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TGF-β signaling in cartilage homeostasis and osteoarthritis

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1. ABSTRACT

Healthy cartilage is maintained by a delicate balance between the anabolic and catabolic activities of articular chondrocytes. This involves actions of numerous cytokines and growth

factors that regulate the synthesis and degradation of extracellular matrix components which maintain the functional integrity of the joint. An imbalance between the activities of these anabolic and catabolic factors leads to cartilage degradation resulting in osteoarthritis (OA), a chronic degenerative joint disorder characterized by destruction of articular cartilage, alterations of subchondral bone and synovial fibrosis. Among the cytokines and growth factors that have been studied in the context of cartilage homeostasis and OA, transforming growth factor-beta-β (TGF-β) has emerged as an important molecule that plays a critical role in the development, growth, maintenance and repair of articular cartilage. Deregulation of its signaling and responses has been shown to be involved in OA. Several components of the TGF-β pathway, including extracellular, cell surface and intracellular molecules, display altered expression or action in OA. In this review, we discuss the regulatory mechanisms of TGF-β signaling and link these mechanisms to cartilage function, highlighting the important role of TGF-β in maintaining cartilage function and integrity. We also summarize the alterations in the molecular events of TGF-β signaling and responses that may contribute to OA progression and discuss the potential of targeting the TGF-β signaling pathway for the development of novel therapies for OA.

2. INTRODUCTION

Transforming growth factor-β (TGF-β) is a multifunctional growth factor that plays a critical role in cartilage development, homeostasis and repair (1-3). Impaired TGF-β signaling has been implicated in a variety of cartilage-related disorders including gout (4, 5), lupus (6-7), rheumatoid arthritis (8) and osteoarthritis (OA) (3, 9, 10). Although much progress in understanding the mechanism of TGF-β action in normal and OA cartilage has been made, this knowledge has not yet led to therapies that slow or reverse the progression of the disease. The purpose of this review is to highlight recent advances in understanding the role of TGF-β signaling in normal cartilage, the deregulation of TGF-β action in osteoarthritis (OA) and the potential of targeting TGF-β signaling pathway as a therapeutic strategy for the treatment of OA.

3. OVERVIEW OF TGF-β SIGNALING

3.1. TGF-β family

The TGF-β superfamily of growth factors, with more than 30 members including TGF-βs, activins and bone morphogenetic proteins (BMPs), are critical for metazoan development and homeostasis (11-13). They regulate diverse cellular processes including proliferation, differentiation, migration and apoptosis as well as extracellular matrix (ECM) production and degradation (11-14). Although the three mammalian TGF-β isoforms (TGF-β1, -β2, -β3) share significant sequence (71-79% identity) and structural similarity (15-19), the non-overlapping phenotypes of TGF-β isoform-specific null mice suggest that they have unique functions *in vivo* (20). Moreover, TGF-β isoforms display distinct spatial and temporal expression in developing tissues, regenerating tissues and in pathologic responses (21).

3.2. TGF-β synthesis and activation of latent TGF-β

TGF-β is synthesized as a homo-dimeric pro-protein (pro-TGF-β). It is cleaved in the trans-Golgi network by furin-like enzymes resulting in the formation of the mature TGF-β dimer

and the pro-peptide, also known as latency associated peptide (LAP). The LAP has a high affinity for the mature TGF-β and remains non-covalently associated with it, rendering it inactive. In most cases, this small latent complex is bound to latent TGF-β binding protein (LTBP) which forms a disulphide bond with the LAP, giving rise to the large latent complex, the most abundant secreted form. Once secreted, the large latent complex associates with the ECM by covalent cross-linking of LTBP with ECM proteins by transglutaminase (22-25).

Activation of latent TGF-β, which represents the liberation of TGF-β from its latent complex, is required for TGF-β binding to its receptors and to exert its biological effects (25). Latent TGF-β can be activated by physical processes such as acidification, alkalization and heat denaturation, as well as biological processes involving proteolysis or protein-protein interactions (22, 24-26). Many serine proteases including plasmin and thrombin, and metalloproteinases such as MMP-2, -9, -13 and -14 have been implicated in TGF-β activation (24). In addition, the matricellular protein thrombospondin-1 (TSP-1) interacts with LAP directly and is thought to activate latent TGF-β by causing a conformational change in LAP that prevents it from conferring latency to TGF-β (27). Also, the integrins, αvβ6 and αvβ8 have been shown to contribute to activation of latent TGF-β via protease-dependent and protease-independent mechanisms (26, 28, 29). Although the precise context and mechanistic details of TGF-β activation by these unrelated groups of factors are ill-defined, the process of activation is seemingly controlled in a temporal, spatial and isoform-specific manner, and is likely to be a critical mechanism for regulating TGF-β bioavailability (22, 24-26).

3.3.TGF-β receptors and signaling pathways

Bioactive TGF-β signals through a pair of transmembrane serine/threonine kinases known as the TGF-β type II (TβRII) and type I (TβRI or activin receptor-like kinase-5; ALK5) receptors (11-13, 30). TGF-β binds TβRII, a constitutively active kinase, which then phosphorylates TβRI/ALK5 resulting in activation of ALK5 kinase activity (31, 32). Activated ALK5 then phosphorylates two receptor-regulated Smad (R-Smad) proteins, Smad2 and Smad3, which bind to the common mediator Smad (co-SMAD), Smad4, and translocate to the nucleus where they interact with various co-activators, co-repressors and transcription factors to regulate gene expression (33, 34). TGF-β has also been shown to activate another TGF-β type I receptor known as ALK1 which phosphorylates three different R-Smads i.e. Smad-1, -5 and -8 (35-37). In addition to Smad signaling, TGF-β also activates several non-Smad pathways including the MAPK kinase pathways (ERK, JNK and p38), Rho-like GTPase signaling pathways and phosphatidylinositol-3-kinase (PI3K)/AKT pathways (38-40).

3.4. Regulation of TGF-β signaling

The pleiotropic effects of TGF-β suggest that its signaling activity requires tight regulation. This is achieved through the action of many extracellular and intracellular proteins that co-ordinately control TGF-β action in a spatial, temporal and cell-type dependent manner.

3.4.1. Extracellular control

The control of TGF-β signaling at the extracellular level includes regulation of TGF-β synthesis and activation of latent TGF-β. Factors regulating TGF-β expression are poorly defined, although auto-induction and hypoxia are well documented (41-47). Activation of latent TGF-β, a process believed to be strictly regulated, is controlled by a variety of factors as discussed above (24-26). In addition to LAP and LTBP, molecules such as lipoproteins have been shown to sequester TGF-β ligand into an inactive pool (48). At the cell surface level, TGF-β co-receptors including endoglin, betaglycan (type III TGF-β receptor) and CD109 are emerging as important regulators of TGF-β signaling. These molecules are critical for normal development and adult tissue homeostasis and their aberrant expression has been reported in many pathological conditions (49-52). Endoglin is a homo-dimeric single-pass transmembrane glycoprotein predominantly expressed on endothelial cells that binds TGF-β1 and TGF-β3 with high affinity in the presence of TβRII but does not bind the TGF-β2 isoform (51). In endothelial cells, this co-receptor has been reported to (i) alter the phosphorylation status of TβRII and TβRI (ALK1 and ALK5), (ii) promote TGF-β/ALK1 signaling, (iii) inhibit TGF-β/ALK5/Smad2/3 signaling and (iv) antagonize TGF-β-induced MAP kinase signaling through a mechanism that involves interaction with β-arrestin-2 (53, 54). These findings suggest that endoglin's regulation of TGF-β signaling occurs at multiple levels involving complex mechanisms. Betaglycan, a homologue of endoglin, is a ubiquitously expressed transmembrane protein that binds all three TGF-β isoforms (TGF-β1, -β2, -β3) with high affinity and enhances their binding to the signaling receptors (51, 55). Betaglycan has been shown to direct clathrin-mediated endocytosis of TGF-β types I and II receptors (56), and enhance TGF-β signaling via Smad and MAP kinase (57-59) pathways. On the other hand, betaglycan has also been reported to promote β-arrestin2-dependent TGF-β receptor internalization and down-regulation of TGF-β signaling (60). Further research is required to fully delineate the mechanisms involved in betaglycan's regulation of TGF-β signaling. CD109 is a GPI-anchored protein and member of the α2-macroglobulin/complement family previously shown to be expressed on activated T-cells and platelets, endothelial cells and a variety of human cancer cell lines (61-64). We have recently identified CD109 as a TGF-β co-receptor and a component of the TGF-β receptor system (65). CD109 binds TGF-β1 with high affinity and forms a heteromeric complex with the TGF-β signaling receptors, inhibiting Smad2/3 signaling in different cell types (65, 66). Our recent results indicate that CD109 inhibits TGF-β signaling by promoting TGF-β receptor internalization and degradation in a Smad7/Smurf2-dependent manner (66). Recent reports by others indicate that CD109 expression is upregulated in several cancers and that its expression is strictly regulated in normal tissues (50, 52, 62, 65, 67-70). Together, these studies demonstrate that endoglin, betaglycan and CD109 play critical roles in regulating TGF-β signaling in different cell types.

3.4.2. Intracellular control

Intracellularly, TGF-β signaling is regulated by a plethora of cytoplasmic proteins including FKBP12, TRIP-1, STRAP, TRAP-1, SARA, HSP90 (71) and nuclear proteins such as TGIF, c-Ski, SnoN and Evi-1 (34) that control TGF-β signaling intensity, duration and termination. The inhibitory Smads or I-Smads, Smad6 and Smad7, play critical roles in negative feedback regulation of TGF-β/BMP signaling by forming stable complexes with the activated type I receptors thereby blocking R-Smad phosphorylation (72). In addition, Smad6 and Smad7 also act as adaptor proteins for E3 ubiquitin ligases such as Smurf1 and Smurf2

that induce ubiquitination and proteosomal degradation of the activated type I receptors (73). Smad7 may also recruit protein phosphatase 1α to ALK1 to reduce TGF-β/ALK1-induced Smad1/5 phosphorylation (74).

4. TGF-β ACTION IN CARTILAGE

Articular cartilage, an avascular tissue, receives its nutrients from the synovial fluid. Chondrocytes, the only cells found in cartilage, are embedded in an extensive extracellular matrix consisting mainly of collagens and proteoglycans (75) Type II collagen makes up about 85-90% of the collagen in articular cartilage and provides tensile strength to the tissue (76, 77). Aggrecan is the major proteoglycan of articular cartilage and provides structural support by retaining water in the matrix (78). Articular cartilage has a hierarchical structure that is divided into four distinct zones: 1) superficial zone, 2) middle zone, 3) deep zone and 4) calcified cartilage zone where the cartilage interfaces with the bone (75). The zones differ in collagen organization, proteoglycan content and chondrocyte morphologies (75).

TGF-β plays multiple roles in the development, growth and maintenance of articular cartilage. TGF-β stimulates early events in chondrogenesis including chondrogenic condensation via stimulation of fibronectin synthesis and N-CAM regulation (79, 80) and chondroprogenitor cell proliferation and differentiation via Smad3-dependent activation of SOX9 transcription (81, 82). It also inhibits terminal differentiation of chondrocytes to the hypertrophic phenotype thereby blocking cartilage matrix calcification, vascular invasion, osteoblastic differentiation and ossification (3, 9), leading to the formation of articular cartilage at the end of the long bones, through a Smad3-dependent mechanism (83).

The maintenance of articular cartilage is critically dependent on a balance between the anabolic and catabolic cytokines and growth factors that it elaborates. TGF-β-mediated anabolic signaling represents a crucial aspect of articular cartilage matrix turnover and homeostasis (1, 3). Not only does TGF-β stimulate the production of ECM proteins such as type II collagen and aggrecan, but it also blocks degradation of ECM proteins by increasing production of protease inhibitors such as TIMP (1, 3). Moreover, TGF-β can counteract the catabolic effects of IL-1 and TNF-α on cartilage degradation (1, 3).

In addition to growth factors, mechanical loading plays a major role in cartilage development and homeostasis (84). Mechanical forces stimulate the synthesis of ECM proteins and are important to maintain cartilage integrity. While excessive load can damage cartilage structure, some mechanical stimulation is necessary to promote chondrogenesis. Also, cartilage that is not mechanically stimulated is known to atrophy (85). An interesting concept in current cartilage research is that chondrocytes act as mechanosensors that alter their metabolism in response to local physico-chemical changes in the microenvironment (86). Such alterations may include changes in gene expression and production of matrix degrading enzymes (86, 87). In this regard, several studies have reported an important role for TGF-β in mediating chondrocyte responses to mechanical forces. For example, shear forces were shown to increase chondrocyte proliferation (88) and stimulate expression of superficial zone protein (SZP) (89) in TGF-β-dependent manners. Accordingly, exogenous TGF-β alters the biomechanical properties of chondrocytes in terms of their 'stiffness' and 'compressibility' (90) and decreases the friction of articular cartilage (91). Moreover, TGF-β3 and mechanical factors appear to act synergistically to enhance the functional properties of

tissue-engineered articular cartilage (92-94). These studies emphasize the importance of considering mechanical factors in the context of TGF-β signaling and chondrocyte biology.

The potent effects of TGF-β on articular cartilage *in vivo* in animal models are well documented. Injection of TGF-β into the periosteum of rat or mouse femur induces chondrocyte differentiation and cartilage formation (95, 96). Local administration of TGF-β into murine knee joints stimulated articular cartilage repair (97) and healing of full thickness cartilage defects (98, 99). Also, inhibition of endogenous TGF-β during experimental OA impaired articular cartilage repair (100). In addition, expression of a dominant negative type II TGF-β receptor in cartilage resulted in OA-like phenotype in the mouse (101). Furthermore, Smad3 knockout mice develop degenerative joint disease resembling human arthritis (83) and Smad3 deficient chondrocytes exhibit enhanced BMP signaling and inappropriate terminal maturation (102). In addition, decreased TGF-β expression and Smad2 signaling are associated with loss of TGF-β protective effect during OA progression (103). Furthermore, evidence for a causal relationship between TGF-β and OA in the human is indicated by the recent identification of asporin (a proteoglycan that sequesters TGF-β in the ECM and inhibits TGF-β function) as an OA susceptibility gene (104-107). However, TGF-β also has been shown to have undesirable effects on cartilage. A number of studies have reported that TGF-β administration or overexpression in normal murine joints is associated with stimulation of osteophyte outgrowth, inflammation or synovial fibroplasia, although proteoglycan synthesis is enhanced (108-110). It is possible that normal cartilage function is dependent on a narrow range of bioactive TGF-β levels and concentrations below or above may lead to alterations in TGF-β signaling pathways and their integration, resulting in abnormal cartilage function.

5. DEREGULATION OF TGF-β SIGNALING IN OSTEOARTHRITIS

Osteoarthritis (OA) is a chronic degenerative joint disorder characterized by destruction of articular cartilage (111, 112). The etiology of OA is unknown but some of the common risk factors include aging, obesity, abnormal mechanical loading, genetics and anatomical abnormalities (113). Although cartilage degradation is a hallmark of OA, studies indicate that other components of the joint tissue, including subchondral bone and synovial membrane, play a significant role in OA pathogenesis. Indeed, subchondral bone alterations may precede cartilage degradation and contribute to the initiation and/or progression of OA by producing catabolic factors that degrade the overlying cartilage (114). Synovial inflammation and synovitis occur in early and clinical stages of OA, respectively, and are thought to be induced by cartilage matrix degradation products that are phagocytosed by synovial lining macrophages which, in turn, secrete pro-inflammatory mediators into the synovial fluid that diffuse into the cartilage, thereby creating a vicious circle of synovial inflammation and cartilage degradation (114). In addition, although moderate mechanical loading maintains the integrity of articular cartilage, both disuse and overuse can contribute to cartilage degradation (115). Thus, abnormal biochemical or biomechanical activity in cartilage, subchondral bone and synovial membrane likely contributes to the imbalance between anabolic and catabolic factors in cartilage, leading to cartilage degradation and OA (1, 3). A detailed analysis of the contributions of abnormal mechanical loading and subchondral bone alterations to the pathogenesis of OA are beyond the scope of this review and the reader is referred to some excellent recent reviews (87, 112, 114-117). The current review is centered on the role of aberrant TGF-β signaling in articular cartilage homeostasis and OA. Available information pertaining to alterations in

the components of the TGF-β signalling pathway in human OA and animal models of OA and the potential of targeting the TGF-β pathway for therapeutic intervention in OA is reviewed below and summarized in Tables 1-3.

5.1. TGF-β isoform expression

Several studies suggest that expression levels of the TGF-β isoforms (TGF-β1, -β2, -β3) are down-regulated in OA. These include reports showing that TGF-β1 protein levels are decreased in human OA cartilage as compared to normal cartilage (118, 119), that TGF-β1 and TGF-β2 mRNA levels are slightly decreased in OA cartilage in a rabbit model (120), and that TGF-β3 levels are reduced in cartilage during OA progression in both spontaneous (STR/Ort) and collagenase-induced mouse models of OA (103). However, several other studies have reported opposite results. For example, TGF-β1, -β2 and -β3 levels have been shown to be increased in human OA (121-123). In addition, TGF-β1 and -β3 levels were elevated in a papain-induced mouse model of OA (100) whereas TGF-β2 was increased in a surgically-induced model of early OA in rats (124). One possible explanation for the discrepancy in these results is that TGF-β isoform expression in OA may vary during the course of the disease, and may depend on factors such as age, gender, genetics and mechanical factors (obesity, trauma). It is possible that TGF-β isoform expression increases in the early stages OA in an attempt to counteract the catabolic effects of inflammatory cytokines such as IL-1β or TNF-α (10, 125) or as an adaptive response to the progressive loss of TGF-β receptor expression but then decreasing due to loss of TGF-β auto-induction (46). While such alterations in TGF-β isoform expression at the transcriptional level undoubtedly play a role in the deregulation of TGF-β action in OA, any changes in the activation of the secreted TGF-β can be of critical importance since TGF-β activation from its latent complexes controls TGF-β bioavailability.

5.2. TGF-β Activation

Several studies indicate that factors involved in latent TGF-β activation display altered expression in OA. Current data suggest that members of the LTBP family are differentially expressed in OA, which may lead to a decrease in TGF-β bioavailability. The expression of both LTBP-1 and LTBP-2 are upregulated in human OA cartilage (124, 126, 127) and in experimental models of OA (124, 126). In contrast, LTBP-3 knockout mice develop OA and display phenotypic changes that are similar to those of mice with impaired TGF-β signaling (128, 129). These observations demonstrating that increased LTBP-1/2 expression in humans and decreased LTBP-3 expression in mice are associated with OA are intriguing. Species-specific differences in the function of LTBP isoforms and/or their different TGF-β isoform-specificities may account for the seemingly discrepant findings (25). Interestingly, the levels of other molecules involved in the activation of latent TGF-β are also upregulated in human OA and in a variety of animal models of OA. Transglutaminase-2 (TG-2), the predominant transglutaminase subtype in hypertrophic chondrocytes are higher in knee (130, 131) and femoral (132) cartilage in human OA and in experimental OA models (124, 126, 133). Whether the enhanced TG-2 expression in OA correlates with increased TGF-β activation or LTBP cross-linking to ECM remains to be determined.

Similarly, TSP-1 levels are increased in the cartilage in mild and moderate OA, but decreased in severe OA (134). F-Spondin (Spondin-1), another member of the TSP-1 family known to activate latent TGF-β, is also elevated in human OA cartilage as compared to normal cartilage (135, 136) and in rat knee cartilage following surgical menisectomy (135). Intra-articular gene transfer of TSP-1 was shown to reduce disease progression in a collagen- or anterior cruciate ligament transection (ACLT)-induced OA in rats (137, 138). This is consistent with the notion that TSP-1 mediates latent TGF-β activation in OA cartilage and that the up-regulation of TSP-1 is an adaptive response in an attempt to increase cartilage repair. In addition, ascorbic acid, which plays a role in redox-mediated activation of latent TGF-β (139), was shown to increase collagen content as well as osteophyte formation, at least in part by TGF-β activation in a in a guinea pig OA model (140). Also, a recent report suggests that alendronate, a potent inhibitor of bone resorption, decreases osteophyte formation in the cartilage in a rat anterior cruciate ligament transection OA model, possibly by reducing activation of TGF-β via inhibition of MMP-13 expression (141).

5.3. TGF-β receptor expression

Increasing evidence indicates that TGF-β receptor expression levels are altered in OA. TβRII levels were shown to be decreased in human OA cartilage as compared to normal cartilage (119). In addition, TβRII mRNA levels in cartilage were also shown to be dramatically reduced at mid and late stages of OA in a rabbit model (120). Moreover, TβRII mRNA expression was decreased in cultured chondrocytes from OA donors as compared to chondrocytes from normal donors (142). These studies suggest that the loss of TβRII expression may contribute to the initiation and/or progression of OA. This notion is supported by the finding that a truncated, kinase-defective TβRII expressed in mouse skeletal tissue was associated terminal chondrocyte differentiation and the development of OA-like features (101). Thus, deregulation of TβRII expression levels likely represents a central component in the pathogenesis of OA.

Emerging evidence indicates that the expression of TGF-β type I receptors is also altered in OA. We have recently shown that, in addition to the canonical TGF-β type I receptor ALK5, human chondrocytes also express ALK1 and that both ALK5 and ALK1 are needed for TGF-β-induced Smad1/5 phosphorylation whereas only ALK5 is essential for TGF-β-induced Smad3 phosphorylation (37). In addition, ALK1 inhibits whereas ALK5 potentiates the expression of type II collagen and PAI-1 in chondrocytes, indicating that ALK1 and ALK5 have opposing functions in chondrocytes (37), as shown previously in endothelial cells (35, 36). More recent data suggest that although both ALK5 and ALK1 expression are decreased in mouse models of OA, ALK1 expression decreases to a lesser extent than that of ALK5, suggesting that the ratio of ALK1/ALK5 increases during OA (143). Also, ALK1 mRNA expression highly correlates with MMP-13 mRNA levels whereas ALK5 mRNA levels correlated with aggrecan and collagen type II levels in human OA cartilage (143). In addition, ALK1 has been identified as one of the genes upregulated in a mensical tear rat model of OA (126) whereas ALK5 levels were dramatically reduced in partial meniscectomy and post-surgery training rat model of OA (144). Together these data suggest that alterations in the expression of TGF-β signaling receptors (TβRII and ALK5/ALK1) play an important role in OA pathogenesis and that an increase in the

TGF-β/ALK1 pathway activation relative to that of the TGF-β/ALK5 pathway activation is likely to be a critical event in OA disease progression.

5.4. Smad2/3 signaling

Since the expression levels of TGF-β signaling receptors are altered in OA, changes in the activity of their signal mediators Smad2/3 in OA can be anticipated. It has been shown that Smad2 phosphorylation levels are reduced in cartilage during OA progression in both spontaneous- (STR/Ort) and collagenase-induced mouse models of OA (103). Also, Smad2 phosphorylation is decreased in cartilage in old mice compared to young mice (145). Although Smad3 phosphorylation was not examined in these models, a recent study has reported decreased Smad3 phosphorylation levels in the Smurf-2 transgenic mice that spontaneously develop an OA-like phenotype (146). Together, these studies suggest that OA is associated with reduced TGF-β/ALK5/Smad2/3 signaling.

The potential importance of Smad3 in OA is underscored by the finding that Smad3 knockout mice develop a degenerative joint disease resembling human OA (83) and intervertebral disc degeneration (147). Furthermore, genetic studies showing that mutations in the human Smad3 gene may be a risk factor for susceptibility to OA. Accordingly, a missense mutation in the Smad3 gene is associated with elevated serum MMP-2 and MMP-9 levels in a patient with knee OA, suggesting that mutations in the Smad3 gene may be involved in the pathogenesis of human OA (148). A recent report indicates that a single nucleotide polymorphism mapping to Smad3 intron 1 is involved in risk of both hip and knee OA in European populations (149). The effects of the missense mutation and SNP on Smad3 function remain to be determined. However, collectively these findings indicate that a decrease or loss of Smad3 signaling likely plays an important role in the pathogenesis of OA. Delineating the mechanisms involved in the deregulation of ALK5/Smad3 signaling in OA is critical for understanding the role of TGF-β in the development of OA.

5.5. Smad1/5 signaling

The hypothesis that a shift in the balance of Smad1/5 versus Smad2/3 signaling plays an important role in OA pathogenesis has recently gained attention. TGF-β signals through both these pathways in human chondrocytes with the Smad1/5 pathway opposing the Smad2/3 pathway (37). This is consistent with the findings in endothelial cells (35, 36), skin fibroblasts (150) and in chondrocyte terminal differentiation (151). The expression levels and subcellular localization *in vivo* of Smad-1, -5, and -8 in human OA were not significantly different from that of normal articular chondrocytes, although two Smad1 gene splice variants are reduced in OA cartilage (152). However, when the reported decrease in ALK5 expression and Smad2/3 signaling (see above) in OA cartilage is taken into account, a shift in the balance of Smad1/5 versus Smad2/3 signaling may occur, contributing to OA progression (9, 143). Factors that regulate the balance of signaling between these two TGF-β signaling pathways are not known. Although chondrocytes from mice null for Sulf-1 (an extracellular sulfatase up-regulated in OA cartilage) show reduced Smad1 protein expression and Smad1/5 phosphorylation (153, 154), it is not known whether this is accompanied by parallel changes in Smad2/3 signaling or whether Sulf-1 expression in human OA cartilage correlates with changes in the activation state of the ALK5/Smad2/3 and ALK1/Smad1/5 pathways. Identification of factors regulating the ALK5/Smad2/3 versus

ALK1/Smad1/5 pathway may lead to the development of strategies to increase ALK5/Smad2/3 signaling while reducing ALK1/Smad1/5 signaling.

5.6. Smad7/Smurf2 activity

As alluded to earlier, Smad7/Smurf2 mediated TGF-β receptor degradation is an important mechanism for the termination of TGF-β signaling (72, 73). The potential role of Smad7 and Smurf2 in the development of OA is beginning to be elucidated. Although no difference in Smad7 expression is detected between normal and OA cartilage in the human (155), inflammatory cytokines such as IL-1β increase Smad7 expression in human chondrocytes, leading to inhibition of TGF-β activity (156-158). Furthermore, murine cartilage displays an age-related increase in Smad7 expression (145), suggesting that Smad7 might be important in the initiation or progression of primary OA where aging is considered an important factor (9, 159). In addition, Smurf2 is increased in human OA cartilage as compared to normal cartilage (160) and Smurf2-transgenic mice spontaneously develop an OA-like phenotype that correlates with decreased TGF-β signaling and increased pSmad3 degradation (160).

5.7. Non-Smad pathways

Although the Smad pathway is central in mediating most TGF-β responses, TGF-β also activates non-Smad pathways including MAPK kinase (ERK, p38, JNK) pathways, Rho-like GTPase signaling pathways and PI3K/Akt pathways (38-40). However, the significance of the non-Smad pathways in mediating TGF-β responses in chondrocytes is largely unknown, despite the fact that such pathways have been shown to play important roles in regulating chondrocyte function (161). TGF-β-activated kinase-1 (TAK1), a MAP3 kinase activated by TGF-β, BMP and other MAP kinase signaling components, is a necessary mediator of cartilage development and function (162). It has been shown to mimic and mediate TGF-β-induced stimulation of type II collagen synthesis in chondrocytes by translational/post-translational mechanisms in a Smad3-independent manner (163). Importantly, extensive cross-talk between the non-Smad and Smad pathways in chondrocytes has been demonstrated. Activation of MAPK kinase activity by cytokines such as interleukin-1β or tumor necrosis factor-α decreases Smad3/4 DNA binding and ECM production in chondrocytes (158). In addition, ATF-2 works synergistically with Smad3 to mediate the inhibitory effect of TGF-β on chondrocyte maturation (164). Moreover, inhibition of PI3K, Akt, or mTOR blocks the ability of TGF-β to stimulate expression of the tissue inhibitor of metalloproteinase-3, an enzyme which is involved in the breakdown of ECM in normal physiological processes (161). Together, these studies demonstrate that TGF-β induced non-Smad signaling and the extensive cross-talk between the multiple signaling pathways play a critical role in maintaining cartilage function and integrity. Further research towards determining how the numerous pathways that are activated in chondrocytes in response to TGF-β are integrated to control chondrocyte behaviour is important to develop approaches to re-establish the balance of signaling that is disrupted in OA.

5.8. TGF-β co-receptors

TGF-β co-receptors such as endoglin, betaglycan and CD109 have emerged as important regulators of TGF-β signaling and responses with critical roles in diseases such as cancer

and organ fibrosis (49-51, 55, 62, 65, 165). Available evidence suggests that these co-receptors may regulate TGF-β action in chondrocytes and that alteration in their expression and activities may contribute to OA progression.

5.8.1. Endoglin: We have previously shown that endoglin is expressed in human articular cartilage *in vivo* and in primary human articular chondrocytes *in vitro* (166). More recently, we demonstrated that endoglin regulates TGF-β signaling in chondrocytes, enhancing TGF-β-induced Smad1/5 signaling and inhibiting Smad2/3 signaling and ECM production in human chondrocytes (167). Furthermore, our results show that endoglin protein levels are increased in human OA chondrocytes as compared to normal chondrocytes *in vivo* (167). This is in agreement with a large-scale gene expression study suggesting that endoglin mRNA expression is increased in OA cartilage as compared to normal cartilage in humans (127), and in a surgically induced model of early OA in rats ((Data from Gene Expression Omnibus (GEO), DataSet Record GDS2809, Reference Series: GSE8077) (124). In addition, soluble endoglin in plasma and synovial fluid correlates with progressive joint damage in knee OA, suggesting that endoglin may be a useful biomarker for determining disease severity and/or play a role in the pathogenesis of OA (168).

5.8.2. Betaglycan: Expression and function of betaglycan in cartilage are only beginning to be explored. We have previously shown that betaglycan is expressed in human chondrocytes and that it forms a complex not only with the signaling receptors but also with endoglin in a ligand- and TβRII-independent manner (166). A recent study has shown that betaglycan expression levels were similar in paired intact and damaged human OA cartilage (169) and did not change in a rat model of experimental OA (124). However, betaglycan expression was shown to be increased in response to mechanical injury to adult human articular cartilage (170) suggesting that deregulated betaglycan expression might play a role in secondary OA when joint trauma is involved. Interestingly, betaglycan expression was shown to be increased in mesenchymal stem cells (MSC) from the femur channel (171) and in trabecular bone from the iliac crest (172) of OA patients as compared to normal controls, suggesting that deregulated expression of betaglycan in non-cartilagenous tissues might play a role in OA pathogenesis.

5.8.3: CD109: This novel TGF-β co-receptor is an important regulator of TGF-β signaling, inhibiting Smad2/3 phosphorylation and enhancing TGF-β receptor degradation in other cell types (65, 173). There is limited information on the expression and function of CD109 in the cartilage. CD109 is present in the conditioned media of human articular chondrocytes in monolayer (174) and of bovine cartilage explants treated with interleukin-1β or TNF-α (175). Our group has detected CD109 in OA and normal chondrocytes in monolayer culture and in their conditioned media (Finnson and Philip, unpublished data). Recently, CD109 was detected in peripheral circulation and synovial fluid as a component of CD146-positive lymphocytes (176).

As discussed above, TGF-β activates multiple signaling pathways. It is now well documented that activation of these different pathways occurs in a context and cell-type specific manner (11-13, 177, 178). All three of the TGF-β co-receptors mentioned above have the ability to bind TGF-β and associate with the TGF-β signaling receptors to form hetero-oligomeric complexes (probably of different subtype composition) on the cell surface and modulate signaling receptor activity (51, 65). Such regulation by the co-receptors may

contribute to the activation of distinct TGF-β signaling pathways in different cell types, resulting in diverse responses of TGF-β.

6. TGF-β SIGNALING PATHWAY AS A THERAPEUTIC TARGET FOR OSTEOARTHRITIS

That several components of the TGF-β signaling pathway display altered expression in human OA cartilage and that genetic manipulation of these components in animal models leads to OA-like phenotypes suggests that the TGF-β pathway represents a suitable molecular target for therapeutic treatment of this disease. A number of studies have focused on the direct use of TGF-β as a therapeutic agent. Injection of TGF-β into the osteoarthritic temporomandibular joint of rabbits was shown to have a protective effect on articular cartilage degradation (179). In addition, TGF-β injection into IL-1 injected murine knee joints was shown to confer a protective effect against IL-1-induced articular cartilage damage (180, 181). Although repeated injections of TGF-β1 into a normal joint leads to prolonged elevation of proteoglycan synthesis and content in articular cartilage, it also induces inflammation, synovial hyperplasia and osteophyte formation (108-110). These studies indicate that although TGF-β has a beneficial effect on cartilage repair, it also has undesirable side effects. Recent reports suggest that adjuvant therapies can be used to circumvent the undesirable effects of TGF-β on cartilage repair. Thus, adenoviral delivery of Smad7 or LAP during experimental OA leads to a reduction in osteophyte formation and synovial thickening, while increasing the loss of proteoglycan in the articular cartilage, by inhibiting endogenous TGF-β action (182). Furthermore, adenoviral overexpression of TGF-β stimulates repair of IL-1- and OA-damaged cartilage and the TGF-β-induced synovial fibrosis is blocked by locally inhibiting TGF-β signaling in the synovial lining by simultaneous overexpression of Smad7 (183). These findings indicate that strategies that take advantage of the beneficial effects of TGF-β and block its adverse side effects will be a fruitful avenue for development of this molecule for OA therapy.

Strategies targeting factors that regulate latent TGF-β activation have also shown promise in pre-clinical studies. For example, intra-articular gene transfer of TSP-1 was shown to reduce disease progression in two different rat models of experimental OA (137, 138). TG inhibitors were shown to suppress calcium pyrophosphate dihydrate (CPPD) crystal formation by porcine chondrocytes (131) suggesting that this approach may be of benefit for the treatment of OA.

An alternative approach will be to target other components of the TGF-β signaling pathway in OA chondrocytes. For example, modulating the balance of signaling via ALK1 versus ALK5 might improve the cartilage repair effects of TGF-β. We have shown that ALK1 opposes ALK5/Smad2/3 signaling and collagen type II production in human chondrocytes (37), suggesting that ALK1 would interfere with the chondroprotective effects of TGF-β. Furthermore, others have shown that signaling via ALK1 but not ALK5 stimulates MMP13 expression, a hallmark of OA, in chondrocytes and that ALK5 expression correlates with type II collagen and aggrecan production whereas ALK1 expression correlates with MMP-13 expression in human OA cartilage (143). Thus, a better use of TGF-β for OA treatment will be to include strategies that simultaneously block ALK1 activity in chondrocytes. Such approaches include delivery of ALK1-specific shRNA driven by a type II collagen. Alternatively, molecules that modulate the balance of signaling via ALK1 versus ALK5 in OA

chondrocytes can be targeted. We have shown that endoglin inhibits TGF-β/ALK5/Smad2/3 signaling and ECM production and promotes TGF-β/ALK1/Smad1/5 signaling in human chondrocytes (167) suggesting that abrogating endoglin expression in OA chondrocyte would have therapeutic benefit.

The use of co-receptors to regulate specific TGF-β signaling pathways to control chondrocyte gene expression that favour repair and inhibit undesirable effects is an attractive concept. Recent evidence shows that TGF-β co-receptors can act as dual modulators of TGF-β signaling and responses (51). In addition to their membrane anchored forms, the three TGF-β receptors also exist in soluble forms due to enzymatic shedding of their ectodomain and these soluble forms have been shown to bind and sequester TGF-β (65, 184-186). Thus administration of these proteins locally to the synovial lining may inhibit TGF-β's undesirable side effects such as synovial fibroplasia as has been shown for Smad7-mediated inhibition of this process.

7. CONCLUDING REMARKS

TGF-β is an important growth factor in the development, maintenance and repair of articular cartilage. Studies to date indicate that several components of the TGF-β pathway including extracellular, cell surface and intracellular molecules display altered expression or activity in OA suggesting that they might represent potential targets for the development of novel therapeutic agents for the treatment of this disease. A schematic diagram showing the various sites for potential therapeutic intervention is depicted in Figure 1.

TGF-β has been shown to have a chondroprotective effect and its therapeutic utility might be improved by blocking its unwanted side effects by local inhibition of TGF-β activity in synovial tissues in an attempt to halt or reverse synovial fibrosis and osteophyte formation. TGF-β co-receptors such as endoglin, betaglycan and CD109, whose functional significance is only beginning to be explored in OA, have been shown to act as dual modulators of TGF-β action in cancer and other diseases (51). Application of these concepts to regulate TGF-β action in the cartilage may present exciting new possibilities to manipulate TGF-β signaling and action for the treatment of OA. Moreover, recent advances such as the discovery of a growing list of factors altered in OA including neogenin (187), Sulf-1 (154) and HIF-2α (188-191) with potential to regulate specific TGF-β signaling pathways (153, 188-193) provide new research avenues for the development of novel therapies for OA.

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