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Osteoarthritis-related fibrosis is associated with both elevated pyridinoline cross-link formation and lysyl hydroxylase 2b expression

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SUMMARY

Objective: Fibrosis is a major contributor to joint stiffness in osteoarthritis (OA). We investigated several factors associated with the persistence of transforming growth factor beta (TGF-β)-induced fibrosis and whether these factors also play a role in OA-related fibrosis.
Design: Mice were injected intra-articularly (i.a.) with an adenovirus encoding either TGF-β or connective tissue growth factor (CTGF). In addition, we induced OA by i.a. injection of bacterial collagenase into the right knee joint of C57BL/6 mice. mRNA was isolated from the synovium for Q-PCR analysis of the gene expression of various extracellular matrix (ECM) components, ECM degraders, growth factors and collagen cross-linking-related enzymes. Sections of murine knee joints injected with Ad-TGF-β or Ad-CTGF or from experimental OA were stained for lysyl hydroxylase 2 (LH2). The number of pyridinoline cross-links per triple helix collagen in synovium biopsies was determined with high-performance liquid chromatography (HPLC).
Results: Expression of collagen alpha-1(I) chain precursor (Col1a1), tissue inhibitor of metalloproteinases 1 (TIMP1) and especially procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2b (Plod2b) were highly upregulated by TGF-β but not by CTGF. Elevated expression of Plod2b mRNA was associated with high lysyl hydroxylase 2 (LH2) protein staining after TGF-β overexpression and in experimental OA. Furthermore, in experimental OA the number of hydroxypyridinoline cross-links was significant increased compared to control knee joints.
Conclusions: Our data show that elevated LH2b expression is associated with the persistent nature of TGF-β-induced fibrosis. Also in experimental OA, LH2b expression as well as the number of hydroxypyridinoline cross-link were significantly upregulated. We propose that LH2b, and the subsequent increase in pyridinoline cross-links, is responsible for the persistent fibrosis in experimental OA.

Introduction

Important hallmarks of osteoarthritis (OA) are cartilage degeneration, osteophyte formation and fibrosis, resulting in both joint pain and stiffness. Fibrosis is characterized by excess connective tissue accumulation, which can occur in many organs and can lead to organ failure[1,2]. In OA, fibrosis is a major contributor to joint stiffening and is involved in joint pain[3]. Two main players in fibrotic diseases which are also elevated in OA are transforming growth factor beta (TGF-β) and connective tissue growth factor (CTGF)[4,5].

TGF-β controls cell proliferation, differentiation and fulfills different roles in immunity and wound healing but also plays a role in fibrosis in different organs[6–12]. We have previously shown that TGF-β plays an important role in synovial fibrosis in experimental OA, as blocking TGF-β could prevent synovial fibrosis[13]. However, blocking TGF-β in OA is not an option as it is crucial for cartilage maintenance and repair[14,15]. Besides TGF-β, CTGF has been proposed as a major player in fibrotic diseases. CTGF can be induced by TGF-β through a TGF-β response element, however it can also function independent of TGF-β[16–18]. During adulthood, CTGF is

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expressed in endothelia and neurons in the cerebral cortex where it promotes angiogenesis and tissue integrity[19–22]. When it is expressed in other tissues it is mostly associated with wound healing, vascular diseases and fibrosis[22].

By injecting an adenovirus overexpressing CTGF in murine knee joint we previously showed that CTGF can induce synovial fibrosis[23]. However, in contrast to TGF-β-induced synovial fibrosis, which is persistent, CTGF overexpression resulted in fibrosis that was resorbed by day 28. Others have also found that adenoviral expression of TGF-β induced pronouncedly prolonged fibrosis, while adenoviral expression of CTGF induced only transient fibrosis[10,24]. This raised the question: What causes this difference between TGF-β and CTGF-induced fibrosis, and specifically which factor(s) are responsible for the persistent nature of TGF-β-induced fibrosis?

Fibrosis results from an imbalance between matrix synthesis and matrix degradation. Therefore, we assessed the effect of either TGF-β or CTGF overexpression on mRNA expression levels of various matrix turnover related genes and whether these give an indication of the observed differences. Thereafter we examined whether these factor(s) play a role in OA-related fibrosis.

Here we report that lysyl hydroxylase 2b is strongly induced in TGF-β-induced persistent fibrosis and not in CTGF-induced transient fibrosis. Lysyl hydroxylases are collagen modifying and cross-linking enzymes that convert lysine into hydroxylysine, thereby leading to cross-links that make collagen harder to degrade[25–29]. Especially LH2b, which hydroxylases the telopeptides and so induces the formation of pyridinoline collagen cross-links, makes collagen harder to degrade. LH2b and the pyridinoline cross-links it induces were both significantly elevated in OA-related 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 committee on animal research and ethics has approved this study.

CTGF and TGF-β overexpression

We injected murine knee joints intra-articularly (i.a.) with $1 \times 10e^7$ pfu virus in the right knee joint, thereby transfecting the synovial lining with an adenovirus overexpressing active porcine TGF-β1 (Ad-TGF-β223/225) (gift from Dr. C.D. Richards) or human CTGF (Ad-CTGF) (FibroGen, Inc., South San Francisco, CA, USA). As a viral control Ad-luc was used. The mice were sacrificed on day 3, 7 and 21. Synovial biopsies from at least five individual mice per group, were taken from the right knee joint for RNA isolation and subsequently Q-PCR. The right knee joints of mice injected with Ad-luc were used as controls for TGF-β1 and CTGF injected joints. This experiment was repeated and whole knee joints were isolated for histology ($n = 6$ per group). Since the adenoviruses overexpress either porcine TGF-β or human CTGF we could distinguish these from endogenous murine TGF-β and CTGF with specific primer sets.

Collagenase-induced OA

To induce OA, five units of bacterial collagenase in a total volume of 6 μl were injected i.a. into the right knee joint as previously described[30]. Mice were sacrificed on day 7, 21, and 42 and synovial biopsies were taken for RNA isolation ($n = 4$ per group*). This experiment was repeated for pyridinoline cross-link measurement and synovial biopsies were taken at day 2, 7, 21, and 42 ($n = 12$ per group*). Whole knee joints were isolated for histology ($n = 10$ per group*) (day 7, 28 and 42). The left non-injected knee joints served as controls. The injection of bacterial collagenase leads to joint laxity and subsequent OA lesions resembling those occurring naturally in old mice. This model represents an equivalent to human secondary OA resulting from joint instability. *Each number represents an individual mouse.

Quantitative PCR (Q-PCR)

RNA was isolated from the synovial biopsies with an RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) after which an reverse transcriptase PCR (RT-PCR) was performed. Q-PCR was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) according to manufacturers protocol. The used primer sets are displayed in Table I. Ct values of the genes of interest were corrected for glyceraldehyde-3-phosphate dehydrogenase (Gapdh) and there corresponding controls (delta delta Ct (ΔΔCt)). Each biological sample was measured once.

Histology and immunohistochemistry

The murine knee joints were processed and immunohistochemically stained as previously described[14]. Specific primary Abs against LH2 (1/100) (Proteintech Group Inc., Chicago, IL, USA) were incubated overnight at 4°C. The second antibody biotinylated

Table I
Primers used for Q-PCR. 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. Efficiencies (*E*) for all primer sets were determined using a standard curve of five serial cDNA dilutions in water in duplicate

Gene	R^2	*E*	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
Gapdh	0.997	2.05	GGCAAATTCAACGGCACA	GTTAGTGGGGTCTCGCTCCTG
Col1a1	0.997	2.10	TGACTGGAAGAGCGGAGAGTACT	CCTTGATGGCGTCCAGGTT
Mmp3	0.997	2.21	TGGAGCTGATGCATAAGCCC	TGAAGCCACCAACATCAGGA
Mmp9	0.998	2.15	GGAACTCACACGACATCTTCCA	GAAACTCACACGCCAGAAGAATTT
Mmp13	0.992	1.93	ACCTTGTGTTTGCAGAGCACTAACTT	CTTCAGGATTCCCGCAAGAGT
Adamts4	0.996	2.10	CACTGACTTCCTGGACAATGGTTAT	GGAAAAGTCGTCGGTAGATGGA
Adamts5	0.994	1.97	GATGATCACGAAGAGCACTACGA	TCACATGAATGATGCCCACAT
Timp1	0.972	1.93	CAACTCGGACCTGGTCATAAGG	CATCTTGATCTTATAACGCTGGTATAAGG
Tgf-β1	0.996	2.00	GCAGTGGCTGAACCAAGGA	AAGAGCAGTGAGCGCTGAATC
Ctgf	0.998	2.15	CCGCCAACCGCAAGATC	ACCGACCCACCGAAGACA
Plod1	0.997	1.90	GGAGCCGAACAGCCTAAGG	TGAGTGGCGCAATGACATTC
Plod2	0.997	2.08	GAGCAGCCTTGTCCAGATGTC	CCAACTCATCACAGGCTCGTT
Plod2b	0.999	1.93	CCGCAATGCTAGAGATATGACCTT	CATTTGGAATGTTTCCGGAGTAG
Plod3	0.993	2.02	AAGACACGGGCAGTGATGAACT	AAGCGAGGGCTGCTCATCT
Human CTGF	0.999	2.03	GCCCTCGCGGCTTACC	AGGCAGTTGGCTCTAATCATAGTTG
Porcine Tgf-β1	0.997	1.99	TGACCTGGCCACCATTCAT	TCCGTGGAGCTGAAGCAATA

goat anti-rabbit IgG (Vector Laboratories) was incubated for 1 h. A biotin–streptavidin detection system was used according to the manufacturer's protocol (Vector Laboratories). Sections were counterstained with hematoxylin and mounted with Permount.

The presence of fibrosis was determined by the synovial thickening, due to the accumulation of extracellular matrix and increase in fibroblasts (histology), and at the increase in collagen alpha-1(I) chain precursor (Col1a1) gene expression (Q-PCR).

Cross-link measurement

Synovial tissue samples ($n = 12$) were hydrolyzed in an oven with 6 M HCl at 110°C for 20 h. After drying (Speed Vac) samples were dissolved in 100 µl internal standard. The amount of hydroxyproline (Hyp) and the cross-links lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP) in these samples were determined by reversed-phase high-performance liquid chromatography (HPLC) as described by Bank *et al.*[31]. To calculate the amount of HP per triple helix the amount of HP (pmol) in the sample was divided by the total amount of collagen (=Hyp/300). Values are expressed as total amount of residues per collagen molecule, assuming 300 Hyp residues per triple helix.

Statistical analysis

First all data were checked for normality with the Shapiro–Wilk test. To determine significant ($P < 0.05$) differences between groups that were normally distributed a One-Way ANOVA with Games–Howell *post hoc* test for multiple comparison was performed. Significant ($P < 0.05$) differences between groups that were not normally distributed were determined with the Wilcoxon Signed Ranks Test. The statistical analyses were performed with the statistical software package SPSS 18.0 (SPSS, Chicago, IL, USA).

Results

To address the question which factors could be responsible for difference in TGF-β-induced persistent and CTGF-induced transient fibrosis, 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, which were intra-articular injected in the right murine knee joint, induced expression of human CTGF and porcine TGF-β. This showed clear expression of both factors until at least day 21 (Table II).

Ad-TGF-β induces both endogenous TGF-β and CTGF mRNA expression

We examined the effect of adenoviral overexpression of CTGF or TGF-β on the endogenous expression of CTGF and TGF-β. Adenoviral overexpression of TGF-β induced relatively high levels of endogenous (murine) TGF-β1 and CTGF, with maximal induction levels of 3.5 ΔΔCt ($P < 0.0001$) for TGF-β1 at day 7 and 3.8 ΔΔCt ($P < 0.0001$) for CTGF on day 3 [Fig. 1(A)]. Adenoviral overexpression of CTGF only resulted in a small increase (1.3 ΔΔCt) of TGF-β on day 7. Further there were hardly any changes induced by Ad-CTGF with regard to the endogenous expression levels of CTGF and TGF-β [Fig. 1(B)].

Elevated gene expression of Mmp3/9/13, Adamts4 and Timp1 by Ad-TGF-β

TGF-β induced the expression of most of the studied matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) and tissue inhibitor of metalloproteinases 1 (Timps). Expression of Mmp13 and Adamts4 were strongly elevated on all measured days [Fig. 2(A)]. Timp1 expression was highly elevated by TGF-β overexpression with a maximum at day 7 of 5.8 ΔΔCt ($P < 0.0004$) compared to normal expression. Compared to TGF-β, CTGF only significantly induced

Table II
Ct values of human CTGF and porcine TGF-β. Mice were injected i.a. 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 expression was still clearly detectable at day 21

Sample	Human CTGF	Porcine TGF-β
Control virus day 3	Nd	Nd
Control virus day 7	Nd	Nd
Control virus day 21	Nd	Nd
Ad-TGF-β day 3	Nd	9.6
Ad-TGF-β day 7	Nd	11.2
Ad-TGF-β day 21	Nd	13.6
Ad-CTGF day 3	7	Nd
Ad-CTGF day 7	10.2	Nd
Ad-CTGF day 21	13.0	Nd

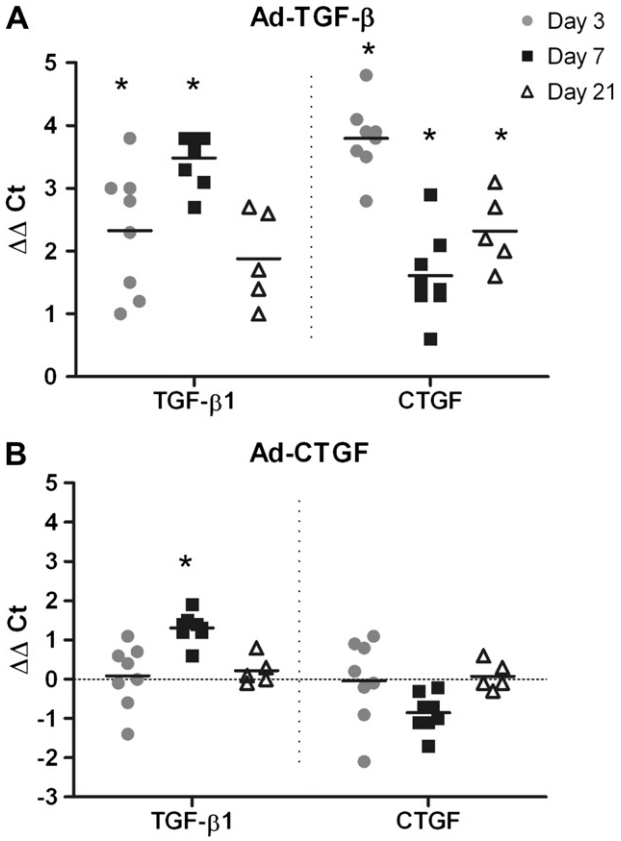

Fig. 1. mRNA expression (ΔΔCt) of endogenous TGF-β and CTGF in murine synovial tissue. Mice were injected i.a. with an adenovirus encoding either TGF-β1 or CTGF. Three, 7 and 21 days after injection of the adenovirus, synovial biopsy punches were taken of which RNA was isolated. ΔΔCt values were calculated by correcting the Ct values of the genes of interest for GAPDH and there corresponding controls. (A) TGF-β induced elevated levels of TGF-β1 RNA. In addition, Ad-TGF-β overexpression resulted in elevation of CTGF RNA expression. (B) Ad-CTGF only resulted in a significant ($P = 0.02$) increase of TGF-β on day 7. The endogenous levels of CTGF were not affected by Ad-CTGF.

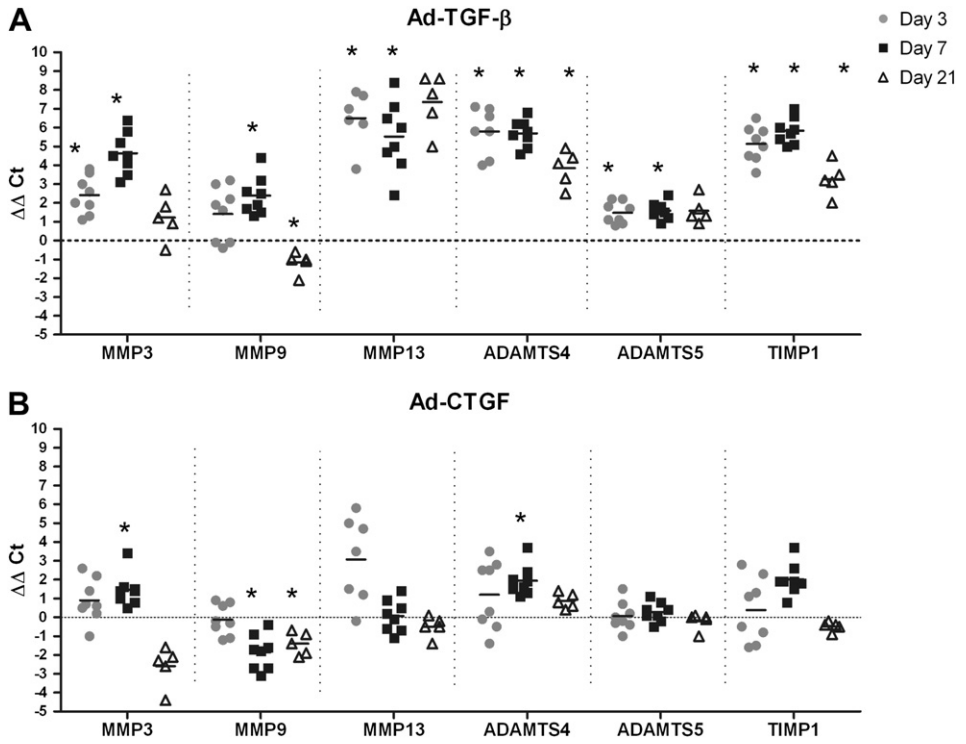

Fig. 2. mRNA expression (ΔΔCt) of various enzymes in the synovial tissue. Mice were injected i.a. with an adenovirus encoding either TGF-β or CTGF. Three, 7 and 21 days after injection of the adenovirus synovial biopsy punches were taken of which RNA was isolated. ΔΔCt values were calculated by correcting the Ct values of the genes of interest for GAPDH and for control samples. (A) Ad-TGF-β induced the highest changes on day 7, with most marked changes in expression of MMP3, MMP13, ADAMTS4 and TIMP1. (B) Overexpression of Ad-CTGF resulted in a significant induction of MMP3 ($P = 0.008$) and ADAMTS4 ($P = 0.017$) at day 7. MMP9 was downregulated by Ad-CTGF at day 7 ($P = 0.003$) and 21 ($P = 0.011$).

Adamts4 (1.9 ΔΔCt; $P = 0.0168$), further it did not significantly change any of the measured expression levels more than 1.5 ΔΔCt [Fig. 2(B)].

Ad-TGF-β, but not Ad-CTGF, induces procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2b (Plod2b) mRNA expression in murine synovium

Since, collagen cross-linking can have a major impact on the degradation of collagen we also evaluated the levels of mRNA expression of the lysyl hydroxylases (enzymes involved in collagen cross-linking). The family of lysyl hydroxylases consists of: LH1, LH2 and LH3 which are coded by *Plod1*, *Plod2*, and *Plod3* respectively. LH2 has two alternative splice-variants: LH2a and LH2b. TGF-β induced all Plods with the highest expression on day 7 [Fig. 3(A)]. Strikingly, TGF-β overexpression resulted in very high Plod2b expression levels, with an induction of approximately 4 ΔΔCT ($P < 0.003$) on all measured days. Adenoviral overexpression of TGF-β also resulted in elevated expression levels of Col1a1 mRNA in synovium on all measured days, with a maximum increase (4.7 ΔΔCt; $P < 0.0001$) on day 7 compared to controls [Fig. 3(A)]. CTGF had no effect on Plod gene expression levels on day 3 and day 7, at day 21 Plod2(b) and Plod3 were somewhat downregulated but this was not significant [Fig. 3(B)]. In contrast to TGF-β, and rather unexpected, CTGF did not induce the expression of Col1a1.

Ad-TGF-β-induces persistent fibrosis which is accompanied by elevated LH2 protein expression

Having found a particularly high Plod2(b) gene expression in the synovium, we investigated if this translates into LH2 protein expression. To this end, we stained paraffin sections of the murine knee joint transduced with either CTGF or TGF-β for LH2. First we investigated if the synovium was thickened. After TGF-β overexpression there was a strong increase in the thickness of the synovium over time, which was still present on day 21 [Fig. 4(C)]. CTGF overexpression resulted in synovial fibrosis with a peak on day 7, which was completely resolved on day 28 [Fig. 4(B)]. These results support our earlier published observations that overexpressing TGF-β leads to persistent fibrosis whereas CTGF induces transient fibrosis. Similar to LH2b mRNA expression after TGF-β overexpression, LH2 protein expression was the highest on day 21 [Fig. 4(C)]. However, on day 3 and 7 there was also a clear increase in LH2 protein expression compared to the control knee joints. As expected, CTGF did not influence LH2 protein expression [Fig. 4(B)].

Plod2b mRNA is elevated in collagenase-induced OA

Because Plod2b was strongly upregulated in TGF-β-induced persistent fibrosis we investigated if Plod2b was also elevated in OA-related synovial fibrosis in collagenase-induced OA. We found that in collagenase-induced OA there was a significant and long-lasting increase of Plod2b gene expression. The highest induction of 6.3 ΔΔCt ($P < 0.0001$) was measured on day 7, however Plod2b was still significant increased on day 21 and 42 [Fig. 5(A)]. Thus, comparable to TGF-β-induced fibrosis, Plod2b gene expression is also elevated in experimental OA-related fibrosis.

LH2 protein expression is strongly induced in OA-related fibrosis

Histological sections of murine knee joints were stained for LH2, to assess if LH2 protein expression was induced in OA-related fibrosis. A clear increase in LH2 protein expression was observed in

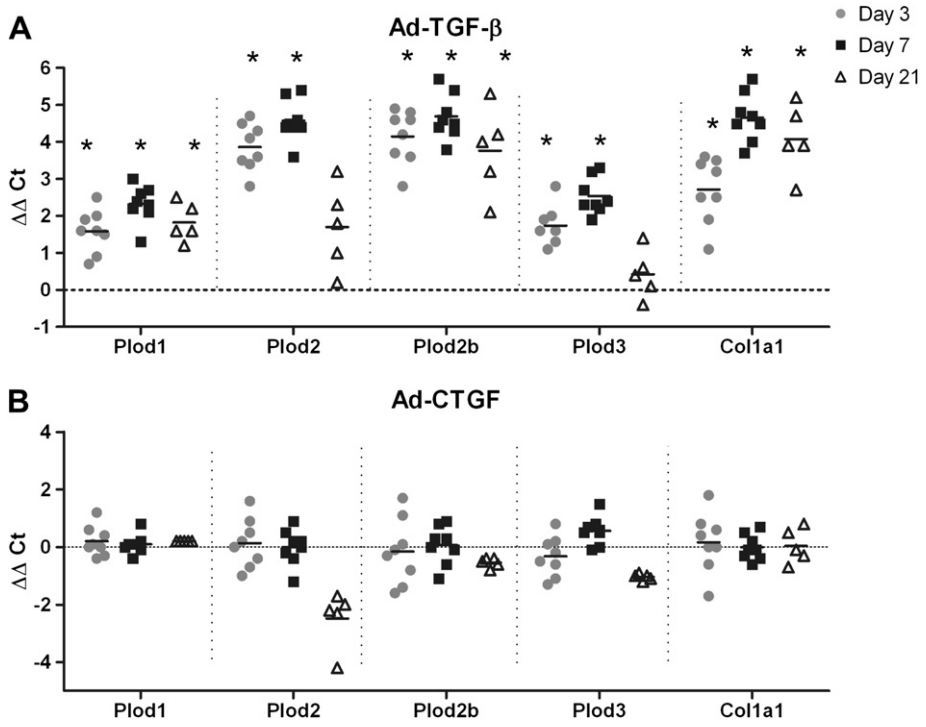

Fig. 3. mRNA expression (ΔΔCt) of various lysyl hydroxylases and Col1a1 in the synovial tissue. Mice were injected i.a. with an adenovirus encoding either TGF-β or CTGF. At day 3, 7 and 21 after injection of the adenovirus synovial biopsy punches were taken for RNA isolation. ΔΔCt values were calculated by correcting the Ct values of the genes of interest for GAPDH and for control samples. (A, B) In contrast to CTGF, TGF-β induced expression levels of all measured Plods. The most marked change was observed in Plod2(b) expression, which was strongly induced on all measured days. Col1a1 expression was also induced after Ad-TGF-β overexpression but not after Ad-CTGF overexpression.

the synovium at all measured time points [Fig. 5(B)]. Especially on day 7 there was a very strong increase in LH2 expression, while on both day 21 and 42 LH2 protein expression was still elevated but less intense than on day 7. The thickness of the synovium of the murine knee joints with collagenase-induced OA was mildly increased at day 7, while a large increase was seen on day 28 and 42. LH2 protein expression being strongly elevated in collagenase-induced OA, indicating LH2(b) may play a key role in OA-related fibrosis.

Elevated number of HP cross-links in synovium in experimental OA

To determine whether a higher LH2b expression level results in an increase of pyridinoline cross-links, the number of LP and HP per triple helix was measured in the synovium of mice with collagenase-induced OA. The amount of LP was under the detection limit at all measured time points. However, there was a significant increase in the number of HP cross-links per triple helix on both day 21 and 42, with a 1.8-fold increase on day 21 and a 2.6-fold increase on day 42 compared to unaffected left control joints [Fig. 5(C)]. This indicates that elevated levels of LH2b expression during experimental OA indeed result in more pyridinoline cross-links per triple helix. Suggesting the formation of more degradation-resistant collagen[27].

Discussion

Synovial fibrosis is thought to contribute significantly to joint stiffness[3]. Previously we have shown that TGF-β induces persistent fibrosis whereas CTGF induces reversible fibrosis[23,32,33]. In this study we evaluated several factors to get an indication why TGF-β induces persistent fibrosis and CTGF induces only transient synovial fibrosis and whether the factors are involved in the persistent nature of OA-related fibrosis. The most pronounced and abundant induction we detected was the induction of LH2b by TGF-β. In addition, we found that LH2b gene expression and LH2 protein expression as well as the number of pyridinoline cross-links per triple helix were strongly induced in experimental OA.

First we explored if the endogenous levels of TGF-β1, and CTGF were altered by overexpressing either TGF-β or CTGF. Overexpression of TGF-β resulted in the induction of endogenous TGF-β and CTGF, whereas overexpressing CTGF had no noteworthy effects on endogenous CTGF and TGF-β. TGF-β autoinduction could contribute to the potentiation of TGF-β induced effects. However, TGF-β-induced fibrosis persists for months whereas TGF-β autoinduction strongly diminished in time. Therefore it is unlikely that TGF-β autoinduction is the cause of the persistence of TGF-β-induced fibrosis.

As expected, endogenous CTGF was induced by overexpressing TGF-β. In contrast to our finding, several other groups have established that fibrosis induced by TGF-β alone was not persistent and that only simultaneous application of CTGF and TGF-β resulted in long-term fibrotic tissue formation in skin of mice[34–36]. In our system, TGF-β was sufficient to maintain fibrosis maybe supported by the induced CTGF. Our results show that Ad-CTGF overexpression, which induces high CTGF expression, gives mild and transient fibrosis. Since CTGF alone only induces transient fibrosis, CTGF is most likely not the factor responsible for the persistence of TGF-β induced fibrosis.

Expression of Col1a1 mRNA levels was highly upregulated by TGF-β when compared to CTGF or controls. We expected Col1a1 upregulation by both TGF-β and CTGF, but we found almost no upregulation after Ad-CTGF exposure. This was unexpected as type 1 collagen is the major component of fibrosis and has frequently been found upregulated by CTGF[17,24,37,38]. However, Bonniaud *et al.* found a 3-fold upregulation of Col1a1 mRNA at day 14 after Ad-

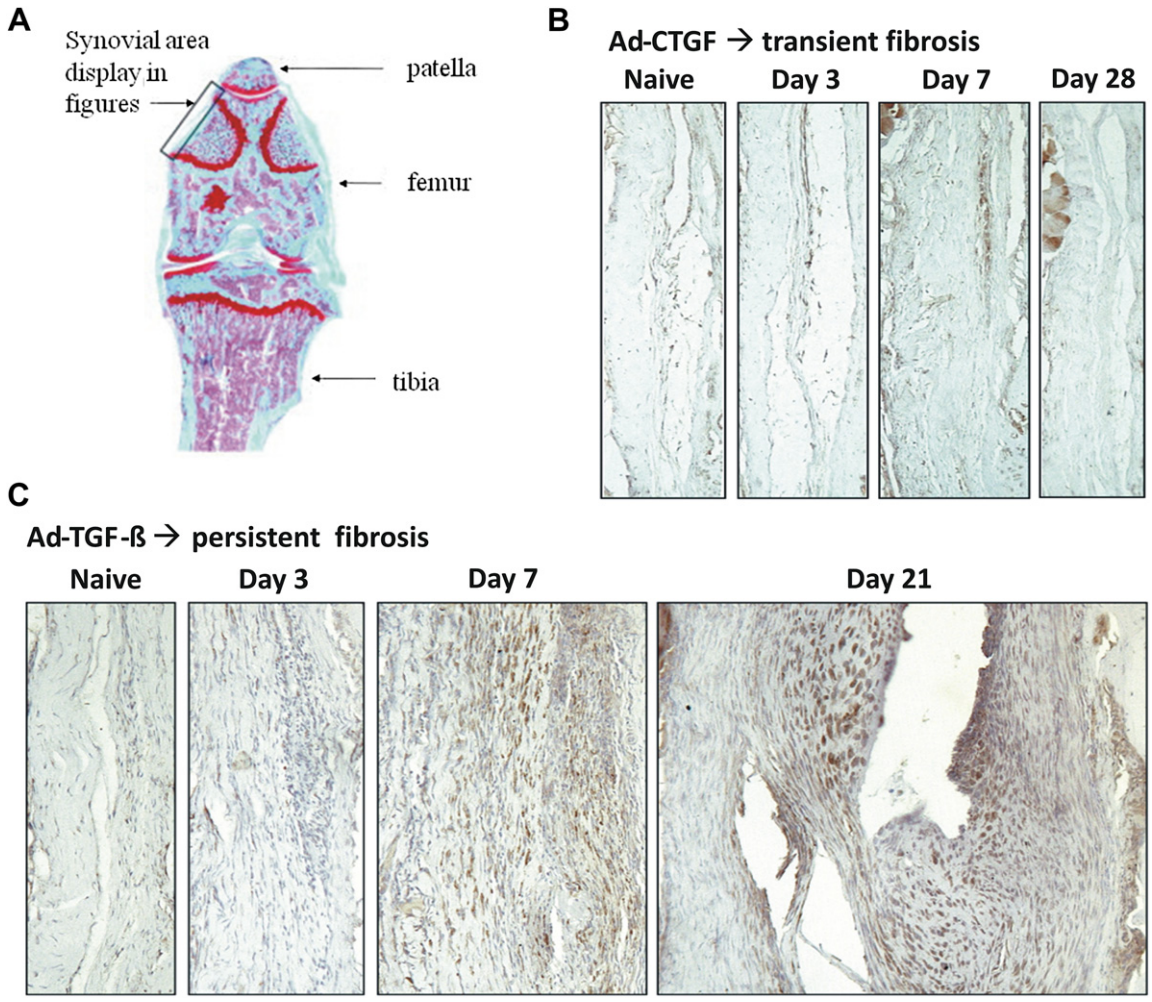

Fig. 4. (A) Normal murine knee joint, with the area that is displayed in the histology figures. (B, C) Immunohistochemically staining for LH2 protein expression in the synovial tissue of murine knee joints after i.a. of CTGF or TGF-β. TGF-β overexpression resulted in a strong increase over time in the thickness of the synovial membrane starting at day 3. CTGF-induced fibrosis was most abundant at day 7 and was completely resorbed at day 28. In contrast to CTGF, overexpressing of TGF-β strongly induced LH2 expression in the synovium, especially at day 21 (Original magnification ×100).

CTGF exposure but they did find only a small increase (approximately 1.5-fold) in Col1a1 mRNA expression on day 3 and 7 and approximately 0.75 at day 21[24]. Thus, we might have missed the window in which this increased expression could have been present.

High type 1 collagen production maybe crucial in the building up process of fibrosis. However type 1 collagen, without an elevation in the number of pyridinoline cross-links can be degraded by different enzymes and may therefore have a limited role in the persistence of fibrosis. We expected that the reversibility observed

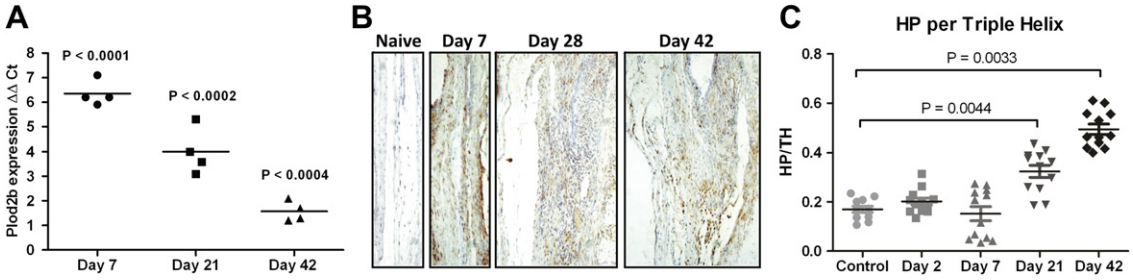

Fig. 5. (A) Plod2b mRNA expression (ΔΔCt) in the synovial tissue of murine knee joints with collagenase-induced OA. Plod2b mRNA expression was significantly induced on all measured days in the synovium of OA-affected joint compared to the healthy control joints. This increase was with an induction of 6.3 ΔΔCt the strongest on day 7, but was still 1.6 ΔΔCt increased at day 21. (B) Immunohistochemically staining for LH2 protein on murine knee joints with collagenase-induced OA (Original magnification ×100). The picture is focused at the synovial tissue lateral of the growth plate (see [Fig. 3(A)]). At day 7 a mild increase in the thickness of the synovial membrane was seen whereas on day 28 and 42 a strong increase in synovial thickening was observed. LH2 expression was strongly induced in the synovium at day 7, on day 28 and 42 LH2 was still elevated but less intense compared to day 7. (C) HPLC cross-link measurement in synovial punches, obtained from mice with collagenase-induced OA ($n = 12$ per group). The number of pyridinoline cross-links per triple helix was significantly elevated on day 21 and 42 in the synovium of murine knee joints with collagenase-induced OA compared to unaffected left control joints.

in CTGF-induced fibrosis might be due to a higher degree of ECM degradation, but in fact TGF-β led to elevated levels of MMP expression, whereas CTGF hardly influenced the expression of the proteases explored. TGF-β also induced elevated Timp1 expression, proteins that are able to inhibit MMPs and might prevent or reduce MMP-mediated matrix degradation. The outcome of the effect of Timp1 on TGF-β-induced synovial fibrosis is probably determined by the balance between the TIMPs and MMPs. The increase at day 21 in Mmp13 mRNA expression was higher than Timp1 mRNA expression, suggestive for Timp1 not being responsible for the persistence of TGF-β-induced fibrosis. The net effect of this balance on matrix turnover however is hard to predict.

Since the previous described genes did not clarify the persistent nature of TGF-β induced fibrosis, we also examined differences in collagen cross-linking enzymes. A prominent observation was that TGF-β induced high expression of Plod2 in the synovium, especially Plod2b, whereas CTGF did not induce any significant changes in Plod mRNA expression levels. That TGF-β is able to induce Plod2/LH2b was earlier shown by van der Slot *et al.* in skin fibroblasts[25]. They also showed that fibroblasts of several fibrotic disorders had increased levels of Plod2/LH2b mRNA expression. Induced LH2b results in overhydroxylation of lysine residues within collagen telopeptides which leads to increased formation of pyridinoline cross-links, making collagen harder to degrade by proteases[27,39,40]. This could 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 at least for months.

We investigated if Plod2 is also involved in OA-related synovial fibrosis in collagenase-induced OA. In this OA model that is accompanied by fibrosis both Plod2b mRNA and LH2 protein expression levels were strongly elevated. We postulated that the strong induction of LH2b in the beginning of collagenase-induced OA lead to overhydroxylation of lysyl groups in the telopeptides of procollagen molecules. During the maturation process these overhydroxylated procollagen molecules will form more pyridinoline cross-links per triple helix compared to not overhydroxylated procollagen. We measured the amount of pyridinoline cross-links per triple helix and as hypothesized these were significantly elevated on day 28 and 42. This finding was not unexpected because collagen cross-link maturation may take several weeks[41]. This increase in pyridinoline cross-links will result in collagen that is harder to degrade, and lead to persistent fibrosis after TGF-β exposure and in experimental OA.

We have shown that LH2b gene expression and LH2 protein were induced in both TGF-β-induced fibrosis and collagenase-induced OA-related fibrosis. For different fibrotic diseases an increase in pyridinoline cross-links is reported, for instance in systemic sclerosis, alcoholic cirrhosis, and glomerulosclerosis[26]. Since these pyridinoline cross-links are increased due to the elevated LH2b expression levels, we propose that LH2b is responsible for the persistence of fibrosis in OA. Most likely TGF-β, that is elevated in OA, is the driving force of enhanced LH2b expression in the OA process. We and other groups have shown that blocking TGF-β prevents fibrosis[13,42]. However, complete blockage of TGF-β in OA is not an option as it is crucial for cartilage maintenance and repair. Selective blocking of LH2b in an early stage of OA may prevent the formation of pyridinoline cross-links, and therefore persistent fibrosis. This makes LH2b an interesting target for the treatment of OA-related synovial fibrosis.

Contributions

Each author mentioned on the title page contributed sufficient to the study design, analysis of the data or acquisition of data and revising the article. All contributing authors approved the article to be published.

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The authors have no conflict of interest.

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