be generated. However, for BMP-2 to have a reparative effect it is crucial that the degradational properties do not exceed the production of extracellular matrix. Overall, BMP-2 increased proteoglycan content in patellar cartilage, showing that although there was degradational activity, BMP-2 had an overall anabolic effect.

The cartilage surfaces that were measured responded differently in the magnitude of their response. The conformation of the cartilage is likely to be different, as their weight-baring properties require different stiffness of the cartilage. This might also influence the properties of the chondrocytes in the cartilage, hence their response to a stimulus. However, since the nature of the response is the same, this indicates that the response that was found is predictive for different cartilage surfaces.

Although the overall effect was anabolic, an altered appearance of the chondrocytes was observed, which was expected to be an alteration towards a hypertrophic state. On PCR levels no up regulation of collagen type X was found, nor an up regulation in MMP13 expression. This indicates that the altered appearance is not a hallmark of terminal differentiation. Therefore the possibility of an altered proliferation rate causing the altered appearance was explored, poten-

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**Figure 5B + 5C**

Mice were injected i.a. with IL-1β to induce cartilage damage. After 2 days an adenovirus expressing either BMP-2, BMP-2 + gremlin or a control virus were injected. After 4 days patellae were isolated and incubated in medium with 35SO₄²⁻ to assess proteoglycan synthesis (B), or whole knee joints were isolated to measure proteoglycan content of the cartilage (C). This showed that BMP-2 boosts proteoglycan synthesis and that blocking of BMP activity results in an abrogation of the natural reparative response after cartilage damage. (B) Moreover, blocking of BMP activity with gremlin resulted in an overall outcome of proteoglycan depletion. (C) Ad-gremlin injection alone, without IL-1, has no effect (data not shown).
ially causing the cartilage to appear more cellular. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) showed no difference between BMP-2 and controls resulting in dismissal of this theory (data not shown). The chondrocytes displayed a highly increased proteoglycan production, but also a high degree of degradational activity. VDIPEN and NITEGE staining was particularly intense in the pericellular area surrounding the unusually large chondrocytes. It could be speculated that the degradational activity led to resorption of the pericellular matrix, combined with a potential activated state of the chondrocyte this could have given to the impression of cell enlargement.

BMPs are growth factors that are necessary for cartilage formation during embryonic skeletal development (26). Lack of BMP signaling in mice will result in loss of cartilage as it wears away in BMP-receptorαb deficient mice. These data show that BMP is necessary for cartilage maintenance (27). Besides cartilage maintenance, BMP-2 is also beneficial for cartilage repair. This has been demonstrated by the fact that BMP-2 stimulates cartilage repair in defects filled with collagen sponges (28). In addition, utilization of rh-BMP-2 in full thickness defects improves the properties of the newly synthesized cartilage (29). Our group previously found that BMP-2 was low in normal cartilage, but was expressed in areas surrounding cartilage damage or in osteoarthritic cartilage in mice (12). Nakase et al found a similar localization in humans (30). This indicates that BMP-2 is up regulated in injured areas. BMP up regulation was also found in other kinds of injury like in mechanically injured cartilage explants but also in chondrocytes stimulated with either IL-1 or TNF-α (13,31). In this study the importance of BMP for cartilage repair was confirmed blocking of BMP activity after IL-1 induced cartilage damage resulted in an abrogation of the natural reparative response by chondrocytes. These data confirm those of Fukui et al, who demonstrated a similar effect in chondrocytes exposed to TNF-α (32). When BMP activity was blocked by noggin during TNF-α exposure, the proteoglycan synthesis was reduced. BMP-2 is apparently necessary for cartilage integrity and improves its repair. Our group has previously shown that, like BMP, TGF-β increased proteoglycan synthesis and that blocking of TGF-β abolished the proteoglycan overshoot after IL-1 induced cartilage damage, in a similar fashion as BMPs ability to do so shown in this paper (33). Blocking either TGF-β or BMP is apparently sufficient to block the natural reparative response. This indicates that intrinsic TGF β and BMP act synergistically in IL-damaged articular cartilage. During experimental OA TGF-β signaling decreases, whereas BMP-2 expression is induced (12). Taking into account the present data, one could speculate that the increase in BMP-2 is a means of compensation for the lack of TGF-β and thus a functional response to injury. The eventual cartilage loss observed in OA shows that BMP activity alone is not sufficient to adequately protect cartilage against destruction. Overall these findings imply that the expression of BMP in OA cartilage is an anabolic response to injury in an attempt of the chondrocytes to compensate the catabolic effects of both cytokine induced and mechanically induced injury. The BMP-2-induced elevated degradational activity is most likely an attempt to clear away old matrix molecules to make room for the newly synthesized molecules, indicating a role for BMP-2 in cartilage remodeling. Alternatively it can be that the newly synthesized aggrecan molecules are more vulnerable for degradation leading to increased presence of VDIPEN and NITEGE epitopes in the BMP-2 exposed cartilage.

Conclusions

These data show that BMP-2 exposure resulted in a strong stimulation of proteoglycan synthesis, both in healthy and in damaged cartilage. Blocking endogenous BMP activity compromised cartilage repair. Moreover, BMP-2 clearly elevated degradation of aggrecan, mediated by MMP’s and ADAMTS. Thus, BMP activity appears to be involved in cartilage repair and the replacement of damaged matrix molecules.

Reference List


Chapter 8

Osteophytes in Experimental Osteoarthritis Resemble TGF-β-induced Osteophytes. Limited role of BMP in Early Osteoarthritic Osteophyte Formation.

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Introduction

Osteoarthritis (OA) is a major problem amongst the ageing population. The main characteristics are cartilage degeneration, synovial fibrosis, and osteophyte formation. The formation of osteophytes is thought to be a response to either the altered joint loading or to the changed metabolic environment in osteoarthritic joints. Osteophytes are considered an attempt of the joint to redistribute the altered weight bearing by widening the loading area. Unfortunately this attempt is often a waste as the osteophytes result in joint deformation, movement inhibition and can compress neighboring tissue resulting in pain and loss of joint function. Osteophytes develop either at the insertions site of tendons (enthesophytes) or arise from the periost covering the bone. This process starts with chondrogenesis resulting in a cartilage-like structure termed a chondrophyte. Thereafter, the core of the chondrophyte undergoes hypertrophy followed by ossification (chondro-osteophyte). Eventually the entire structure turns into bone (osteophyte).

It is unknown what initiates the osteophyte formation, but it involves both mechanical and humoral stimuli. Transforming Growth Factor-β1 (TGF-β1) and Bone Morphogenetic Protein-2 (BMP-2) are factors that can induce de novo osteophyte formation in normal murine knee joints (1-4). The chondrogenic phase of osteophyte formation is accompanied by high levels of proteoglycan synthesis (3). Both TGF-β and BMP-2 are able to induce chondrogenesis in stem cells in vitro (5-8). In addition, both factors are very potent stimulators of proteoglycan synthesis in cartilage, but with different kinetics. TGF-β induces a lower, but longer lasting stimulation, while BMP-2 induces a short, but very strong increase in proteoglycan synthesis (3).

Both growth factors are expressed in osteophytes in murine OA (9). Blocking TGF-β during murine osteoarthritis has been shown to reduce osteophyte formation indicating that not only exogenous TGF-β but also endogenously produced TGF-β is able to induce osteophytes (10;11). Our group has demonstrated that TGF-β is involved in osteophyte formation during experimental OA, but the role for BMP during this process remained to be elucidated.

We set out to compare location and nature of TGF-β1 and BMP-2 induced osteophytes to those developing in experimental murine OA. We found that osteophyte formation that occurs during experimental OA resembles osteophyte formation induced by TGF-β. In this paper we show that blocking BMP activity during osteophyte development in experimental OA and induced by TGF-β could not abrogate osteophyte development indicating a very limited involvement for BMP in early osteophyte formation.

Abstract

Objective

Osteoarthritis (OA) is characterized by cartilage damage, synovial fibrosis and osteophyte formation. TGF-β and BMP-2 can both induce the formation of osteophytes during OA but their specific role in this process is unclear. Therefore, we investigated the respective contribution of TGF-β and BMP-2.

Methods

Murine knee joints were injected with Ad-TGF-β or Ad-BMP-2 and compared histologically to knee joints of murine models of OA: collagenase injection and STR/ort mice. In addition, Ad-gremlin was injected into knee joints injected with Ad-TGF-β or collagenase to further investigate the role of BMP during osteophyte formation.

Results

BMP-2 induced early osteophytes bulging out of the growth plates on the femur and growing on top of the patella, whereas TGF-β induced early osteophyte formation on the bone shaft beneath the collateral ligament on the femur, and also on top of the patella. Osteophyte formation during experimental OA closely resembled TGF-β induced osteophyte formation, but differed from the pattern induced by BMP-2.

Ad-gremlin proved to be able to totally block BMP-2 induced osteophyte formation. However, blocking BMP activity did neither inhibit TGF-β-induced nor experimental OA associated osteophyte formation.

Conclusion

We demonstrate that the role for BMP during onset of TGF-β and experimental OA-induced osteophyte formation is limited. The latter does not rule out a role for BMP during osteophyte maturation.
Materials and Methods

Construction of the BMP-2 adenovirus
A PCR was performed on cDNA of synovial fibroblast cells isolated from a human knee joint biopsy samples. As primers (Biolegio, Nijmegen, the Netherlands) we used 5’-CCCGTGAAGAGAGAC-3’ as a forward primer and 5’-AAATCTAGACTGCGA-3’ as the reversed primer, thereby introducing the XbaI restriction site. The PCR-product was ligated blunt into the Srf restriction site of the PCR-Script vector (Statagene, La Jolla, CA, USA). The vector containing the product was introduced into JM109 cells via heat shock and plated on ampicillin resistant agar plates. Several colonies were cultured and the vector was isolated by miniprep (QIAGEN, Venlo, the Netherlands) according to manufacturers protocol, followed by restriction analysis. The miniprep product of one of the colonies that contained the BMP-2 PCR-product was cut with restriction enzymes XbaI and SalI (New England Biolabs, Ipswich, MA, USA). The same restriction was performed on the pShuttle-CMV vector (Statagene, La Jolla, CA, USA). Thereafter, the PCR-product that was isolated from the PCR-Script vector was ligated into the pShuttle-CMV vector using T4 DNA Ligase. (Invitrogen, Grand Island, N.Y., USA). The adenovirus was then produced with the AdEasy Adenoviral Vector System (Statagene, La Jolla, CA, USA) by co-transfection of the vector with the plasmid in N52E6 cells according to manufacturers protocol.

Construction of the gremlin adenovirus
To investigate the involvement of BMP in osteophyte formation we needed a BMP-inhibitor. Therefore, we constructed an adenovirus that overexpressed the BMP-inhibitor gremlin. A PCR was performed on cDNA of 3T3 cells. As primers (Biolegio, Nijmegen, the Netherlands) we used 5’-ACACCATGAAATCGACGC-3’ as a forward primer and 5’-GTCAAGCAGGCGACATTCA-3’ as the reversed primer. The PCR-product was ligated blunt into the Srf restriction site of the PCR-Script vector (Statagene, La Jolla, CA, USA). The vector containing the product was introduced into JM109 cells via heat shock and plated on ampicillin resistant agar plates. Several colonies were cultured and the vector was isolated by miniprep (QIAGEN, Venlo, the Netherlands) according to manufacturers protocol, followed by restriction analysis. The miniprep product of one of the colonies that contained the gremlin PCR-product was used for PCR again in order to introduce the XhoI and XbaI restriction sites. Therefore we used the primers 5’-CCGCTCGAGACCCATGAAATCGACGC-3’ as a forward primer and 5’-GCTCTAGATGAATGTGCCCGCTTGAC-3’ as the reversed primer. The PCR product was cut with XhoI and XbaI. The same enzymes were used to cut the pShuttle-CMV vector (Statagene, La Jolla, CA, USA). Thereafter, the PCR-product was ligated into the pShuttle-CMV vector using T4 DNA Ligase (Invitrogen, Grand Island, N.Y., USA). The adenovirus was then produced with the AdEasy Adenoviral Vector System (Statagene, La Jolla, CA, USA) by co-transfection of the vector with the plasmid in N52E6 cells according to manufacturers protocol.

Animals
Male C57Bl/6N mice or male STR/ort mice 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 study has been approved by the Local Animal Experimentation Committee, Nijmegen, The Netherlands.

Testing Ad-BMP-2 and Ad-gremlin in vivo
To test the ability of the BMP-2 adenovirus to induce osteophytes as expected from previous results with BMP-2 protein and the ability of the gremlin adenovirus to block the formation of these osteophytes, we injected the adenoviruses intra-articularly into murine knee joints, thereby transfecting synovial lining cells. Therefore, C57Bl/6 mice (n = 48) were injected with either a control virus, Ad-BMP-2 + control virus, Ad-BMP-2 + Ad-gremlin or Ad-gremlin + control virus at a pfu of 0.5 x10^7 per virus. Knee joints were isolated 14 and 28 days after viral injection for histology.

Animal models
Ad-TGF-β in mice
C57Bl/6N mice (n = 24) were injected intra-articularly with Ad-TGF-β223/225 (an adenovirus expressing active TGF-β1) (gift by Dr. C.D. Richards) at a pfu of 1x10^7. Knee joints were isolated 3, 7, 14 and 21 days after injection for histology.

Ad-BMP-2 in mice
C57Bl/6N mice (n = 54) were injected intra-articularly with either Ad-BMP-2 (an adenovirus expressing human BMP-2) or Ad-luc as a control virus (an adenovirus expressing the luciferase gene) at a pfu of 2x10^6. Knee joints were isolated on day 3, 7, 14, 21 or 28 for histology.

Instability-induced OA
To induce OA, C57Bl/6N mice (n = 30) were injected with collagenase (5 units) to induce joint instability. After 3, 7, 14 and 28 days knee joints were isolated for histology.
Scoring of osteophyte size
As no differences in osteophyte incidence were found upon exposure to gremlin, we set out to investigate whether we overlooked more subtle changes in osteophyte formation. Therefore, we decided to investigate whether the size of the osteophyte had been altered. Therefore, we chose to select the osteophyte on the medial side of the joint on the femur just below the collateral ligament (number 5). This osteophyte, to our experience, is almost always present in our OA models. Per joint 3 sections were scored containing the (chondro-)osteophyte on the medial side of the joint on the femur just below the collateral ligament. We captured a picture of this (chondro-)osteophyte with a computerized imaging system (Leica) and measured the area of the (chondro-)osteophyte. This was averaged per joint.

Results

Testing Ad-BMP-2 and Ad-gremlin in vivo
To verify the activity of BMP-2 we injected Ad-BMP2 intra-articularly into murine knee joints. To test whether Ad-gremlin was able to block BMP-2-induced osteophyte formation we co-injected Ad-BMP-2 and Ad-gremlin. This showed that Ad-BMP-2 injection induced osteophyte formation and that co-injection with Ad-gremlin could totally block osteophyte development induced by BMP-2 (Figure 1). Intra-articular injection of Ad-gremlin alone did not result in any effects observed by histology. RNA isolation of the transfected synovial tissue for Q-PCR showed that co-injection of the adenoviruses did not interfere with the expression of viral BMP-2 or gremlin mRNA (data not shown).

Osteophytes induced by adenoviral TGF-β overexpression
To investigate the role of BMP during TGF-β-induced osteophyte formation we evaluated if blocking BMP could abrogate the osteophyte formation induced by TGF-β. Therefore, we co-injected Ad-TGF-β with the BMP-inhibitor Ad-gremlin i.a. into knee joints of C57Bl/6 mice and compared to Ad-TGF-β + control virus, Ad-gremlin + control virus or control virus alone at a pfu of 0.5 x10^7 per virus (n=48 total). Knee joints were isolated 7 and 14 days after viral injection for histology.

Role for BMP during OA-induced osteophyte formation
To investigate the role of BMP during osteophyte formation as seen during experimental OA knee joints of C57Bl/6 mice (n = 16) were injected intra-articularly with 5 units collagenase. One day after collagenase injection either Ad-gremlin or a control virus was injected i.a. Knee joints were isolated after 14 days for histology.

Scoring of osteophyte incidence
To investigate patterns of osteophyte formation, we assessed at which locations chondrophytes, chondro-osteophytes, osteophytes and enthesophytes developed in all knee joints. These were all termed osteophytes in this scoring system. Every joint was scored for osteophyte formation at all locations. Locations are numbered 1-9 both on the medial and on the lateral side of the joint. Locations are: cruciate ligaments (1), on top of the bone of the tibia beneath the collateral ligament (2), in the collateral ligament (3), on the rim of the meniscus (4), on top of the bone of the femur beneath the collateral ligament (5), at or around the root of the collateral ligament on the femur (6), protruding from growth plate on the femur located furthest from the patella, sometimes extending towards the insert from the collateral ligament (7), protruding from the growth plate on the femur located close to the patella (8), on top of the patella (9). The percentage of mice having an osteophyte at each particular location was depicted per time point. (Figure 2)
Osteophyte induction by adenoviral expression of BMP-2

Adenoviral overexpression of BMP-2 in murine knee joints typically induced chondrophytes bulging out of the growth plate on the femur by day 3 (Figure 2). By day 7 chondrophytes were found on top of the patella and protruding from the growth plate on the tibia (Figure 2). After 14 days the chondrophytes had enlarged and some had developed into chondro-osteophytes. Twenty-one days after Ad-BMP-2 injection other locations displayed chondro-osteophytes as well, with a higher incidence on the medial side of the joint. This resulted in restriction of joint movement as the chondro-osteophyte developing in the collateral ligament eventually fused to the ones in the meniscus and tibia gradually fixing the joint. By day 28 most of the chondrophytes had developed into chondro-osteophytes (Figure 1).

Osteophytes during experimental OA

To compare growth factor induced (chondro-)osteophytes to those developing during OA, we evaluated osteophyte formation in two of our OA models: the instability-induced OA model (collagenase) and the spontaneous OA model (STR/ort). After induction of instability the first chondrophytes were found beneath the collateral ligament, clearly present on day 7. At this time point we also found chondrophytes adjacent to the insertion sites of the collateral ligament. In addition, we found initial chondrogenesis in the collateral and cruciate ligaments. At day 7 apparition of osteophytes was restricted to the medial side of the joint. By day 14 chondrophytes were observed protruding from the growth plates opposite of the patella and on the patella itself both on the medial and on the lateral side of the joint. In addition, chondrogenesis on the outer border of the meniscus could be found, which evolved into a chondro-osteophyte by day 28. (Figure 2).

In the spontaneous OA model the earliest chondrophyte formation was found on the medial femur beneath the collateral ligament close to the joint space. This could be observed already in mice aged 8 weeks. In some cases chondrophyte formation adjacent to the insertion site of the collateral ligament on the femur could be seen as well. By 6 months of age and older (chondro-)osteophytes were observed at all locations, but due to the great variability between mice in this model not all mice displayed osteophyte formation and the locations varied. (Figure 2)

Some mice hardly showed any alterations by 1 year of age whereas others had completely deformed joints.

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**Figure 1**

Histology of knee joints injected with a control virus (A, E, I, M, Q, U), Ad-BMP-2 + control virus (B, F, J, N, R, V), Ad-BMP-2 + Ad-gremlin (C, G, K, O, S, W) or Ad-gremlin + control virus (D, H, L, P, T, X) (n=48 total, n=12 per group). Knee joints were isolated 14 (A-D; I-L; Q-T) or 28 days (E-H; M-P; U-X) after viral injection. Sections were stained with Safranin O and Fast Green.

Panels represent the patella-femoral area (A-H), the tibial-femoral area on the medial side of the joint (I-P) and on the lateral side of the joint (Q-X). These pictures clearly demonstrate that Ad-BMP-2 induces osteophyte formation (B, F, J, N, R, V), which can totally be blocked by co-injection with Ad-gremlin (C, G, K, O, S, W).
instability-induced OA (collagenase model) (n = 30 in total, n = 10 per group), Ad-TGF-β (n = 24 in total, n = 8 per group) and Ad-BMP-2 (n = 54 in total, n = 18 per group) induced osteophytes. The numbers on the Y-axis correspond to the numbers assigned to the various locations of osteophyte formation. Bars on the left side of the Y-axis represent osteophytes on the lateral side of the joint whereas bars on the right side represent those on the medial side of the joint. Location nr 1 is a central location, therefore its depiction on the medial side is arbitrary. The incidence indicates the % of mice having an osteophyte at a particular location, regardless of the size or developmental stage of the osteophyte. This graph shows that there are differences in osteophyte formation at early developmental stages of osteophyte formation (black bars) between BMP-2 and OA-induced osteophytes, whereas TGF-β shows a great deal of overlap with OA-induced osteophytes (black rectangle). BMP-2 and TGF-β also show some overlap (dotted rectangle).

Figure 2
Locations of osteophyte formation in the murine knee joint. Top: Graphical representation of the location of early osteophyte formation corresponding to the black bars depicted in the graphs in the bottom part of the picture. Bottom: Incidence of osteophyte formation at various locations in the joint, numbered 1-9 both on the medial and on the lateral side of the joint. We evaluated spontaneous OA (STR/ort model) (aged 8 weeks (n = 18), 6 months (n = 9) and 1 year (n = 11)), instability-induced OA (collagenase model) (n = 30 in total, n = 10 per group), Ad-TGF-β (n = 24 in total, n = 8 per group) and Ad-BMP-2 (n = 54 in total, n = 18 per group) induced osteophytes. The numbers on the Y-axis correspond to the numbers assigned to the various locations of osteophyte formation. Bars on the left side of the Y-axis represent osteophytes on the lateral side of the joint whereas bars on the right side represent those on the medial side of the joint. Location nr 1 is a central location, therefore its depiction on the medial side is arbitrary. The incidence indicates the % of mice having an osteophyte at a particular location, regardless of the size or developmental stage of the osteophyte. This graph shows that there are differences in osteophyte formation at early developmental stages of osteophyte formation (black bars) between BMP-2 and OA-induced osteophytes, whereas TGF-β shows a great deal of overlap with OA-induced osteophytes (black rectangle). BMP-2 and TGF-β also show some overlap (dotted rectangle).
Comparison of osteophyte development

To compare osteophyte patterns, the incidence of osteophyte formation at all known locations of osteophyte formation in the murine knee joint were scored regardless of developmental stage of the osteophyte (Figure 2).

When comparing the pattern of early osteophyte formation we observed that the location of origin of the osteophytes induced by TGF-β were very similar to those during experimental OA. Hardly any overlap between BMP-2 and OA-induced early osteophyte formation was observed (Figure 2). We found that at late time points almost every location showed osteophytes in some stage of development, some in chondrophyte phase, others already turned into bone (Figure 2).

The differences that were found at early stages of osteophyte formation were no longer apparent at later stages.

These data suggest that early osteophyte formation as seen during experimental OA more closely resembled that of TGF-β induced osteophyte formation, but is not comparable to BMP-2 induced osteophyte formation. This indicates that the role for BMP-2 in early osteophyte development during OA might be very limited.

Involvement of BMP in TGF-β induced and OA-induced osteophyte formation

To further investigate the role of BMP-2, we blocked BMP activity with Ad-gremlin. As there was overlap between TGF-β and BMP-2 osteophyte formation we first investigated whether BMP activity plays a role in TGF-β induced osteophyte formation. Blocking BMP during TGF-β-induced osteophyte formation by co-injection of Ad-TGF-β with Ad-gremlin did not result in changes in the incidence of osteophyte formation (data not shown). Therefore, we decided also to investigate whether any differences were found in (chondro-)osteophyte size. We scored the size of the (chondro-)osteophyte at location 5 (see Figure 2) on the medial side of the joint 14 days after viral injection, but found no alterations either (Figure 3A). These data suggest that BMP did not play a role in the initiation of TGF-β induced osteophyte formation.

To evaluate the important question whether BMP is involved in the formation of osteophytes developing during experimental OA we blocked BMP during this process by intra-articular injection of Ad-gremlin. We found no alterations in incidence of osteophyte formation, nor did we find changes in (chondro-)osteophyte size (Figure 3B). These data strongly suggest a limited role for BMP in the early formation of OA-induced osteophytes.
In this study we compared osteophyte formation induced by TGF-β and BMP-2 to OA-induced osteophyte formation. The ability of TGF-β to induce osteophyte formation had been demonstrated by our own group earlier and was confirmed by others. We showed that TGF-β protein injections resulted in osteophyte formation and no differences were found between the 3 TGF-β isoforms. In this paper we demonstrated that there is a significant overlap in location of TGF-β and experimental OA-induced osteophyte formation. This confirms our previous findings in which we demonstrated that TGF-β plays a role in osteophyte development during experimental OA.

In contrast to TGF-β, the pattern of osteophyte formation that is displayed upon BMP-2 exposure is different from that during experimental OA. The pattern of Ad-BMP-2 induced osteophyte formation was similar to that induced by protein injections. The role for BMP during OA-induced osteophyte formation had not yet been studied. We showed that blocking BMP activity did not abrogate the osteophyte formation during experimental OA, nor did it interfere with TGF-β induced osteophyte formation. These data show that TGF-β does not need BMP to induce osteophytes and that osteophyte formation during experimental OA do not require the presence of BMP either. However, we blocked BMP during onset of osteophyte formation and therefore cannot rule out involvement of BMP in osteophyte maturation.

We could speculate that distinct cell types that are involved in osteophyte formation might cause the differences we found in location of origin of osteophyte formation. The cells that form the osteophytes induced by TGF-β and during OA are probably mesenchymal stem cells that reside in the perist. These cells are thought to be the important originating cells for osteophyte formation during OA. Also in vitro they have the capacity to condensate and form cartilage-like tissue. De Bari et al have shown that cells from human perist, even from aged individuals, are still able to undergo chondrogenesis. In contrast a typical location of osteophyte formation that was observed upon BMP-2 exposure is cartilage-like tissue bulging out of the growth plate. De Bari et al have shown that BMP-2 stimulates the cells that already have a chondrocyte-like phenotype, like those in the growth plate whereas TGF-β stimulates the cells that originate in the perist. If this holds true, this might be an explanation for the differences in time needed to develop an osteophyte. In this case TGF-β might require a cell to differentiate and condensate prior to the development of cartilaginous tissue, whereas BMP initiates outgrowth of more mature tissue, thus requiring less time to develop.

The developmental pattern of osteophyte formation starts with chondrogenic differentiation within fibrous, mesenchymal tissue followed by the appearance of fibrocartilagenous cells. Then chondrocytes appear and eventually hypertrophic chondrocytes. We often find these developmental stages within one (chondro-) osteophyte extending from the outer, fibrous layer towards the core, which is eventually turned into bone. We have shown that TGF-β expression in chondro-osteophytes is strong in the outer fibrous layer, whereas active TGF-β signaling was found also in the cartilaginous layer of the chondro-osteophyte, as observed by Smad-2P staining. These data are comparable to those of Horner et al and Dodds et al. Horner et al show abundant TGF-β and TGF-β receptor staining in the proliferating layer of the chondro-osteophyte and the staining decreases towards negative in the mineralized zone, with the exception of TGF-β2, which is abundantly expressed in all layers. Dodds et al also showed that TGF-β1 mRNA expression in chondrocytes and that the expression was lost with the progression towards calcifying cartilage. In contrast to TGF-β expression, we found that BMP-2-staining mainly resided in the pre-hypertrophic chondrocytes. Zoricic did an extensive study of osteophytes from human knee and hip joint, studying BMP-2, 3, 5 and 7 expression. They found no expression in proliferating and mature chondrocytes, only BMP-3 in the hypertrophic chondrocytes and high expression in osteoblasts. Given the information from the studies mentioned above one could speculate that both growth factors might be important for different processes within osteophyte formation. Where TGF-β seems to be very important in the initiating process, BMPs seems to be involved in later stages in the process of endochondral ossification. Thus, the osteophytes at late time points will not solely be a response to one specific growth factor stimulus. The actual role of osteophytes during the OA process is still unknown. It seems to be a way of compensating for instability, but apparently they can be a side effect of an elevated growth factor level as well. In the latter case we should focus on blocking these growth factors locally to prevent this problem. However, we have previously shown that both TGF-β and BMPs are beneficial for cartilage. We demonstrated that TGF-β expression in cartilage is correlated with healthy cartilage, and that BMP seems to play a role in cartilage repair, implicating that the blocking should be local to prevent progression of cartilage damage.


Chapter 9

Concluding Remarks
Concluding remarks

Osteoarthritis

Osteoarthritis is a complex disease of which the cause still has to be discovered. Nowadays several important players in the OA process have been identified, but until now none of them have provided a solid target for therapy. Research has revealed that genetic defects in TGF-β or downstream in the TGF-β signaling cascade can lead to OA-like or OA-related phenotypes (reviewed in chapter 1). In a contribution to unraveling the process underlying OA, we set out to investigate the role of TGF-β in this complex disease.

TGF-β in cartilage

We demonstrated that there is a correlation between loss of TGF-β signaling via Smad2P in cartilage and cartilage damage (1) (Chapter 2). Reduced TGF-β signaling is already apparent in ageing mice, which precedes OA (2) (Chapter 3). We have shown that abrogating the TGF-β signal has a functional consequence: aggravation of cartilage damage (2) (Chapter 3). In other words, lack of TGF-β makes cartilage susceptible to damage. TGF-β is a potent inducer of cartilage ECM synthesis and a very potent counteracting agent of IL-1 actions (3;4). Therefore, lack of TGF-β causes a reduction in ECM deposition and suppression of catabolic stimuli is drastically reduced. Thus, the balance between catabolic and anabolic factors that maintains cartilage integrity is shifted towards the catabolic side in OA. Not only through elevation of catabolic stimuli, but also through a dramatic decrease in anabolic stimuli, like TGF-β.

Our new findings are in line with older observations at our laboratory in which we found that reduction in TGF-β receptor expression in chondrocytes that we found during ageing, thereby making them numb to TGF-β presence (2) (Chapter 3). When OA patients come to the clinic, they already have cartilage damage. Thus it was important to see whether damaged cartilage still has the ability to respond to TGF-β supplementation. We have shown that STR/ort mice, which already have OA, still have the ability to respond to TGF-β supplementation by up regulating their proteoglycan content (6) (Chapter 4). This shows that there are ways to overcome the problem of TGF-β side-effects in the joint. However, Smad7 is not an easy tool to use in the clinic. Therefore we searched for secondary mediators that could be blocked thereby abrogating TGF-β-induced fibrosis. In many fibrotic disorders, CTGF has been proposed as a downstream mediator of TGF-β that is involved in fibrosis. Therefore, we investigated the potential role of CTGF in synovial fibrosis. We demonstrated that CTGF is able to induce synovial fibrosis on its own, but it did not compare to the magnitude and persistence of fibrosis induced by TGF-β. (9) (Chapter 5). This was most likely caused by TGF-β induced lysyl hydroxylase 2b, which causes cross links in collagen which are very hard to degrade.

In skin-fibrosis, Mori et al suggested that TGF-β is needed for the initial impulse to induce fibrosis and that CTGF is important for maintenance (10). We have found that TGF-β is expressed in OA synovium mainly in early stages, whereas in later stages of OA CTGF was more abundantly expressed (1) (Chapter 2). Thus although CTGF cannot induce persistent fibrosis on its own, its role is not canceled out by our studies. One must keep in mind that during TGF-β overexpression, there is always continuous CTGF expression. To investigate the sole contribution of TGF-β it would be necessary to block CTGF during TGF-β induced fibrosis.

Besides its role in fibrosis, CTGF has been found to have chondrogenic effects (11-16). In spite of these findings, adenoviral expression of CTGF in murine knee joints resulted in reduction of proteoglycan content of the cartilage indicating deleterious effects (9). Moreover, CTGF overexpression did not induce osteophyte formation (9) (Chapter 5). Although it is still possible that CTGF can elicit chondrogenesis under explicit conditions, CTGF injection into murine knee joints had opposite effects. This might be due to CTGF-induced fibrosis, which can result in excretion of catabolic factors into the joint, ultimately leading to loss of proteoglycans in cartilage. If indeed CTGF mediates or aggravates TGF-β-induced fibrosis it would be very beneficial to block CTGF to get rid of the TGF-β induced fibrosis while maintaining TGF-β effects on cartilage.

Synovial fibrosis: role for TGF-β and CTGF

Besides being an important factor for maintaining cartilage integrity, TGF-β can induce pathology. TGF-β is a major player in fibrotic disorders. One of the symptoms of OA is synovial fibrosis and TGF-β has been shown not only to be able to induce synovial fibrosis de novo, but also plays a role in experimental OA-induced synovial fibrosis. We showed that it is possible to isolate the beneficial effect of TGF-β on cartilage from its fibrotic side effect on synovium by simultaneous transfection of the synovial lining with both a TGF-β and a Smad7 adenovirus. This way the synovial lining was protected from TGF-β as the cells expressed Smad7 and did not respond to TGF-β. The TGF-β that they produced was secreted into the synovial fluid and could reach the cartilage where it induced elevation of proteoglycan content, even in a murine OA-model (6) (Chapter 4). This shows that there are ways to overcome the problem of TGF-β side-effects in the joint. However, Smad7 is not an easy tool to use in the clinic. Therefore we searched for secondary mediators that could be blocked thereby abrogating TGF-β-induced fibrosis. In many fibrotic disorders, CTGF has been proposed as a downstream mediator of TGF-β that is involved in fibrosis. Therefore, we investigated the potential role of CTGF in synovial fibrosis. We demonstrated that CTGF is able to induce synovial fibrosis on its own, but it did not compare to the magnitude and persistence of fibrosis induced by TGF-β. (9) (Chapter 5). This was most likely caused by TGF-β induced lysyl hydroxylase 2b, which causes cross links in collagen which are very hard to degrade.

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BMP effects in cartilage

We found that expression of BMP-2 is low in normal cartilage, but elevated staining is seen around osteoarthritic lesions (1) (Chapter 2). We investigated what the effect of BMP-2 was on cartilage. We found that BMP-2 induced high levels of proteoglycan synthesis, but also induced increased levels of ECM breakdown. However, overall this resulted in an elevation of proteoglycan content in the cartilage. Moreover, when endogenous BMP was blocked, this led to aggravation of IL-1 induced cartilage damage. This showed that the presence of BMP-2 during OA is most likely an attempt to repair the damaged cartilage (Chapter 7).

Osteophyte formation: TGF-β versus BMP-2

TGF-β is a potent inducer of osteophytes, but so is BMP-2 (17;18). Our group has previously established that TGF-β is an important factor in OA-induced osteophyte formation (19). The role for BMP during this process has been studied in this thesis and found to be absent during the onset of osteophyte formation (Chapter 8). The osteophytes that were induced by BMP-2 showed a different pattern in location of development when compared to TGF-β and OA-induced osteophytes. It seems that BMP induces bone formation in cells that are more differentiated towards a chondrocyte-like state, whereas TGF-β can already induce bone formation in a more stem cell-like cells in the periost. We have shown that BMP is not important in the onset of TGF-β- or OA-induced osteophyte formation, but it might still play a role in the outgrowth of the osteophyte towards a bone-structure (Chapter 8). However, if osteophyte formation during human OA follows the same regimen as during experimental OA, blocking BMP is not useful as a therapy to prevent osteophyte formation.

Conclusions

We have shown that TGF-β is important for cartilage maintenance, but also for cartilage repair and that lack thereof predisposes cartilage to develop damage. This suggests that TGF-β is a potential tool for repair of OA cartilage or prevention of further degradation. However, TGF-β application also implies side-effects like fibrosis and osteophyte formation. We have shown that a potential solution to this problem is compartmentalized inhibition of TGF-β signaling, in non-articular tissues. But the application of selective inhibition is not easy. We identified secondary mediators of TGF-β effects on synovial fibrosis and osteophyte formation, but these were not the sole mediators of the eventual effects and therefore blocking these mediators is not sufficient for therapeutic applications. However, they have contributed to better understand the underlying process.

Final remarks

There are many questions raised by the “OA disease” that still seek an answer: what causes chondrocytes to lose their ability to maintain cartilage integrity? What is the factor, or are the combinations of factors, which force chondrocytes to respond? Does the cartilage break down through factors secreted elsewhere in the joint or is it the chondrocyte itself that decides it needs a change of scenery? Or maybe a combination of both? What triggers the events? Changes in synovial tissue or changes in the cartilage? Much research has to be performed to find answers to these questions. This thesis has contributed to understanding the mechanism underlying OA, and will provide a stepping-stone towards the development of new OA therapies.


Chapter 10

Nederlandse Samenvatting
Dankwoord
Curriculum Vitae
List of Publications
Osteoartritis (OA), ook wel osteoarthrose genoemd, is een veel voorkomende ziekte die met name bij de oudere bevolking voorkomt. Het kan echter ook veroorzaakt worden door gewrichtstrauma. Ouderdom en trauma zijn enkele van de bekende risicofactoren voor OA, maar de onderliggende oorzaak van het ontstaan van OA is nog onbekend. De belangrijkste symptomen van OA zijn kraakbeenschade, fibrose van het synoviale weefsel en osteofytvorming (ontstaan van nieuw bot op de botrand). De schade die aan het kraakbeen ontstaat is een groot probleem omdat kraakbeen niet de capaciteit heeft zichzelf te herstellen. Kraakbeen bevat weinig kraakbeencellen en bestaat grotendeels uit het omgevende weefsel (kraakbeennatrix). Deze kraakbeennatrix bestaat vooral uit collageen type II en proteoglycanen die samen voor de veerkrachtige en schokdempende eigenschappen van het kraakbeen zorgen. De kraakbeencellen liggen geïsoleerd in de kraakbeennatrix zonder bloedvaten en zenuwen, waardoor ze volledig afhankelijk zijn van hun omgeving: de kraakbeennatrix. En juist deze matrix is beschadigd in OA. Kraakbeencellen kunnen echter wel door groeifactoren aangezet worden tot verhoogde productie van nieuwe kraakbeennatrix. Een van de groeifactoren die dit kan doen is TGF-β (Transforming Growth Factor-β). Wanneer het signaal dat TGF-β aan de chondrocyten geeft onderbroken wordt kan dit leiden tot OA-achtige veranderingen in het kraakbeen. (1-4) (hoofdstuk 3) Hieruit blijkt dat TGF-β een duidelijke afname in TGF-β signaal waarnemen kan. Deze expressie van TGF-β en de moleculen die van belang zijn voor het TGF-β signaal (TGF-β receptor, BMP-2 en TGF-β receptor, BMP-2) kan afneemden, waardoor het uiteindelijk bij het kraakbeen terecht kan komen. Door deze cel tegelijk te transfecteren met een adenovirus dat de intracellulaire remmer van TGF-β, Smad7, tot expressie brengt, gaan deze cellen tevens TGF-β produceren en uitscheiden in de gewrichtsholte, waardoor het uiteindelijk bij het kraakbeen terecht kan komen. Dit systeem is helaas niet direct toepasbaar in de kliniek. Daarom zou het veel voordelen hebben als we secundaire mediatoren konden identificeren die door TGF-β aangezet worden tot het veroorzaken van de bijwerkingen (fibrose en osteofytvorming). In andere ziektebeelden waarin fibrose een groot probleem is, is aangetoond dat CTGF, een factor die sterk door TGF-β geïnduceerd wordt, een belangrijke speler is in het fibrose proces. Dit zou in synoviale fibrose ook het geval kunnen zijn en daarom hebben we onderzocht of CTGF in staat is synoviale fibrose te veroorzaken. Een van de belangrijkste cytokinen die in staat is kraakbeenschade te veroorzaken is Interleukine-1 (IL-1). TGF-β is normaliter in staat om IL-1 tegen te werken. (5) Bij veroudering is deze capaciteit van TGF-β echter sterk afgenomen. (6) Aangezien veroudering een risicofactor is voor OA zou deze afname van IL-1 tegen te werken. (7) Bij veroudering is deze capaciteit van TGF-β echter sterk afgenomen. (6) Aangezien veroudering een risicofactor is voor OA zou deze afname van IL-1 tegen te werken. (7) Bij veroorzaakt door een afname in fibrose in OA. Trauma. Ouderdom en trauma zijn enkele van de bekende risicofactoren voor OA, maar de onderliggende oorzaak van het ontstaan van OA is nog onbekend. De belangrijkste symptomen van OA zijn kraakbeenschade, fibrose en osteofytvorming (ontstaan van nieuw bot op de botrand). De schade die aan het kraakbeen ontstaat is een groot probleem omdat kraakbeen niet de capaciteit heeft zichzelf te herstellen. Kraakbeen bevat weinig kraakbeencellen en bestaat grotendeels uit het omgevende weefsel (kraakbeennatrix). 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Het is zelfs in staat om nieuwe osteofyten te laten ontstaan in normale muizenknieën. (hoofdstuk 5) BMP-2 in het beschadigde OA kraakbeen hoogstwaarschijnlijk een poging is tot modelleren en sterke bevordering van kraakbeenherstel. (hoofdstuk 7) Dit toont aan dat de aanwezigheid van BMP-2 aanwezig is in OA-kraakbe en. Wat de functie hiervan is is onbekend. In hoofdstuk 2 vonden we dat de groeifactor BMP-2 weinig voorkomt in normaal kraakbeen, maar juist verhoogd aanwezig is in OA-kraakbeen. Wat de functie hiervan is is onbekend. Daarom hebben we onderzocht wat BMP-2 doet op kraakbeen. (hoofdstuk 7) BMP-2 zorgde voor een verhoogde productie van proteoglycanen in het kraakbeen, maar daarnaast zorgde het ook voor verhoogde afbraak van proteoglycanen. Netto bleef er een verhoging van de proteoglycaanhoud van het kraakbeen over. Om uit te zoeken of dit mogelijk een poging kon zijn van het kraakbeen tot herstelling van onstane schade hebben we schade geïnduceerd door IL-1 in muizen kniegewrichten. Vervolgens hebben we BMP geblokkeerd met een remmer of extra BMP-2 toegevoegd. Hieruit bleek dat wanneer BMP werd gemeden, de IL-1 geïnduceerde schade aan het kraakbeen erger werd. Wanneer extra BMP-2 werd toegevoegd resulteerde dit in een sterke bevordering van kraakbeenherstel. (hoofdstuk 7) Dit toont aan dat de aanwezigheid van BMP-2 in het beschadigde OA kraakbeen hoogstwaarschijnlijk een poging is tot modelleren en herstellen van het beschadigde kraakbeen. Behalve in OA kraakbeen was BMP-2 ook sterk verhoogd aanwezig in osteofyt en. (hoofdstuk 2) Het is zelfs in staat om nieuwe osteofyt en te laten ontstaan in normale muizenknieën. (hoofdstuk 8) TGF-β is in staat sterk BMP-2 op te reguleren. BMP-2 is dus potentieel een secundaire mediator van TGF-β geïnduceerde osteofyt en speelt mogelijk ook een rol in OA-geïnduceerde osteofyt en. Om deze rol nader te studeren hebben we een vergelijk gemaakt tussen osteofyt en zoals ze ontstaan gedurende OA in onze muismodellen, TGF-β geïnduceerde osteofyt en BMP-2 geïnduceerde osteofyt en. Hieruit bleek dat de locaties waarop deze osteofyt en ontstaan verschillen tussen de verschillende condities. TGF-β en OA-geïnduceerde osteofyt en toonden veel overlap in osteofyt enpatroon, maar BMP-2-geïnduceerde osteofyt en weken sterk af. Deze ontstonden op locaties waar reeds kraakbeen-achtige cellen aanwezig waren bij de groeisij, terwijl TGF-β en OA-geïnduceerde osteofyt en vanuit het periost ontstonden. De verschillende groeifactoren sturen andere celsoorten aan tot het ontwikkelen van een osteofyt. Eerder heeft ons laboratorium al aangetoond dat TGF-β een rol speelt in OA-geïnduceerde osteofyt vorming en dat wordt door deze data nog eens onderstreept. Dit was echter nog onbekend of BMP-2 een rol speelt in TGF-β geïnduceerde osteofyt en of BMP-2 een rol speelt in OA-geïnduceerde osteofyt en. Door BMP te remmen gedurende het ontstaan van TGF-β en OA-geïnduceerde osteofyt en hebben we aangetoond dat in deze vroege fase geen rol voor BMP is weggelegd. Dit neemt echter niet weg dat BMP mogelijk belangrijk is bij het omzetten van de vroege kraakbeennachtige-osteofyt tot bot. (hoofdstuk 8) Dit toont echter aan dat het remmen van BMP niet kan zorgen voor het blokkeren van TGF-β geïnduceerde osteofyt en en dus niet als target gebruikt kan worden om positieve en negatieve TGF-β effecten te scheiden. In dit proefschrift hebben we laten zien dat TGF-β erg belangrijk is voor het beschermen van het kraakbeen, maar ook in staat is herstel van beschadigd kraakbeen te bevorderen. Om TGF-β als therapeutische interventie toe te kunnen passen moeten eerst de bijwerkingen hiervan (fibrose en osteofyt vorming) uitgeschakeld worden. Wij hebben aangetoond dat het mogelijk is om fibrose uit te schakelen en daarbij het positieve effect op het kraakbeen te behouden. Dit is echter niet direct toepasbaar in de kliniek. Daarom is er gezocht naar secundaire mediatoren van TGF-β die mogelijk uit te schakelen zijn om zo de negatieve effecten te voorkomen. CTGF en BMP-2 dragen echter onvoldoende bij aan respectievelijk TGF-β geïnduceerde osteofyt en osteofyt vorming of daarmee de negatieve bijwerkingen uit te kunnen schakelen. Onderzoek naar deze factoren heeft echter wel bijgedragen aan een beter begrip van de onderliggende mechanismen van fibrose en osteofyt vorming. Ons onderzoek toont aan dat TGF-β een belangrijke speler is in het OA proces en dat beter begrip van de werking hiervan kan bijdragen tot het ontwikkelen van therapeutische interventies. 
Reference List


Dankwoord

Hij is af! Mijn proefschrift is ECHT AF!!! Het leek erg ver weg, maar het is nu toch echt zover. De tijd is voorbij gevlogen, en dat heb ik vooral te danken aan de mensen van lab reuma. Jullie hebben ervoor gezorgd dat ik vijf jaar met erg veel plezier mijn werk kon doen!

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Curriculum Vitae


List of Publications

6. Blaney Davidson EN, Vitters EL, van Lent PLEM, van de Loo FAJ, van den Berg WB, van der Kraan PM. Elevated extracellular matrix production and degradation upon BMP-2 stimulation point towards a role for BMP-2 in cartilage repair and remodeling. (Conditionally Accepted Arthritis Research and Therapy)
7. Blaney Davidson EN, Vitters EL, van Beuningen HM, van de Loo FAJ, van den Berg WB, van der Kraan PM. Osteophytes in murine experimental osteoarthritis resemble TGF-beta-induced osteophytes. The role for BMP in early osteoarthritis osteophyte formation appears to be limited. (Accepted for publication in Arthritis & Rheumatism)
10. van der Kraan PM, Blaney Davidson EN, van den Berg WB, Change in TGF-beta signaling, from ALK5 to ALK1, during ageing as a cause of aberrant chondrocyte differentiation and osteoarthritis. (Manuscript in preparation)
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