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Increased formation of pyridinoline cross-links due to higher telopeptide lysyl hydroxylase levels is a general fibrotic phenomenon

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Abstract

Fibrosis is characterized by an excessive accumulation of collagen which contains increased levels of pyridinoline cross-links. The occurrence of pyridinolines in the matrix is an important criterion in assessing the irreversibility of fibrosis, which suggests that collagen containing pyridinoline cross-links significantly contributes to the unwanted collagen accumulation. Pyridinoline cross-links are derived from hydroxylated lysine residues located within the collagen telopeptides (hydroxyallysine pathway). Here, we have investigated whether the increase in hydroxyallysine-derived cross-links in fibrotic conditions can be ascribed to an increased expression of one of the lysyl hydroxylases (LH1, LH2 with its splice variants LH2a and LH2b, or LH3) and/or to an increased expression of lysyl oxidase (LOX). In fibroblast cultures of hypertrophic scars, keloid and palmar fascia of Dupuytren's patients, as well as in activated hepatic stellate cells, increased levels of LH2b mRNA expression were observed. Only minor amounts of LH2a were present. In addition, no consistent increase in the mRNA expression levels of LH1, LH3 and LOX could be detected, suggesting that LH2b is responsible for the overhydroxylation of the collagen telopeptides and the concomitant formation of pyridinolines as found in the collagen matrix deposited in long-term cultures by the same fibrotic cells. This is consistent with our previous observation that LH2b is a telopeptide lysyl hydroxylase. We conclude that the increased expression of LH2b, leading to the increased formation of pyridinoline cross-links, is present in a wide variety of fibrotic disorders and thus represents a general fibrotic phenomenon.

Keywords: Fibrosis; Pyridinoline cross-links; Telopeptide lysyl hydroxylase

1. Introduction

Fibrosis is a common pathophysiological process as a response to chronic damage or inappropriate tissue repair. The main characteristic of fibrosis is an excessive accumulation of collagen which is the result of increased collagen synthesis and of decreased protease activity responsible for collagen degradation (Trojanowska et al., 1998). Another factor that seems to be involved in the excessive accumulation of collagen is the altered mode of collagen cross-linking found in fibrotic lesions.

Collagen cross-linking is the final step in the biosynthesis of collagen fibrils. Two related cross-link routes are responsible for the formation of collagen cross-links, the allysine route and the hydroxyallysine route. In the allysine route, a lysine (Lys) residue within the telopeptide is converted by lysyl oxidase (LOX) into the aldehyde allysine, whereas in the hydroxyallysine route, a hydroxylysine (Hyl) residue within the telopeptide is converted into the aldehyde hydroxyallysine. In both routes, the aldehyde

Abbreviations: α-SMA, α-smooth muscle actin; B2M, β2-microglobulin; COL1A2, collagen type I α2 chain; HSCs, hepatic stellate cells; Hyl, hydroxylysine; HP, hydroxylysylpyridinoline; Hyp, hydroxyproline; LH, lysyl hydroxylase; LOX, lysyl oxidase; LP, lysylpyridinoline; Lys, lysine; SSc, systemic sclerosis; TLH, telopeptide lysyl hydroxylase.

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subsequently reacts with a Lys, Hyl and/or histidine (His) residue within the triple helix to form di-, tri-, or tetrafunctional cross-links (Eyre et al., 1984b; Herbage et al., 1985; Reiser et al., 1992; Robins, 1982). Cross-links derived from the hydroxyallysine route occur in a variety of connective tissues including bone, cartilage and tendon (Eyre et al., 1984a), whereas collagen in the skin is mainly cross-linked via the allysine route. Interestingly, during the fibrotic process, a switch from the allysine route to the hydroxyallysine route occurs (Brinckmann et al., 1999) which results in the increased formation of pyridinoline cross-links derived from the hydroxyallysine route. This switch seems to be a general fibrotic phenomenon, as increased formation of hydroxyallysine cross-links (such as pyridinolines) has been found in various fibrotic tissues and organs, such as the skin (hypertrophic scars, keloid, systemic sclerosis (SSc) and lipodermatosclerosis; Bailey et al., 1975; Brinckmann et al., 1999, 2001; Istok et al., 2001; Moriguchi and Fujimoto, 1979; Ricard-Blum et al., 1993; Uzawa et al., 1998), the lung (infant/adult respiratory distress syndrome and interstitial lung disease; Last et al., 1990; Reiser and Last, 1987), the liver (parasitic, viral, and alcoholic cirrhosis; Brenner et al., 2000; Ricard-Blum et al., 1992, 1995), the kidney (glomerulosclerosis and interstitial fibrosis; Di Donato et al., 1997) and the palmar fascia (Dupuytren's disease: Brickley-Parsons et al., 1981; Gelberman et al., 1980). The observation that the increased formation of hydroxyallysine cross-links is related to the irreversible accumulation of collagen in fibrotic lesions suggests that collagen containing these kind of cross-links is more difficult to degrade, thereby contributing to the unwanted collagen accumulation (Bailey et al., 1975; Bailey and Light, 1985; Last et al., 1990; Ricard-Blum et al., 1992, 1993).

The increased formation of pyridinoline cross-links in fibrotic lesions is the result of an overhydroxylation of Lys residues within the collagen telopeptides. Lysyl hydroxylase 2 (LH2) has been identified as a telopeptide lysyl hydroxylase (TLH; Mercer et al., 2003; van der Slot et al., 2003). LH2 is a member of the lysyl hydroxylase family consisting of LH1, LH2a, LH2b and LH3 (EC 1.14.11.4; Hautala et al., 1992; Passoja et al., 1998; Valtavaara et al., 1997, 1998). LH2a and LH2b are alternative RNA splice variants of the LH2 gene (LH2b contains an extra exon; Ruotsalainen et al., 2001; Yeowell and Walker, 1999). Whereas LH2 hydroxylates telopeptide Lys residues, LH1 catalyzes the conversion of triple helical Lys residues into Hyl (Steinmann et al., 1995; Yeowell and Walker, 1997); the substrate specificity of LH3 is unknown. We have found in fibroblasts cultured from the fibrotic skin of systemic sclerosis patients (SSc) an increased level of LH2b mRNA expression; this was associated with elevated levels of pyridinolines in the matrix deposited by these fibroblasts (van der Slot et al., 2003). These data indicate that an increased level of LH2b is responsible for the overhydroxylation of the collagen telopeptides as found in the fibrotic skin of SSc patients. Here we investigate whether an increase in LH2b expression, in concordance with the general increased level of hydroxyallysine cross-links seen in fibrosis, is a general phenomenon. If so, LH2b would be an attractive target with respect to the treatment of fibrosis. In addition, we investigated whether the expression of three other collagen-modifying enzymes, namely LH1, LH3 and LOX, is generally increased in fibrosis as well. The expression of these genes was studied in fibroblasts cultured from hypertrophic scars, keloid, fibrotic palmar fascia of Dupuytren's disease patients and in activated hepatic stellate cells (HSCs). The latter cells are responsible for liver fibrosis (Friedman, 2003). We found an increased LH2b mRNA expression in all cultured fibrotic fibroblasts. In contrast, no consistent increase was found in the expression levels of LH1, LH3 and LOX, indicating that LH2b is responsible for the overhydroxylation of the collagen telopeptides in the extracellular matrices deposited by the fibrotic fibroblasts.

2. Results

To examine whether LH2b mRNA levels are universally increased in fibrotic tissues, we investigated LH2b mRNA expression levels in myofibroblasts isolated from keloid, hypertrophic scars and the palmar fascia of Dupuytren's disease patients, as well as in activated human NSCs cells. mRNA expression of α-smooth muscle actin (α-SMA) was analyzed to ensure that a significant part of the fibroblasts isolated from the different fibrotic tissues exhibits the myofibroblasts phenotype; an increased α-SMA expression is the main characteristic of myofibroblasts (Darby et al., 1990; Tomasek et al., 2002). Fig. 1 reveals that α-SMA expression was significantly increased in all fibrotic cell populations compared to the controls. Therefore, we conclude that a significant part of the fibroblasts isolated exhibits a myofibroblast phenotype.

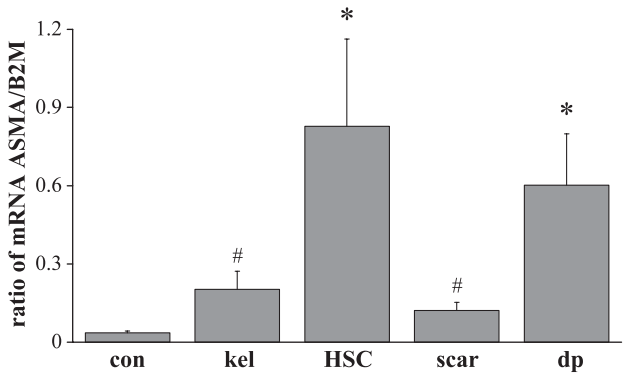

Fig. 1. Increased expression of α-SMA mRNA in fibrotic fibroblasts. Shown is the α-SMA mRNA expression relative to B2M mRNA expression in keloid (kel; $n=6$), hepatic stellate cells (HSC; $n=3$), hypertrophic scar (scar; $n=7$), Dupuytren (dp; $n=4$) and control fibroblasts (con; $n=7$). $^{*}P \leq 0.01$ and $^{\#}P \leq 0.05$ for fibrotic cells compared to controls. Data are presented as mean ± S.E.M.

The next step was to analyze LH2b mRNA expression in the different fibroblast cultures. Real-time PCR analysis showed that LH2b mRNA expression was dramatically increased in all fibrotic cells compared to normal skin fibroblast populations (Fig. 2b). Furthermore, we found elevated levels of LH2b mRNA expression relative to collagen type I α2 chain (COL1A2) mRNA expression (Fig. 3b). This increase was due to the changes in LH2b mRNA levels and not due to changes in COL1A2 expression, which was in our culture system the same in all fibroblasts except for the Dupuytren fibroblasts. These latter fibroblasts showed a threefold increase compared to controls (Fig. 2e). LH2a, the short splice variant of LH2, was slightly elevated in all fibrotic cells, but LH2a expression was still less than 5% of the LH2b mRNA expression (Fig. 2c). Analysis of the collagen matrix deposited by the keloid, hypertrophic scar and Dupuytren fibroblasts, as well as the activated HSCs, revealed increased pyridinoline cross-link

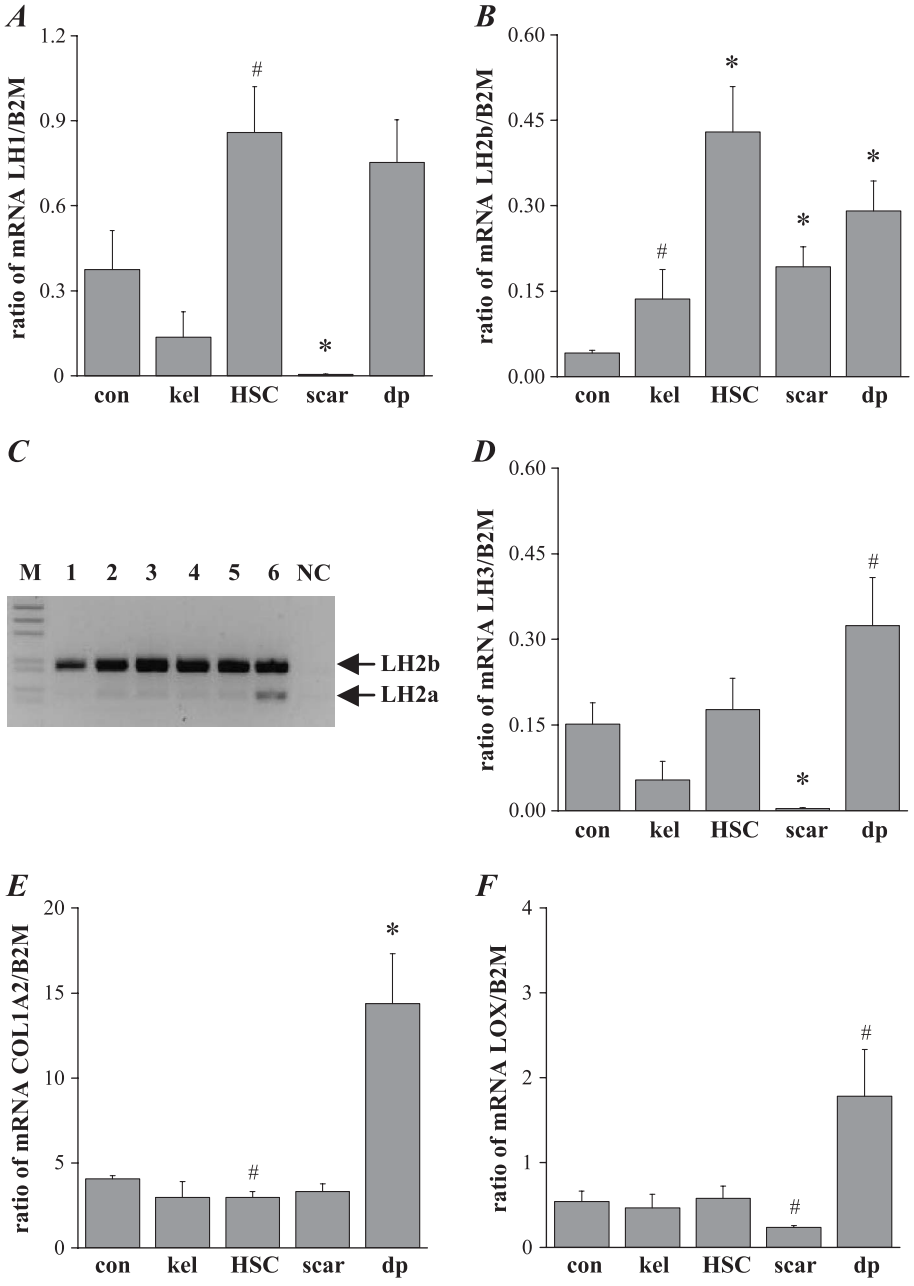

Fig. 2. Increased expression of LH2b mRNA in fibrotic fibroblasts. Shown is the mRNA expression of LH1/B2M (A), LH2b/B2M (B), LH3/B2M (D), COL1A2/B2M (E) and LOX/B2M (F) in keloid fibroblasts (kel; $n=6$), hepatic stellate cells (HSC; $n=3$), hypertrophic scar fibroblasts (scar; $n=7$), Dupuytren fibroblasts (dp; $n=4$) and controls (con; $n=7$). $^{*}P \leq 0.01$ and $^{\#}P \leq 0.05$ for fibrotic cells compared to controls. (C) Expression of LH2a en LH2b in control (1), keloid (2), HSC (3), hypertrophics scar (4) Dupuytren (5) and in kidney fibroblasts (6). M, molecular weight marker; NC, negative control. Data are presented as mean ± S.E.M.

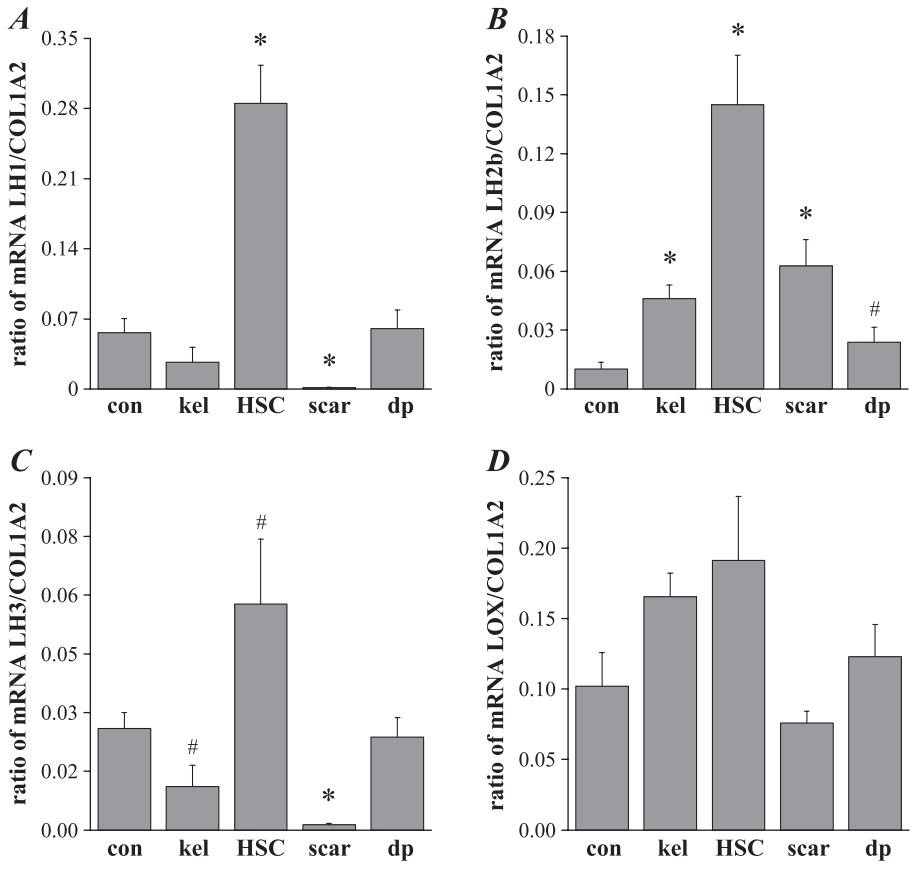

Fig. 3. Increased expression of LH2b mRNA relative to COL1A2 mRNA in fibrotic fibroblasts. Shown is the mRNA expression of LH1/COL1A2 (A), LH2b/COL1A2 (B), LH3/COL1A2 (C) and LOX/COL1A2 (D) in keloid fibroblasts (kel; $n=6$), hepatic stellate cells (HSC, $n=3$), hypertrophic scar fibroblasts (scar; $n=7$), Dupuytren fibroblasts (dp; $n=4$) and controls (con; $n=7$). $^{*}P\leq 0.01$ and $^{\#}P\leq 0.05$ for fibrotic cells compared to controls. Data are presented as mean ± S.E.M.

levels (Fig. 4), indicating that the hydroxylation of Lys residues within the collagen telopeptides is increased in all fibrotic cells. This is in line with previous in vivo data (Bailey et al., 1975; Brenner et al., 2000; Brickley-Parsons et al., 1981; Gelberman et al., 1980; Moriguchi and Fujimoto, 1979; Ricard-Blum et al., 1992; Uzawa et al., 1998).

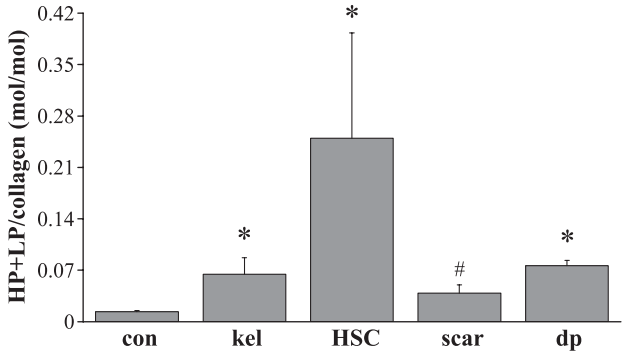

Fig. 4. Increased pyridinoline cross-link levels in the matrices deposited by the fibrotic fibroblasts. Shown is the amount of hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) cross-links relative to the amount of collagen in the extracellular matrix deposited by keloid fibroblasts (kel; $n=6$), hepatic stellate cells (HSC; $n=3$), hypertrophic scar fibroblasts (scar; $n=8$), Dupuytren fibroblasts (dp; $n=4$) and controls (con; $n=10$). $^{*}P\leq 0.01$ and $^{\#}P\leq 0.05$ for fibrotic cells compared to controls. Data are presented as mean ± S.E.M.

To ensure that the increased pyridinoline cross-link levels are not the result of factors other than the increased expression of LH2b, we measured LH1 (Fig. 2a), LH3 (Fig. 2d) and LOX (Fig. 2f) mRNA expression in all fibrotic fibroblasts. Furthermore, the expression of LH1 (Fig. 3a), LH3 (Fig. 3c) and LOX (Fig. 3d) mRNA expression relative to COL1A2 mRNA expression was calculated.

Keloid fibroblasts did not reveal differences in LH1, LH3 and LOX mRNA expression compared to control levels. However, LH3 mRNA expression relative to COL1A2 mRNA expression was slightly decreased in keloid fibroblasts whereas LH1 and LOX mRNA expression relative to COL1A2 did not show significant differences. HSCs showed a twofold increase in LH1 mRNA expression. Furthermore, LH1 mRNA expression relative to COL1A2 mRNA expression was increased fourfold, because COL1A2 expression was slightly decreased. There were no differences in mRNA expression of LH3 and LOX relative to β2-microglobulin (B2M) mRNA expression in HSCs. However, LH3 expression relative to COL1A2 was

significantly increased due to a slight decrease of the COL1A2 expression. LOX expression relative to COL1A2 was unchanged. Interestingly, hypertrophic scar fibroblasts revealed a dramatic decrease in LH1 and LH3 expression compared to controls resulting in a 40-fold decrease in LH1 mRNA expression relative to COL1A2 mRNA expression and a 20-fold decrease in LH3 mRNA expression relative to COL1A2. Although LOX expression was slightly decreased in hypertrophic scar fibroblasts, the LOX expression relative to COL1A2 expression was not changed. Dupuytren fibroblasts did not show differences in LH1 expression or in the LH1 relative to COL1A2 expression. LH3 and LOX expression relative to B2M expression was increased around twofold and threefold in Dupuytren fibroblasts, respectively. However, LH3 and LOX expression relative to COL1A2 expression did not show significant differences.

3. Discussion

Fibrosis is characterized by an excessive deposition of collagen because of a misbalance between collagen synthesis and degradation. Furthermore, the accumulated collagen in fibrotic lesions shows an increase in hydroxyallysine cross-link levels (Bailey et al., 1975; Brenner et al., 2000; Brickley-Parsons et al., 1981; Brinckmann et al., 1999, 2001; Di Donato et al., 1997; Gelberman et al., 1980; Istok et al., 2001; Last et al., 1990; Moriguchi and Fujimoto, 1979; Reiser and Last, 1987; Ricard-Blum et al., 1992, 1993, 1995; Uzawa et al., 1998). The increased amount of these cross-links in fibrosis is the result of an overhydroxylation of the Lys residues within the telopeptides. The enzyme responsible for this remained for a long time unidentified (Bank et al., 1999).

We have recently shown that the overhydroxylation of the collagen telopeptides found in the fibrotic skin of SSC patients is associated with increased TLH (LH2b) mRNA expression in cells present in fibrotic lesions (van der Slot et al., 2003). Interestingly, the presence of elevated hydroxyallysine cross-link levels appears to be a common phenomenon in irreversible fibrotic processes. We have therefore analyzed the expression of LH2b in other fibrotic tissues including hypertrophic scars, keloid and the palmar fascia of Dupuytren's patients. In addition, we have examined LH2b expression in activated HSC which are responsible for liver fibrosis (Friedman, 2003). All fibrotic cells evaluated revealed elevated levels of LH2b mRNA expression relative to COL1A2 mRNA expression. In addition, the short splice variant LH2a was slightly increased in all fibrotic cell cultures. However, the expression was less than 5% of the total LH2b expression, suggesting that this splice variant does not play a significant role in fibrotic conditions. The other collagen-modifying enzymes LH1 and LH3 did not show a consistent increase in mRNA expression relative to COL1A2 mRNA expression. In fact, in some cases, the expression was even dramatically decreased. Furthermore, in all fibrotic cell types, we found normal expression levels of LOX, being the enzyme catalyzing the formation of allysine and hydroxyallysine, a step necessary for the formation of cross-links. The increase in pyridinoline cross-links that we have found in the collagen matrix, deposited in our long-term cultures, is therefore, most likely, not the result of an increased aldehyde formation. At the expression level, the induction of LH2b exceeds that of COL1A2, indicating that the synthesized collagen will become differently modified. LH2b has been previously identified as a telopeptide lysyl hydroxylase (Mercer et al., 2003; van der Slot et al., 2003) and overexpression will thus lead to overhydroxylation of the Lys residues in the telopeptides. Hydroxylation of the telopeptide Lys residues is a necessary step in the formation of hydroxyallysine cross-links. It is therefore plausible that the increased levels of hydroxyallysine cross-links in fibrotic disorders are the consequence of induced LH2b expression. Our studies show that an induction of LH2b expression, and as a consequence, the increased formation of pyridinoline cross-links, is a universal process in fibrosis.

The presence of increased levels of hydroxyallysine cross-links in fibrotic tissues is associated with irreversible accumulation of collagen, suggesting that collagen containing hydroxyallysine cross-links is more difficult to degrade than its counterpart containing allysine-derived cross-links (Bailey et al., 1975; Bailey and Light, 1985; Last et al., 1990; Ricard-Blum et al., 1992, 1993). It is reasonable to assume that inhibition of TLH activity in fibrosis will lead to a decrease in hydroxyallysine cross-links, resulting in a decrease in collagen accumulation. We speculate that TLH is an attractive target to interfere with the irreversible accumulation of collagen in a wide variety of fibrotic disorders.

4. Experimental procedures

4.1. Cell cultures

To obtain primary fibroblast cultures, split skin biopsies of healthy donors, which serve as controls in all experiments ($n = 10$), and specimens of different fibrotic tissues (hypertrophic scar, $n = 8$; keloid, $n = 6$; and the palmar fascia of Dupuytren's disease patients, $n = 4$) were collected. Tissues were incubated in serum-free Dulbecco's Modified Eagles Medium (DMEM) containing ultraglutamine I, 0.8 mg/ml collagenase type II (CLS2 Worthington Biochemical), 100 units/ml penicillin and 100 units/ml streptomycin, for 3 h at 37 °C under continuous stirring, in order to isolate the cells. Subsequently, the suspension was filtered through a nylon gauze and the filtered cells were washed and cultured in DMEM containing 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin and 100 units/ml streptomycin at 37 °C in 5% CO_2. Cells were passaged by trypsinisation (0.05% trypsin/0.02% EDTA; Gibco) after

they reached subconfluence. Primary fibroblasts of one keloid donor were obtained from the American Type Culture Collection (ATCC; CRL-1762).

Hepatic stellate cells (HSCs; $n=3$) were obtained either from tumor-free human liver tissue obtained during hepatectomy performed to remove a tumor from patients, with no previous liver disease, or from a healthy donor liver unsuitable for transplantation because of the age of the donor. The cells were isolated following the collagenase/pronase method as described earlier (Tiggelman et al., 1995), and cultured in the same medium as the above-mentioned fibroblasts cultures. Quiescent HSC from healthy individuals cultured on plastic spontaneously transform into activated HSC, having a similar phenotype as activated HSC in vivo in fibrotic livers (de Leeuw et al., 1984; Gressner, 1996). HSC cultured on plastic therefore provides a good model system to examine the gene expression in cells responsible for liver fibrosis. All tissue collection was approved by the Local Ethical Committee.

4.2. Real-time PCR analysis

Fibroblasts obtained from a 25- or 75-cm^2 culture flask were used for total RNA isolation (RNeasy kit; Qiagen). The mRNA was subsequently reverse transcripted into cDNA (Roche). mRNA levels of α-SMA, LH1, LH2b, LH3, LOX and COL1A2 were measured using real-time PCR as described previously (van der Slot et al., 2003); forward primer α-SMA: 5′ CGTGTTGCCCCTGAAGAGCAT3′ ; reverse primer α-SMA: 5′ ACCGCCTGGATAGCCACATACA3′ ; probe α-SMA: 5′ FAM–cgtcgCCAAGGCCAACCGGGAGAAAATGACgcgacg–DABCYL 3′) and expressed relative to B2M mRNA levels to standardize for differences in the total amount of RNA between the samples. LH2a expression was measured using a PCR amplifying both LH2a and LH2b (van der Slot et al., 2003).

4.3. Cross-link analysis

Measurements were performed on the extracellular matrix deposited by the fibrotic and control fibroblasts. Cells were cultured for 4 weeks in DMEM containing 10% FCS, 100 units/ml penicillin, 100 units/ml streptomycin and 50 μg/ml ascorbic acid. The culture medium was refreshed twice a week. After 4 weeks of culture, the deposited matrix was washed in PBS; the cells present in the matrix were killed by two freeze–thaw cycles. Finally, the remaining matrix was incubated in PBS at 37 °C for another week. We have found that during the latter incubation, the di-functional collagen cross-links further mature into the tri-functional pyridinoline cross-link (data not shown). By doing so, the level of pyridinoline cross-links per collagen molecule reflect more closely the in vivo situation. Hydroxyproline (Hyp) and the cross-links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) were measured in acid hydrolysates of unreduced samples by reversed-phase high-performance liquid chromatography (Bank et al., 1997; Robins et al., 1996) and expressed as total amount of residues per collagen molecule, assuming 300 Hyp residues per triple helix.

4.4. Statistical analysis

Statistical analysis was performed using SPSS 11.0 software. Cross-link analysis and real-time PCR data are presented as mean ± S.E.M. Fibrotic cells and control cells were compared by Mann–Whitney's nonparametric test.

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