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Review

Roles of TGF-beta 1 signaling in the development of osteoarthritis

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Summary. Osteoarthritis (OA) is a degenerative joint disorder characterized by the destruction of articular cartilage, subchondral bone and other joint tissues. Although multiple growth factors and cytokines have been shown to be involved in articular cartilage degeneration and subchondral bone destruction, which eventually leads to OA, the molecular mechanisms underlying the pathogenesis of OA are largely unknown. The canonical transforming growth factor beta 1 (TGF-β1) signaling functions as one of the key factors in cartilage and bone formation, remodeling, and maintenance. However, the effects of TGF-β1 signaling on the development of OA are unclear. Numerous studies provide evidence that TGF-β1 is required for the formation of articular cartilage at early stages of joint development. In contrast, other investigations indicate that TGF-β1 may, in fact, be a factor in joint destruction. Therefore, we, in this review article, discuss the "conflicting" roles of TGF-β1 signaling in the development of OA.

Key words: TGF-β1, Articular cartilage, Knee joint, Osteoarthritis, Mouse model

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Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by the destruction of articular cartilage, subchondral bone and other joint tissues (Lane et al., 2011). Articular cartilage is a highly specialized connective tissue with a structure particularly suitable for load bearing. The tissue primarily consists of a large expanded extracellular matrix, in which chondrocytes are embedded. Two major components of the extracellular matrix are collagen and proteoglycans. Based on morphological characteristics, articular cartilage in human knee joints can be divided into four zones, including the superficial, the intermediate, the hypertrophic, and the calcified zones (Hamerman, 1989). In mice, there are two different layers, superficial (un-calcified) and basal (calcified) layers. The characteristics of articular cartilage degeneration include over-production of proteoglycans at early stages of degeneration, particularly in the pericellular matrix of chondrocytes, chondrocyte clustering, fibrillation, proteoglycan and collagen degradation, missing cartilage, and osteophyte formation (Xu et al., 2003). Results from many investigations demonstrate that multiple growth factors and cytokines play significant roles in the development and maintenance of articular cartilage. However, the function of these factors in the development of OA are largely unknown. For example, the role of transforming growth factor beta 1 (TGF-β1) in the development of OA are contradictory in many reports. In this review article we will discuss current "controversial" observations of the biological effect of the canonical TGF-β1signaling on articular cartilage in

developing (immature) and adult (mature) joints.

Many reports indicate that TGF-β1 can stimulate chondrocytes to synthesize collagen and proteoglycans *in vitro* and *in vivo* (Galera et al., 1992; van Beuningen et al., 1994; van der Kraan and van den Berg, 2012). Thus, TGF-β1 has been considered as a therapeutic reagent to treat OA. In contrast, other investigations demonstrate that TGF-β1 can induce extracellular matrix-degrading enzymes, such as serine protease HTRA1 (high temperature requirement A1) in chondrocytes (Urano et al., 2010; Xu et al., 2014). This suggests that TGF-β1 may also be a factor in joint destruction. What is the explanation for the "conflicting" roles of TGF-β1 signaling in the development of OA?

The canonical TGF-β1 signaling

TGF-β1 is a secreted homodimeric protein that initiates its diverse cellular responses by binding to cell surface receptors, known as TGF-β type I (TGFBR1) and TGF-β type II (TGFBR2) serine/threonine kinase receptors. So far seven of the TGFBR1 receptors (also termed Activin-receptor-like kinases, ALKs) and five of the TFGBR2 receptors, have been identified in vertebrates. During signal transduction, TGF-β1 is first presented to TGFBR2 through accessory receptors such as homodimeric betaglycan. Subsequently, TGFBR1 is recruited to form a TGFBR1 and TGFBR2 complex within which TGFBR2 phosphorylates TGFBR1 in the juxtamembrane region, or 'GS domain'. The activated TGFBR1 propagates the signal downstream by directly phosphorylating R-SMAD2 and 3 (receptor-regulated SMAD). The phosphorylated SMAD2/3 form the heterotrimeric complex with SMAD4 (a common-mediator SMAD). The complex is then translocated into the nucleus and interacts with other transcription factors, co-activators and co-repressors, to regulate gene expressions (Shi and Massague 2003; ten Dijke and Hill 2004). However, SMAD7 (inhibitory SMAD) acts in an opposing manner to R-SMADs and antagonizes TGF-β1 signaling. SMAD7 competes with R-SMAD2/3 for binding to the activated TGFBR1, thus inhibiting the phosphorylation of R-SMAD2/3. This, in turn, recruits the SMAD ubiquitination regulatory factor 1 (SMURF1) and SMURF2 (E3-ubiquitin ligases) to TGFBR1. Consequently, the phosphorylated TGFBR1 and 2 are degraded by the ligases, resulting in the termination of the signaling (Di Guglielmo et al., 2003; Shi and Massague, 2003). Moreover, SMAD7 can also recruit a complex of the growth arrest and DNA damage-inducible protein 34 and the catalytic subunit of protein phosphatase 1 to dephosphorylate TGFBR1 (Shi et al., 2004).

Role of TGF-β1 signaling in the articular cartilage of developing knee joints

It has been shown that TGF-β1 is required for the formation of articular cartilage in developing knee joints in mice. Results from three independent research groups demonstrate that the articular cartilage of knee joints cannot be formed properly in mice without TGF-β1. First, transgenic mice with a truncated *Tgfbr2* develop early onset OA-like knee joints. Compared with wild-type littermates, the level of the collagen type X is increased in the articular cartilage of the growth plate in transgenic mice at the age of 4 weeks. However, the level of proteoglycan is decreased in the growth plate. Skeletal defects are apparent in the transgenic mice at the age of 3 months, and the abnormalities become worse as the mice age. By 6 months of age, chondrocyte clusters and fibrillation are evident in the articular cartilage of knee joints in the transgenic mice. As degeneration progresses, hypertrophic-like chondrocytes (chondro-clasts) appear and osteophytes are formed in joints (Serra et al., 1997). It is suggested that the transgene, truncated *Tgfbr2*, acts as a dominant-negative inhibitor to the endogenous *Tgfbr2* and interrupts TGF-β1 signaling. (Serra et al., 1997). Second, *Tgfbr2* is deleted in type II-collagen expressing cells ($Tgfbr2^{Col2ER}$) in mice at 2 weeks of age. The *Tgfbr2*-deficient mice also exhibit the OA-like phenotype similar to what is seen in the *Tgfbr2*-truncated transgenic mice, including increased hypertrophic-like chondrocytes (Shen et al., 2013). Third, *Smad3* is deleted in every cell during mouse development ($Smad3^{ex8/ex8}$). Again, the *Smad3*-deficient mice develop OA-like phenotypes, including increased number of type X-collagen expressing chondrocytes in articular cartilage (Yang et al., 2001). Moreover, a human genetic study reports that a two-nucleotide deletion, 741-742del AT (a nonsense mutation), in *SMAD-3* causes early-onset OA in a human family. This mutation results in the removal of a majority of the MH2 domain of SMAD3, including the TGFBR1 target site for SMAD3 phosphorylation and the residues that are involved in complex formation of SMAD3 with SMAD4. The lack of the canonical TGF-β1 signaling causes the early-onset OA in this human family (van de Laar et al., 2011).

What is the mechanism by which the lack of TGF-β1 signaling causes early onset articular cartilage degeneration, which eventually leads to OA? Data from these *in vivo* studies suggest that TGF-β1/SMAD2/3 signaling may play a significant role in the control of chondrocyte hypertrophic differentiation in immature articular cartilage. Other independent investigations indicate that bone morphogenetic protein (BMP) signaling is enhanced and p38 is down-regulated in cartilage without TGF-β1 (Li et al., 2006, 2010). Results from *in vitro* experiments indicate that TGF-β1 can inhibit chondrocyte hypertrophy by regulating the expression of some cartilage matrix proteins and metalloproteases (Ballock et al., 1993; Dreier, 2010). In addition, the lack of TGF-β1/SMAD2/3 signaling may activate Runx2-inducible expression of matrix metalloproteinase 13 (MMP-13), which leads to the degeneration of articular cartilage (Chen et al., 2012).

All of the aforementioned results support the

argument that TGF-β1 signaling is an indispensable protector in developing knee joints against the development of OA. However, there is no information to suggest that this is also the case in the articular cartilage of mature knee joints.

Role of TGF-β1 signaling in the articular cartilage of adult knee joints

Contrary to developing knee joints, results from other investigators indicate that TGF-β1 may, in fact, be a factor in the destruction of adult knee joints. Triple TGF-β1 injections into adult mice knee joints induce osteophyte formation, suggesting a role of TGF-β1 during joint pathology (van Beuningen et al., 1994). Local injection of TGF-β1 into 30-day-old Sprague Dawley rats results in a decrease in the height of the hypertrophic zone, as well as the reduction in the volume of the collagens and number of chondrocytes. Furthermore, the chondrocytes are smaller and contain abundant rough endoplasmic reticulum. The long-term effect of the local administration of TGF-β1 on the rats is to accelerate articular cartilage maturation and aging, resulting in the early onset of OA (Itayem et al., 1999). Another study reports that the constitutive overexpression of active TGF-β1 in adult mouse knee joints causes OA-like changes. In this study, an adenoviral vector is used to over express TGF-β1 in joint tissues. This leads to OA-like pathological changes, including over-production of proteoglycans in articular cartilages, synovium hyperplasia, and chondro-osteophyte formation at the chondro-synovial junctions (Bakker et al., 2001). It is worth mentioning that increased production of proteoglycans in mature articular cartilage is not necessarily beneficial, even during the progressive process of the articular cartilage degeneration. In fact, the over-production of the proteoglycans, particularly in the pericellular matrix of chondrocytes, is one of the early pathological signs of articular cartilage degeneration (Xu et al., 2009). A study using a mouse model of Camurati-Engelmann Disease demonstrates that a high concentration of active TGF-β1 can initiate early onset OA. In this mouse model, significant proteoglycan loss is seen in the calcified articular cartilage in the mutant mice. The thickness of the calcified cartilage layer is also significantly increased, whereas the thickness of the superficial layer is decreased with hypocellularity (Zhen et al., 2013).

Inhibitors of TGFBR1 or TGFBR2 can protect the articular cartilage of knee joints in animal models of OA. For example, an inhibitor of TGFBR1 slows down the progressive process of the cartilage degeneration of knee joints in an anterior cruciate ligament transection (ACLT) mouse model of OA (Zhen et al., 2013). Another example is that OA progression is attenuated by the application of halofuginone locally and systemically in an ACLT rat model of OA (Zhen et al., 2013). It is believed that halofuginone can inhibit TGF-β1/SMAD2/3 signaling (Cui et al., 2015).

In our investigations, we found that the protein expression of TGF-β1 and p-SMAD2/3 were elevated in the articular cartilage of knee joints of both genetic and non-genetic mouse models of OA (Xu et al., 2014). The increased expression of p-SMAD2/3 was also co-localized with the up-regulated expression of a serine protease, HtrA1 (high temperature requirement A1) in the knee joints. These results suggest that the canonical TGF-β1 signaling is activated to induce HtrA1 in the mouse models of OA. Results from our *in vitro* experiments indicated that TGF-β1 induced HTRA1 in human and mouse chondrocytes. This is consistent with data from another independent research group (Urano et al., 2010). HTRA1 is the most abundant protease in human OA cartilage (Wu et al., 2007) and an increase in the expression of HTRA1 is detected in human OA synovial fluids (Grau et al., 2006). HTRA1 is one of four HTRA family members in human and mouse genomes (Zumbrunn and Trueb, 1996; Clausen et al., 2002). Substrates of HTRA1 have been identified and include decorin, biglycan, fibromodulin, aggrecans, and fibronectin (Oka et al., 2004; Tsuchiya et al., 2005). All of these molecules are pericellular components of chondrocytes in articular cartilage. Very recently, we conditionally deleted *Tgfbr2* in the articular cartilage of knee joints of mice at the age of two months. We then euthanized the mice at the age of 12 months. We found that there were no overt structural changes in the articular cartilage of the knee joints. We did not detect the presence of type X collagen in the mature articular cartilage of knee joints in the cartilage specific *Tgfbr2*-deficient mice, suggesting that the removal of *Tgfbr2* may not have any significant effect on chondrocyte differentiation in mature articular cartilages. This observation is opposite to what is seen in the immature articular cartilage. In addition, we subjected the cartilage specific *Tgfbr2*-deficient mice (two months old) to destabilization of the medial meniscus (DMM) surgery. We euthanized the mice at 8 and 16 weeks after DMM for the collection of knee joints. We found that the articular cartilage of knee joints in the *Tgfbr2*-deficient mice was protected from being degraded. To validate this observation, we also treated mice with the Tgfbr2 inhibitor losartan immediately after DMM surgery. We found that the articular cartilage of knee joints in the losartan-treated mice was protected. Based on these results, we conclude that inhibition of TGF-β1 signaling in mature articular cartilages protects knee joints against the development of OA (Chen et al., 2015).

In line with the animal model studies, numerous investigations report that the level of TGF-β1 is significantly higher in human OA tissues than in healthy articular cartilage (Schlaak et al., 1996; Kawamura et al., 2012). The activation of TGF-β1 signaling causes the up-regulated expression of MMP-13 in human OA cartilages (Aref-Eshghi et al., 2015). Results from a human genetic study demonstrate that a nucleotide change (missense mutation), 859C>T or 782C>T in SMAD3, increases the level and activity of the TGF-β1

signaling pathway in two human families associated with early-onset OA (van de Laar et al., 2011).

Taken all together, these data suggest that the activation of the TGF-β1 signaling may have detrimental effects on adult joints during the development of OA.

Plausible explanations of the "conflicting" roles of TGF-β1 signaling in the pathogenesis of OA

How can TGF-β1 signaling play "conflicting" roles in the pathogenesis of OA? The most plausible explanation is that TGF-β1 signaling acts in a developmental stage-dependent manner. In a developing joint, TGF-β1 is required for the formation of articular cartilage; however, once the joint is formed, TGF-β1 is no longer needed. Therefore, the induction of TGF-β1 signaling results in the destruction of adult joints (Fig. 1).

If this is the case, TGF-β1 must turn on different genetic programs in chondrocytes in immature and mature articular cartilages. Data from the mouse genetic studies indicate an increase in the number of hypertrophic-like cells in the calcified layer of the immature articular cartilage. Thus, it has been speculated that TGF-β1 is required for inhibition of chondrocyte hypertrophy in the immature articular cartilage. Additional studies show that the expression of type X collagen is evident in cells without TGF-β1 signaling. The lack of TGF-β1 signaling can also activate Runx2, which eventually induces MMP-13 expression (Chen et al., 2012). Based on the observation of the pre-mature chondrocyte hypertrophy during the development of OA joints, some investigators suggest that chondrocyte hypertrophy may play significant roles in the pathogenesis of OA in general (van der Kraan and van den Berg, 2012). However, a question remains as to why cells only in the calcified layer become hypertrophic. Also, there is little evidence of chondrocyte hypertrophy at the early stages of articular cartilage degeneration in animal models of OA. If chondrocyte hypertrophy is not a cause of the early onset articular cartilage degeneration, and instead is a consequence of cartilage degeneration, then, inhibition of hypertrophy cannot be a

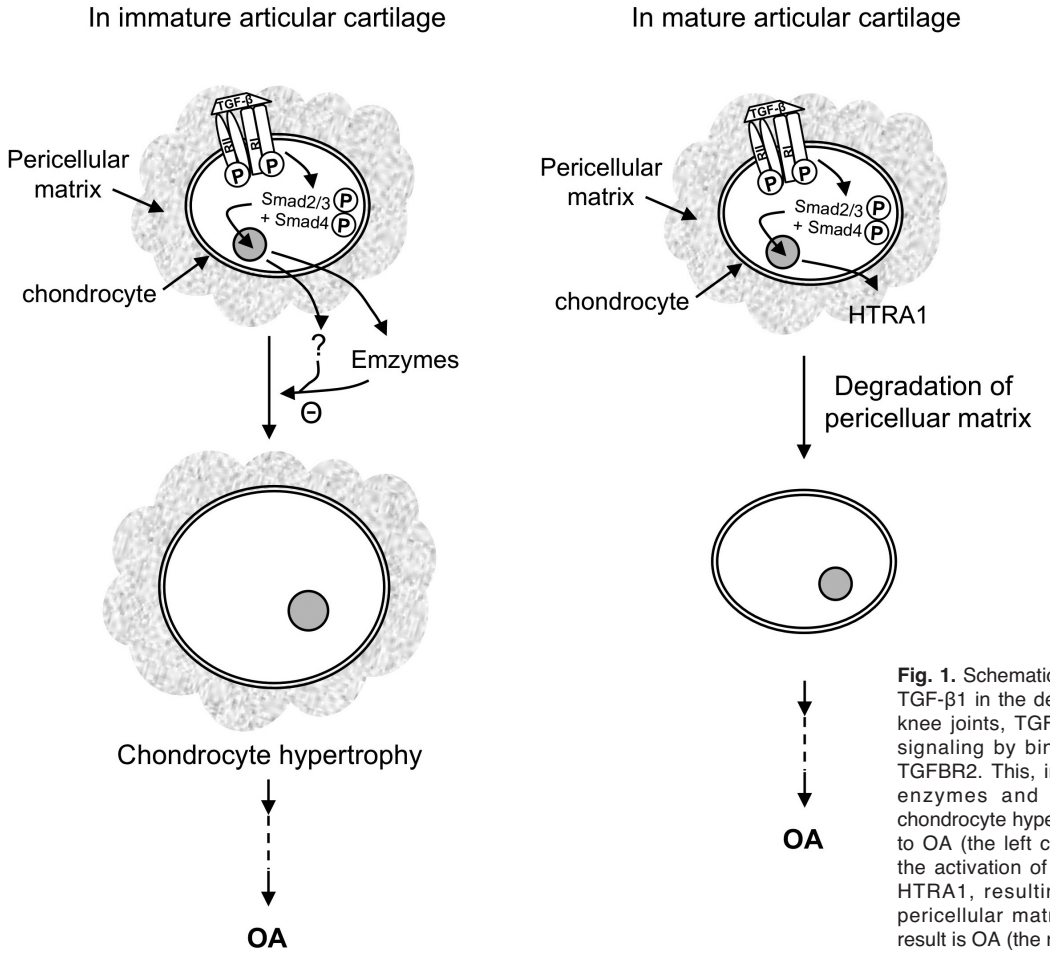

Fig. 1. Schematic illustration of possible roles of TGF-β1 in the development of OA. In immature knee joints, TGF-β1 activates the p-SMAD2/3 signaling by binding to its cognate receptor TGFBR2. This, in turn, induces expressions of enzymes and other factors, resulting in chondrocyte hypertrophy, which eventually leads to OA (the left column). In mature knee joints, the activation of p-SMAD2/3 signaling induces HTRA1, resulting in the degradation of the pericellular matrix of chondrocytes. The end result is OA (the right column).

therapeutic target for the development of disease-modifying OA drugs. The role of pre-mature chondrocyte hypertrophy in the development of OA remains to be further investigated.

In mature chondrocytes, we find that the presence of TGF-β1 is hardly detected. However, the expression of TGF-β1 and its signaling are significantly increased in OA cartilage, in particular at the early stages of development of OA in animal models. More importantly, we find that the activation of TGF-β1 signaling induces HTRA1. The enzyme degrades the pericellular matrix of chondrocytes. In normal articular cartilage, chondrocytes, pericellular matrix, and a capsule surrounding the pericellular matrix, form the primary structural and functional units of articular cartilage, termed chondrons. The capsule and the pericellular matrix separate chondrocytes from the adjacent interterritorial or territorial matrices containing type II collagen fibrils (Poole et al., 1988a-c, 1990, 1991; Hunziker et al., 1997; Poole, 1997). Results from studies of ours and other investigators demonstrate that the degradation of the pericellular matrix enhances exposure of chondrocytes to type II collagen (Polur et al., 2010) and alters chondrocyte metabolism by the interaction of the collagen with its cell surface receptor, such as discoidin domain receptor 2 (Xu et al., 2005, 2010; Sunk et al., 2007; Klatt et al., 2009; Vonk et al., 2011; Holt et al., 2012). The alteration of the chondrocyte metabolism ultimately leads to the destruction of the cartilages.

There is another plausible alternative explanation. During the development of articular cartilage in joints, multiple signaling pathways are needed to coordinate molecular events in chondrocytes. TGF-β1 signaling plays a role in conjunction with other signaling pathways. Cartilage tissue turnover is a fundamental process involved in removal of existing extracellular matrix, and deposition of neo-extracellular matrix, in the formation of articular cartilage. It is possible that TGF-β1 signaling plays a role in the removal of the matrix, and other signaling pathways act on the deposition of the matrix. Therefore, the sole activation of the TGF-β1 may cause the degeneration of articular cartilage. Obviously, further investigations are needed to elucidate the precise role of TGF-β1 signaling in the development of OA.

Conclusion

Based on results from our studies and other investigations, we suggest that the canonical TGF-β1 signaling acts in a developmental stage-dependent manner in the development of OA. In developing joints, TGF-β1 signaling is required for joint formation. However, once a joint is formed, TGF-β1 is not required in joint maintenance. In any case, the induction and activation of TGF-β1 signaling in adult joints initiate and accelerate articular cartilage degeneration, which eventually leads to OA. Inhibition, not the application, of TGF-β1 should be considered for the treatment of OA in adult joints.

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