Hepatic stellate cells and portal fibroblasts are the major cellular sources of collagens and lysyl oxidases in normal liver and early after injury

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Abstract

Liver fibrosis is characterized by the excessive deposition of extracellular matrix proteins by myofibroblasts derived from hepatic stellate cells and portal fibroblasts. Activation of these precursors to myofibroblasts requires mechanical tension, which results in part from increased collagen cross-linking mediated by lysyl oxidase (LOX) family proteins. The aims of this study were to characterize the mechanical changes of early fibrosis, to identify the cells responsible for LOX production in early injury, and to determine which cells in the normal liver produce collagens and elastins that serve as substrates for LOXs early after injury. Hepatocytes and liver non-parenchymal cells were isolated from normal and early-injured liver and examined immediately for expression of LOXs and matrix proteins. We found that stellate cells and portal fibroblasts were the major cellular sources of fibrillar collagens and LOXs in the normal liver and early after injury (day 1 after bile duct ligation, days 2 and 7 after carbon tetrachloride). Activity assays using stellate cells and portal fibroblasts in culture demonstrated significant increases in LOX family enzymatic activity as cells became myofibroblastic. LOX family-mediated deoxypyridinoline and pyridinoline cross-links increased after carbon tetrachloride-mediated injury. There was a significant association between liver stiffness (as quantified by the shear storage modulus $G'$) and deoxypyridinoline levels; increased deoxypyridinoline levels were also coincident with significantly increased elastic resistance to large strain deformations, consistent with increased cross-linking of the extracellular matrix. These data suggest a model in which the liver is primed to respond quickly to injury, activating potential mechanical feed-forward mechanisms.

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The abbreviations used are: ECM, extracellular matrix; $\alpha$-SMA, $\alpha$-smooth muscle actin; LOX, lysyl oxidase; BDL, bile duct ligation; BAPN, $\beta$-aminoproprionitrile; SEC, sinusoidal endothelial cells

Key Words: Collagen cross-linking, liver fibrosis, pyridinoline, deoxypyridinoline, extracellular matrix
Pathological fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM) proteins, most notably fibrillar collagens. The majority of ECM in organ fibrosis is deposited by myofibroblasts, proliferative and motile cells characterized by the expression of \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA), which either migrate to the site of injury or differentiate from preexisting cells in the organ. In the liver, the major myofibroblast precursor cells are hepatic stellate cells and portal fibroblasts.

Myofibroblast differentiation from precursor cells requires mechanical tension. This has been shown \textit{in vitro} for general fibroblast to myofibroblast activation (14) as well as for both hepatic stellate cell and portal fibroblast activation to myofibroblasts in the liver (19, 36) and suggests that increases in liver stiffness precede myofibroblast activation. We have used \textit{ex vivo} livers to show that this is true in a rat carbon tetrachloride (CCl\(_4\)) model of liver fibrosis and that the mechanical heterogeneity of the liver also increases early after injury in this model (12, 17).

Increases in liver stiffness are multifactorial and may include increased interstitial pressure from edema, increased ECM protein expression, and collagen stiffening due to cross-linking. Potentially relevant collagen cross-linking agents include tissue transglutaminases, which are increased in residual septae after fibrosis resolution (but have recently been shown to have minimal impact on the progression of fibrosis in rodent models (27)), and lysyl oxidase (LOX) family enzymes. The LOXs are secreted copper-dependent amine oxidases that initiate the process of covalent intra- and intermolecular cross-linking of collagens and elastin (34, 35). There are five isoforms: LOX itself, and the LOX-like enzymes LOXL1, LOXL2, LOXL3, and LOXL4, all encoded by separate genes and with overlapping but distinct functions and substrate specificity. Multiple studies have shown that LOX-mediated cross-linking significantly increases ECM stiffness (8, 15, 20, 29).

The LOXs play an important role in liver fibrosis. LOX expression in the liver, as determined by immunohistochemistry, is upregulated as early as 24 hours after bile duct ligation (BDL) and continues to increase through at least 72 hours, preceding increases in \( \alpha \)-SMA (7). In a chronic CCl\(_4\) model of fibrosis, LOX activity increased 30 fold in rat liver and 15 fold in plasma after five weeks (32). Treatment of rats with \( \beta \)-aminoproprionitrile (BAPN), an inhibitor of LOX family cross-linking enzymes, reduced early increases in liver stiffness in the CCl\(_4\) model (12); we and others have shown that inhibition of LOX family activity also results in decreased numbers of myofibroblasts and decreased fibrosis (9, 10, 12). A recent study showed that treatment with a LOXL2 blocking antibody blunted liver fibrosis in CCl\(_4\)-treated rats (2).

The LOXs cross-link \textit{newly synthesized} collagen and elastin fibrils (34, 35). Thus, if the LOXs are important mediators of early fibrosis and the first phases of myofibroblast differentiation (and as part of normal liver matrix homeostasis), there must be non-myofibroblast sources of fibrillar collagens in the normal and early-injured liver. Our goals were to characterize mechanical
changes in the normal liver and the liver very early after injury and to identify the cellular
sources of fibrillar collagens and LOXs in an effort to delineate the earliest events in fibrosis.

Materials and Methods

Animal models of liver injury. Liver injury was induced in 250-300 g Sprague-Dawley rats
(Charles River Laboratories, Wilmington, MA) by CCl₄ intoxication or BDL. Rats were injected
intraperitoneally with 0.2 ml/100g body weight of a sterile-filtered 1:1 mixture of CCl₄ (Sigma-
Aldrich, St. Louis, MO) in mineral oil once (1d and 2d experiments) or twice per week. Cross-
linking analyses were also carried out using previously generated samples (12). Mechanical
measurements (G', shear storage modulus, a measure of elasticity or rigidity and G'', shear loss
modulus, a measure of viscosity) were carried out as previously described (12), and included
measurements at varying strains. BDL was carried out per standard protocols, with the
placement of two ligatures around the bile duct. All animal work was approved by the University
of Pennsylvania Institutional Animal Care and Use Committee.

Primary cell isolation. All cells were prepared from 500-700g Sprague-Dawley rats. Cells were
either lysed immediately after isolation for mRNA analysis or were cultured as noted in the
figure legends.

Hepatocytes were isolated as described, with minor modifications (33). Briefly, livers were
perfused in situ through the portal vein with 0.05% collagenase type IV (Worthington,
Lakewood, NJ). Cells were resuspended in HBSS with Ca²⁺/Mg²⁺ (Gibco, Manassas, VA)
containing 0.003% DNase (Sigma-Aldrich), sedimented at 60 g for 3 min at room temperature,
then resuspended in HBSS with Ca²⁺/Mg²⁺ and washed again. To separate hepatocytes from
dead and contaminating nonparenchymal cells, the cell suspension was brought to 10%
Histodenz (Sigma-Aldrich) and 1% FBS (Gemini Bio-Products, West Sacramento, CA) and was
layered over 7 ml of 30 % Histodenz. After centrifugation at 1400g for 15 min, cells were
collected from the interface between the two layers.

Hepatic stellate cells were isolated by sequential digestion of the liver with 0.4% pronase
(Roche Diagnostics, Indianapolis, IN) and 0.04% type II collagenase (Worthington), followed by
density gradient centrifugation over 9% Histodenz (Sigma-Aldrich), as described (36).

Portal fibroblasts were isolated after in situ perfusion of livers with 0.3% type II collagenase
(Worthington) as described (19).

For sinusoidal endothelial cell (SEC) and Kupffer cell isolations (which were carried out
separately), livers were perfused and digested in situ with 0.05% type IV collagenase
(Worthington). Cell suspensions were separated by density gradient centrifugation using Percoll
gradients (11). Kupffer cells were purified by rapid adherence to plastic (15-30 min at 37°C).
SEC were purified via centrifugal elutriation using a Beckman-Coulter Avanti J-201 centrifuge with a JE 5.0 elutriator rotor and large chamber. Cells were eluted in HBSS without Ca\(^{2+}\)/Mg\(^{2+}\). Centrifugation speed was kept constant (2500 rpm) with a variable pump flow rate. SEC were collected at a pump speed of 60 - 80 ml/min (with increases of 2 ml/min). One liter of cell suspension was collected and centrifuged at 1900 rpm for 7 min.

The purity of all primary cells was assessed by immunostaining. Cells were seeded on uncoated glass slides (Kupffer cells, hepatic stellate cells, and portal fibroblasts) or on slides coated with collagen I (SEC, hepatocytes; BD Falcon, Bedford, MA). Cells were stained using antibodies against HNF4\(\alpha\) (1:50, Santa Cruz, CA) for hepatocytes, CD68 (1:200, Abcam, Cambridge, MA) for Kupffer cells, desmin (1:1000, Sigma-Aldrich) for hepatic stellate cells, elastin (1:500, Cedarlane Laboratories, Burlington, NC) for portal fibroblasts, and CD32b (1:100, Santa Cruz) for SEC. Cell viability was determined by 0.4% trypan blue exclusion. Only cell preparations found to be ≥ 95% pure and viable were used for experiments.

Quantitative PCR. Total RNA was isolated from primary cells immediately after isolation, without intervening culture, using the RNeasy micro kit (Qiagen, Valencia, CA). Two-step real-time PCR (qRT-PCR) was performed using SuperScript III reverse transcriptase and random primers (Invitrogen) and Fast SYBR Green (Applied Biosystems, Carlsbad, CA). A relative calibration curve with 10-fold serial dilutions was built for every target. PCR primers were designed using either Vector NTI (Invitrogen) or an online tool from Integrated DNA Technologies (http://www.idtdna.com/Scitools/Applications/RealTimePCR/). All primers were designed to span introns. 12S ribosomal protein mRNA was used for normalization (see Table 1 for sequences).

Lysyl oxidase activity assay. Lysyl oxidase enzymatic activity was determined as described (26) with minor modifications using conditioned media collected over 24 hours from cultures of primary hepatic stellate cells and portal fibroblasts. Cells from a single animal were used for each individual experiment. At least three animals were used for each cell type, with three technical repeats for every time point. Conditioned media were clarified by centrifugation at 10000g for 30 min at 4°C with addition of protease inhibitors without EDTA (Roche) and were concentrated 100-fold by ultrafiltration at 15°C for 1.5 hours at 3700g in Amicon ultra-15 concentrators (10 kDa MW cutoff; Millipore, Carrigtwohill, Ireland). After concentration, equal volumes of a solution containing 4M urea and 20 mM sodium borate pH 8.2 were added to the samples for cryoprotection and they were frozen at -80°C.

LOX activity was assessed fluorometrically using 30 µM Amplex red reagent (Invitrogen, Carlsbad, CA) in 1M urea, 0.5 U/ml horseradish peroxidase (Worthington), 10 mM cadaverine (Sigma-Aldrich), and 45 mM sodium borate pH 8.2. Samples were incubated at 37°C for 1 hour, with readings taken every minute for 20 minutes (excitation 530 nm, emission 590 nm; Synergy 2 plate reader with Gen 5 1.10 software). To avoid interference from possible preexisting H\(_2\)O\(_2\) and confounding from other amine oxidases that also use cadaverine as substrate, activity was
calculated by subtracting fluorescence with BAPN present from fluorescence without BAPN, after normalization to the protein content of the sample. A calibration curve was generated with H$_2$O$_2$ (range 0.125 – 2.5 µM) and background fluorescence was corrected using a no H$_2$O$_2$ control.

Biochemical analyses of collagen cross-links. Liver samples were minced on ice, washed with cold phosphate buffered saline and distilled water, lyophilized and weighed. Aliquots of the dried samples were then hydrolyzed with 6N HCl, and subjected to amino acid and cross-link analyses as described (40). Due to the low hydroxyproline contents in liver samples, non-reducible collagen cross-links (pyridinoline and deoxypyridinoline) were expressed as residues/1,000 total amino acids.

Statistical analysis. Data are expressed as mean ± standard error of the mean (SE) and represent at least three independent experiments with three technical repeats per experiment. For cross-link analysis, values represent mean ± standard deviation (SD). Statistical significance was calculated by t-test (GraphPad Prism) except as noted in figure legends.

Results

Liver mechanics change early after injury. We previously used the rat CCl$_4$ model of liver fibrosis to demonstrate that liver stiffness increased early after injury, before the first major wave of myofibroblast differentiation (12). Treating animals with the LOX inhibitor BAPN suggested that LOX family activity was responsible at least in part for this increase (12). We have now carried out more detailed mechanical measurements and show that they are consistent with increased collagen cross-linking as a cause of increased stiffness. We determined the stiffness (G') of livers from 0 to 14 days after the initiation of CCl$_4$ intoxication. In all cases, livers were removed 3-4 days after an injection of CCl$_4$, when edema was minimal. As we observed previously (12), the livers of CCl$_4$-treated animals were significantly stiffer than normal livers beginning as early as 7 days after the start of treatment (Fig. 1A). When we increased shear strain, there was marked strain softening in samples from all time points (Fig. 1A), although the degree of softening was less pronounced at later time points (Fig. 1B). The reduction in strain softening paralleled a decrease in the ratio of the loss modulus to the storage modulus (G'/G'; Fig. 1C) with strain, consistent with formation of a more elastic tissue due to increased collagen cross-linking.

To rule out edema as a cause of the increased stiffness, we examined livers at 1 and 2 days after CCl$_4$ injection, when there is significant edema. G’ at minimal strain was increased, but there was still pronounced strain softening (Fig. 2A), and the shape of the G'/G' vs. strain curve was similar to that of normal, as opposed to early fibrotic liver (day 14; Fig. 2B). Thus, the strain-dependence of the shear modulus and G'/G' enabled us to differentiate edematous from fibrotic
liver and suggested that matrix cross-linking, rather than edema, was the cause of the significant mechanical changes we observed.

*Increased collagen cross-linking is seen early after injury and correlates with stiffness.* To investigate whether altered mechanics are associated with collagen cross-linking, we measured the LOX-mediated collagen cross-links pyridinoline and deoxypyridinoline directly in livers from rats treated with CCl$_4$ for up to 70 days. These trivalent, mature, non-reducible cross-links are formed involving two hydroxylsine aldehyde (Hyl$^\text{ald}$) residues in the telopeptides and one Hyl (in the case of pyridinoline) or lysine (deoxypyridinoline) residue in the helical domain of collagen (39). We found that the content of pyridinoline increased significantly at late time points (70 days; Fig. 3A), while the content of deoxypyridinoline was significantly elevated by 10 days and continued to increase with time (Fig. 3B). There was no significant correlation between pyridinoline and whole liver stiffness (Fig. 3C); however, deoxypyridinoline cross-links were significantly correlated with liver stiffness ($r^2 = 0.47$, $p=0.0001$; Fig. 3D). Thus, LOX-mediated cross-linking increased early after injury with CCl$_4$, in parallel with mechanical changes suggestive of increased cross-linking.

*Bile duct ligated livers also demonstrate increased stiffness at early points.* Rat livers after bile duct ligation also showed increases in stiffness early after injury, preceding significant increases in collagen expression (Fig. 4A, B, D). Although LOX inhibition experiments are difficult in this model due to biliary excretion of BAPN, LOX, LOXL1, and LOXL2 mRNA expression increased significantly after injury (Fig. 4C). Stiffness continues to increase after the peak of LOX mRNA expression, likely reflecting protein expression and maturation of collagen cross-links as well as (at later points) the deposition of new matrix. Thus, two models of liver fibrosis in rats show early mechanical changes consistent with increased collagen cross-linking.

*LOXs are produced primarily by hepatic stellate cells and portal fibroblasts in the healthy and acutely injured liver.* Whole liver analyses have shown that LOX expression increases after liver injury (7, 32). In order to identify the source of LOXs, we determined ECM expression in five primary cell types from normal livers: portal fibroblasts, hepatic stellate cells, hepatocytes, Kupffer cells, and SEC. We lysed cells immediately after isolation, without intervening, thus avoiding culture artifacts. We found that hepatic stellate cells and portal fibroblasts are the major sources of LOXL1, 2, and 3 (Fig. 5B-D, compare white bars). LOXL4 is widely expressed, and LOX in the normal liver is expressed primarily by hepatocytes and portal fibroblasts (Fig. 5A, E). Cholangiocytes demonstrated minimal expression of any of the LOX family members when compared with normal portal fibroblasts (data not shown).

To determine whether increases in LOX family expression precede whole liver mechanical changes, we examined primary cells isolated at early time points after liver injury induced by BDL (1 day) or CCl$_4$ intoxication (2 days) (Fig. 5). We observed again that expression was limited to hepatic stellate cells and portal fibroblasts, except for LOXL4 which was expressed by
all cells studied except Kupffer cells (Fig. 5E). The only significant increase in expression at
these time points was in LOX expression by hepatic stellate cells after CCl₄ (Fig. 5A).

LOX is the major isoform upregulated in early CCl₄-mediated fibrosis. Further evaluation of LOX
family expression in hepatic stellate cells and portal fibroblasts freshly isolated from livers 7
days after initiation of CCl₄ treatment (2 injections) showed that LOX continues to be the major
isoform increased at this time point, and that its mRNA is increased in both cell types (Fig. 6A).
Other isoforms, with the exception of LOXL1, which increased slightly in portal fibroblasts
(p=0.125), showed decreased mRNA expression (Fig. 6B-E). Thus, while the liver expresses all
5 LOX family members even in the normal liver, LOX (and potentially LOXL1) is the isoform
which increases very early after injury.

Secretion of active LOXs increases as hepatic stellate cells and portal fibroblasts differentiate in
culture. Both hepatic stellate cells and portal fibroblasts undergo differentiation to myofibroblasts
in culture. Analysis of LOX family enzymatic activity in these cells demonstrated measurable
activity from quiescent cells with significant increases as the cells became myofibroblasts (Fig.
7). Activity in hepatic stellate cells increased an average of 22.2 fold after 7 days in culture,
while in portal fibroblasts it increased 4.7 fold.

Collagens I, III and IV are expressed in the normal and early injured liver by hepatic stellate
cells and portal fibroblasts. The LOXs initiate the process of cross-linking, making newly
deposited collagens and elastin insoluble. In order to identify the sources of fibrillar collagen in
the liver at baseline, we determined ECM expression in freshly-isolated primary cells from
normal livers. We found that hepatic stellate cells and, to a lesser extent, portal fibroblasts,
produce measurable amounts of mRNA for collagens I, III, and IV in the normal liver (Fig. 8).
Although there is a small amount of collagen IV mRNA expression by hepatocytes, SEC, and
Kupffer cells, there is no production of the fibrillar collagens I and III by any cells other than
hepatic stellate cells and portal fibroblasts.

We examined expression of these ECM proteins early after injury in representative models of
biliary and parenchymal fibrosis. With the exception of an increase in the α2 chain of collagen I
by portal fibroblasts after BDL, collagen mRNA expression remains stable early after injury
induced by CCl₄ treatment (2 days) or BDL (1 day) (Fig. 8A, B, C). Elastin, which has been used
as a marker of portal fibroblasts in vitro (19), is expressed primarily by portal fibroblasts in the
normal liver but also to a small extent by hepatic stellate cells in the injured liver, and its
expression by portal fibroblasts is significantly increased after BDL (Fig. 8D). Thus, the cells
responsible for pathological ECM deposition in fibrosis also express collagens in the normal
liver and in the injured but non-fibrotic liver. Interestingly, there is mRNA expression of the
myofibroblast marker α-SMA in hepatic stellate cells and portal fibroblasts at baseline,
suggesting that there is a small population of myofibroblasts (or primed myofibroblast
precursors) in the normal liver (Fig. 9).
We demonstrate here that 1) normal livers undergo pronounced strain softening, and that this as well as the $G''/G'$ ratio (reflecting the relationship between viscosity and elasticity in a sample) change markedly within days of liver injury; 2) LOX family-mediated pyridinoline and deoxypyridinoline collagen cross-links increase with injury, and deoxypyridinoline collagen cross-links, which rise at early time points, are significantly correlated with liver stiffness; 3) LOX and to a lesser extent LOXL1 are the major LOX family members with increased expression in early injury; and 4) hepatic stellate cells and portal fibroblasts are the major cellular sources of fibrillar collagens and LOXs in the normal liver and early after injury. These findings have important implications: 1) that mechanical changes are due to collagen cross-linking and may mediate major changes in the behavior of cells of the liver at early time points after injury; 2) hepatic stellate cells and portal fibroblasts, which become the major collagen-secreting cells in the fibrotic liver, are “primed” for this function through their matrix-secreting roles in the normal liver; and 3) hepatic stellate cells and portal fibroblasts, by virtue of their early expression of LOXs, may have an important role in early injury distinct from their role as matrix producing cells.

Our findings that liver stiffness increases early after injury in both the CCl$_4$ and BDL models ((12) and Fig. 4), and that hepatic stellate cells and portal fibroblasts require increased mechanical tension in order to differentiate to myofibroblasts (19, 25) suggest a model whereby increased stiffness shortly after injury drives the myofibroblastic and then the fibrotic response. Potential causes of increased liver stiffness include changes in the matrix (cross-linking or increased matrix deposition) and tension due to edema or vascular pressure. We demonstrate here that edema can be differentiated from other causes of increased liver stiffness, and that the altered mechanics of the liver at day 7 and later are not consistent with edema. The reduction in strain softening as well as flattening of the $G''/G'$ vs. strain curves after injury are most consistent with increased matrix cross-linking. Notably, most studies of stiffness and cell phenotype, including our previous work with stellate cells and portal fibroblasts, has focused on $G'$ (or the closely related Young’s modulus $E$). The implications of strain softening and the $G''/G'$ ratio on cell behavior are not well understood, but may add another layer of complexity to the role of mechanics in disease pathophysiology.

We previously demonstrated that inhibition of LOX-family enzymatic activity resulted in blunting of the liver stiffness response early after injury and in decreased numbers of myofibroblasts (12); others have also reported decreased fibrosis in animal models after treatment with LOX family inhibitors (2, 9, 10). Critical to our model is that cross-linking occurs before the development of significant fibrosis. We therefore examined LOX family and collagen expression within a small window of time. Our data suggest that small perturbations in expression of LOX itself (which likely occur in response to increased TGF-$\beta$) occur at early time points after injury. Surprisingly given the success of an anti-LOXL2 antibody in preventing CCl$_4$-induced fibrosis in
rodents, we did not observe significant changes in LOXL2 at the time points we studied (2); however, it may increase at later time points after injury and play an important role in fibrosis progression rather than initiation. While early fibrosis represents the process of injury repair and is protective (3), later fibrosis could be considered a pathologic process and may involve different subsets of ECM proteins (which undoubtedly contribute to increased stiffness) and ECM modifying enzymes.

Our data show that at early points after the initiation of CCl₄ intoxication, pyridinoline and deoxypyridinoline cross-links begin to increase. This is consistent with findings from cirrhotic human livers, when both types of cross-link are increased (4). These are trivalent, stable cross-links derived from the immature divalent cross-links (39). In the current study, due to the limited amounts of material available, we were unable to measure the precursor aldehydes or immature cross-links that would likely have been increased at earlier time points. Liver stiffness is increased by day 7 (Fig. 1A), while deoxypyridinoline does not increase until 10 days (Fig. 3B), an incongruity likely secondary to immature cross-links that we were unable to measure. We found a significant correlation between deoxypyridinoline but not pyridinoline cross-links and liver stiffness (G’) in the subset of livers for which we had stiffness data. To our knowledge, the relative mechanical impacts of the different forms of cross-links in liver (mature vs. immature, deoxypyridinoline vs. pyridinoline) have not been investigated. Similarly, it is not known what caused a preferential increase in deoxypyridinoline compared to pyridinoline in the early stages of fibrosis. This could be due to the differential expression of specific lysyl hydroxylase isoforms (28, 37). More comprehensive analyses for collagen cross-linking/aldehydes and lysine hydroxylation are warranted to confirm the alteration in cross-linking pattern and to elucidate its molecular mechanisms. We did not evaluate elastin cross-linking, but this may play an important role in liver mechanics and fibrosis, particularly in biliary disease. A better understanding of the gene expression pattern of different LOX and lysyl hydroxylase isoforms and the consequent collagen cross-links and lysine modifications (39) could be important in developing antifibrotic therapies aimed at liver mechanics. Of note, the LOXs may also have functions unrelated to collagen and elastin cross-linking, including gene regulation, modification of receptor function (13, 21), and modulation of growth factors such as fibroblast growth factor (18) and TGF-β (1); these functions may be important in hepatocytes and other cell types that demonstrated decreases in LOX family mRNA expression after injury (Fig. 5).

It would be advantageous for the liver to have the ability to mount a rapid response after injury. Recently published work showed that hepatic stellate cells in the normal liver are heterogeneous and that a subpopulation of these cells express myofibroblast markers, apparently primed to initiate repair processes in the context of liver injury (6). We observed α-SMA expression in both hepatic stellate cells and portal fibroblasts in the normal liver (Fig. 9). It is tempting to speculate that portal fibroblast and hepatic stellate cell subsets expressing α-SMA serve as “rapid responders” and that they are also responsible for the LOX and collagen mRNA expression we observed in the non-injured liver, although this requires further evaluation.
since our studies did not involve single cell analyses. All of the LOXs are upregulated in
response to TGF-β ((31); and data not shown), which is one of the earliest growth factors to
increase after injury; TGF-β-mediated increases in LOX production in early injury, combined
with ongoing deposition of small amounts of collagen, could result in modest regional changes
in stiffness, which we have observed in rodent livers as early as day 6 after initiation of CCl4
treatment (16). Recent reports suggest that small increases in collagen stiffness can be
amplified by cells, suggesting that they could be significant in driving the progression of fibrosis
(38).

Although the cellular source of collagen in fibrosis was at one time the source of significant
debate, definitive studies demonstrated that hepatic stellate cells (as opposed to hepatocytes)
were the major fibrogenic population (22-24). (These studies were carried out before portal
fibroblasts were routinely isolated.) Maher et al. used RNase protection assays to evaluate
mRNA expression in isolated cells, and found that, at 5 days after BDL or 14 days after initiation
of CCl4 treatment, hepatic stellate cells were the major source of types I and III collagen while
SECs were a minor source (22). Our work extends these results by showing that portal
fibroblasts have collagen expression patterns similar to those of hepatic stellate cells.
Additionally, we have demonstrated that portal fibroblasts and stellate cells are the cells
involved in homeostasis of the fibrillar collagens in the normal liver. Unlike Maher et al., we did
not observe significant matrix production by SEC, potentially because we examined cells from
livers early after the two forms of injury we employed; SEC are likely to be a significant source
of at least collagen IV after injury. Although we did not examine cholangiocytes directly, recent
publications have reported that these cells do not produce collagen I in animal models of
fibrosis (5, 30).

In summary, we have identified hepatic stellate cells and portal fibroblasts as sources of both
matrix proteins and their cross-linking agents in the normal liver, and we suggest a model in
which the liver is thereby primed to respond quickly to injury, setting into effect potential feed-
forward mechanisms. LOX and potentially LOXL1 may be potential targets for antifibrotic
therapies, particularly in early disease.

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Figure legends

Fig. 1. Mechanical properties of the liver change in early fibrosis. Livers from normal rats and rats 7, 10 and 14 days after the start of CCl₄ intoxication were analyzed by shear rheometry, with the shear storage and loss moduli G' and G'', respectively, determined over a range of strains. (A) Liver stiffness (G') was increased even by day 7. Livers demonstrated marked softening with increased strain. For G' at minimal strain, all values are significantly different from normal (day 7, p<0.01; day 10 p<0.001; day 14 p<0.001). (B) The data in (A) were normalized to 1 at low (1%) strain to demonstrate the different shapes of the strain curves. For G' at maximum strain, p <0.01 for normal vs. day 7 and normal vs. day 14. (C) Increased elasticity of livers after CCl₄, as manifested by differences in the curves of G''/G' vs. strain (normalized to 1 at starting strain). Significance values shown vs. normal. N= 3-6 for each time point. *, p<0.05; **, p<0.01; ***, p<0.001.

Fig. 2. Mechanical properties of the edematous liver. Livers were removed from rats 1 or 2 days after CCl₄ injection, when edema is pronounced. (A) Liver stiffness (G') is increased compared to normal, although strain softening is unchanged. By one way ANOVA there is no difference between the three curves. At low strain, day 1 and day 2 are both significantly different than normal (p<0.05). (B) Plotting G''/G' against strain demonstrates that, in spite of increased stiffness, edematous livers can be distinguished from livers with early fibrosis (day 14). Normalized curves shown. Similar results are obtained with day 1 livers (not shown). G''/G' at 40% strain, normal vs. day 2, no significant difference; normal vs. day 14, significant at strains >30%. N=3-5 for each time point. *, p<0.05; **, p<0.01.

Fig. 3. LOX-mediated cross-links increase early after CCl₄-mediated injury. Livers from rats with CCl₄-induced fibrosis or from normal, non-injected controls were analyzed for mature LOX-mediated collagen cross-links (pyridinoline (A) and deoxypyridinoline (B)). Deoxypyridinoline cross-links were increased as early as 10 days. N=3-6 per time point. Data shown as mean +/-SD. *, p<0.05; **, p<0.01. (C,D) Correlation between whole liver shear modulus (G') and pyridinoline (A) and deoxypyridinoline (B).

Fig. 4. Liver stiffness and LOX family expression increases rapidly after BDL. Rats underwent BDL or sham surgery. Livers were removed at the times noted. (A) G' was measured by rheometry. (B) α-SMA expression was assessed by immunostaining. Significance for A and B is in reference to sham-operated controls. *, p<0.05; ***, p<0.005. Real-time PCR with liver lysates was used to determine expression of LOXs (C) and collagens (D). For each individual experiment, values were normalized to 18S expression. In the graph, values are normalized to those for samples taken from sham-operated animals from the same time point. Significance for both C and D is in reference to samples taken at 6 hours after surgery. n=5-6 for all data points.*, p<0.05; ***, p<0.005.
Fig. 5. LOX is significantly upregulated in hepatic stellate cells immediately after injury. Primary hepatocytes (Hep), Kupffer cells (KC), hepatic stellate cells (HSC), portal fibroblasts (PF), and sinusoidal endothelial cells (SEC) were isolated from the livers of healthy rats and rats 2 days after a CCl₄ injection or 1 day after BDL. Cells were lysed for mRNA isolation immediately, and real-time PCR was carried out to determine expression of all 5 LOX family members (LOX (A), LOXL1 (B), LOXL2 (C), LOXL3 (D), LOXL4 (E)). All are expressed; however, hepatic stellate cells and, to a lesser extent, portal fibroblasts, are the major producers of LOXs in the normal liver and shortly after injury. *, p<0.05; **, p<0.01; ***, p<0.005.

Fig. 6. LOX but not other family member mRNA expression is upregulated in hepatic stellate cells and portal fibroblasts after injury. Cells were isolated from rat livers 7 days after beginning treatment with CCl₄ (after a total of 2 injections, day 1 and day 4) and were analyzed by real-time PCR for LOX family expression. Only LOX is significantly increased at this time point. *, p<0.05.

Fig. 7. Hepatic stellate cells and portal fibroblasts increase secretion of active LOX family members during myofibroblastic differentiation. Primary cells were placed in culture for up to 7 days. Conditioned media (CCM) were examined by a LOX family activity assay at days 1 and 7 after isolation. *, p<0.05.

Fig. 8. Hepatic stellate cells and portal fibroblasts produce collagens and elastin in the normal and acutely-injured liver. mRNA isolated as for Fig. 5 was analyzed by real-time PCR to determine expression of collagen chains (A-C) or elastin (D). Data represent cells from at least 3 independent isolations per cell type, with 3 technical repeats per isolation.**, p<0.01; ***, p<0.005.

Fig. 9. Hepatic stellate cells and portal fibroblasts from normal liver express α-SMA. mRNA prepared as in Fig. 5 was analyzed by real-time PCR for expression of α-SMA.*, p<0.05.
Figure 1

A. $G'$ (Pa) vs Strain, %

B. Stiffness, normalized vs Strain, %

C. $G'/G$, normalized vs Strain, %
Figure 3

A. Pyridinoline

B. Deoxypyridinoline

C. Pyridinoline and stiffness

D. Deoxypyridinoline and stiffness

$R^2 = 0.47$

$p = 0.0001$
Figure 4

A. Liver stiffness

B. aSMA

C. LOXs

D. Collagens
Figure 5

(A) LOX

(B) LOXL1

(C) LOXL2

(D) LOXL3

(E) LOXL4
Figure 6

A. LOX

B. LOXL1

C. LOXL2

D. LOXL3

E. LOXL4
Figure 7

A. LOX activity in HSC CCM

B. LOX activity in PF CCM
Figure 8

A. Collagen 1a2

B. Collagen 3a1

C. Collagen 4a1

D. Elastin
Figure 9

aSMA

Relative expression

Heps  KC  HSC  PF  SEC
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>Collagen I(α2)</td>
<td>Forward - TTCTCTACTGGGTGAAACCTGC</td>
</tr>
<tr>
<td></td>
<td>Reverse - ACCCCTTCTGCGTGTATTC</td>
</tr>
<tr>
<td>Collagen III(α1)</td>
<td>Forward - GGCCTTGCGTGTTTGTATTC</td>
</tr>
<tr>
<td></td>
<td>Reverse - GAAGTCTCTGAAGCTGATGGG</td>
</tr>
<tr>
<td>Collagen IV(α1)</td>
<td>Forward - AGTTCCCCCGCGTCTCTGT</td>
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<tr>
<td></td>
<td>Reverse - ACAATCACCCCTTCGACAGC</td>
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<tr>
<td>Elastin</td>
<td>Forward - CTTGGAGGCATTGAGGAGT</td>
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<tr>
<td></td>
<td>Reverse - CAATAACCCAGCAAGCCCA</td>
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<tr>
<td>LOX</td>
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<tr>
<td></td>
<td>Reverse - CTACATCCAGGCAATCCACG</td>
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<td>LOXL1</td>
<td>Forward - CCCTTGCTCTCCCTTGAAG</td>
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<td></td>
<td>Reverse - GGTCATGTTCACTCCTTGG</td>
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<td>Reverse - TCCAGATAGCGGTCTTGT</td>
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<tr>
<td>RPS12</td>
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