Increased stiffness of the rat liver precedes matrix deposition: implications for fibrosis

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Georges PC, Hui J-J, Gombos Z, McCormick ME, Wang AY, Uemura M, Mick R, Janney PA, Furth EE, Wells RG. Increased stiffness of the rat liver precedes matrix deposition: implications for fibrosis. Am J Physiol Gastrointest Liver Physiol 293: G1147–G1154, 2007. First published October 11, 2007; doi:10.1152/ajpgi.00032.2007.—Liver fibrosis, the response to chronic liver injury, results from the activation of mesenchymal cells to fibrogenic myofibroblasts. We have recently shown that two key myofibroblast precursor populations, hepatic stellate cells and portal fibroblasts, undergo activation in culture in response to increasing substrate stiffness. We therefore hypothesized that alterations in liver stiffness precede myofibroblast activation and fibrosis in vivo as well. To test this hypothesis, we induced fibrosis in rats by twice weekly injections of carbon tetrachloride (CCl4) and then killed the animals at various time points ranging from 3 to 70 days after the initiation of injury. The shear storage modulus of the whole liver was measured on fresh tissue; fixed and frozen tissue from the same livers was used to quantify fibrosis. We observed that liver stiffness increased immediately and continued to increase, leveling out by day 28. Fibrosis, measured histologically by trichrome staining as well as by quantitative sirius red staining, increased with time, although these increases were delayed relative to changes in liver stiffness. Studies of liver mechanics have been limited by small sample size, variable methodology, and the use of tissue stored for prolonged periods of time. Taken as a whole, however, the literature suggests that liver stiffness increases and may therefore contribute to progressive cycles of myofibroblast activation and matrix deposition, this hypothesis has not been investigated in the intact organ. Similarly, the role of altered stiffness as a primary initiator of myofibroblast activation, preceding fibrosis, is unknown.

LIVER FIBROSIS IS THE WOUND-HEALING response to chronic liver injury, commonly caused by viral hepatitis, ethanol abuse, and biliary obstruction. It is characterized by the accumulation of excess and abnormal extracellular matrix material (ECM) including fibrillar collagens, fibronectin, and proteoglycans. Most abnormal ECM in the fibrotic liver is produced by \( \alpha \)-smooth muscle actin \( (\alpha \text{-SMA}) \)-expressing myofibroblasts. Myofibroblasts result from the “activation” or myofibroblastic differentiation of a variety of cells in the liver, most notably hepatic stellate cells (HSC) and portal fibroblasts (21, 41). The literature contains multiple reports of different factors that induce myofibroblast activation, including growth factors, oxidative stress, matrix components, fragments of apoptotic cells, and the immune response (2, 18).

The role of mechanical factors in myofibroblast activation and fibrosis is a new area of investigation. It is now understood that matrix stiffness (and the mechanical tension that results from cellular adhesion to stiff substrates) is instrumental in determining the phenotype of many cell types in culture (1, 3, 9, 13, 16, 19, 20, 35, 38, 42, 45; for review, see Ref. 11). Relevant to the liver, we have demonstrated in vitro that primary HSC and portal fibroblasts undergo myofibroblastic differentiation as a function of the stiffness of the underlying substrate (31; Chan EP, Gaça MDA, Georges PC, Uemura M, Janney PA, Wells RG, unpublished observations). Fibrillar expression by cultured HSC has also been shown to require mechanical tension (33). The role of mechanical factors in fibrosis in vivo, however, is not well understood. Although it makes intuitive sense that liver stiffness increases with increasing fibrosis and may therefore contribute to progressive cycles of myofibroblast activation and matrix deposition, this hypothesis has not been investigated in the intact organ. Similarly, the role of altered stiffness as a primary initiator of myofibroblast activation, preceding fibrosis, is unknown.

Studies of liver mechanics have been limited by small sample size, variable methodology, and the use of tissue stored for prolonged periods of time. Taken as a whole, however, the literature suggests that the liver is a viscoelastic tissue and that stiffness increases with increasing fibrosis (6–8, 29, 32, 34, 43, 44). In one study, liver stiffness was measured intraoperatively with a tactile sensor in 52 patients with a range of disease, and a positive correlation \( (r = 0.887, \ P < 0.0001) \) was found between stiffness and the fibrosis index (29). More recently, transient elastography (FibroScan; Echosens) and magnetic resonance elastography (MRE) have been used for noninvasive measurements of liver stiffness (7, 17, 23, 39, 46). Notably, although stiffness values have been found to be good predictors of fibrosis, particularly in advanced disease, patients with early-stage fibrosis have not been well studied. There have been no studies, prospective or otherwise, to determine whether there exist patients with minimal fibrosis but stiff livers and whether such patients are at increased risk for the development of fibrosis.

We hypothesized on the basis of our in vitro data that increases in liver stiffness precede myofibroblast activation and
fibrosis. To test this hypothesis, we induced fibrosis in rats by carbon tetrachloride (CCl₄) injection, killed the animals from 3 to 70 days after initiation of treatment, and measured whole liver stiffness (shear storage modulus; G′), α-SMA expression, and various measures of fibrosis.

**MATERIALS AND METHODS**

**Rat CCl₄ treatment and liver harvest.** All animal studies were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (275–300 g; Charles River Laboratories, Wilmington, MA) were injected intraperitoneally twice weekly with 0.2 ml/100 g of a 1:1 mixture of CCl₄ (Sigma-Aldrich, St. Louis, MO) and olive oil or olive oil alone; after 2 wk, the dose was reduced to 0.1 ml/100 g. Animals were euthanized by CO₂ inhalation at the times noted, in all cases 3 or 4 days after the last injection of CCl₄, when inflammation, necrosis, and apoptosis have been shown to be minimal (Table 1) (24). After euthanasia, the abdomens were opened, and the livers were removed and systematically divided for rheometry, formalin fixation, or freezing. Six uninjected animals were used as controls. Three or six CCl₄-injected or CCl₄-injected/BAPN-treated rats per time point, and five rats treated with BAPN alone for 14 days. Six uninjected animals were used as controls. Three animals died unexpectedly during the study shortly after injection.

**Rheometry.** For rheometry, cylindrical samples were prepared from the posterior lobe of freshly removed liver using a scalpel and a 25-mm-diameter stainless steel punch (McMaster-Carr, New Brunswick, NJ). Samples with a thickness of ~3 mm (measured by a micrometer built into the rheometer) were placed between two 25-mm serrated stainless steel parallel plates. Serrated plates were used to eliminate slipping of the sample on the plate, and the tissue was found to stick well to the plate in the absence of any surgical glue. Tissue samples were relatively uniform in thickness when examined by eye. A normal force sensor attached to the rheometer was used to determine thickness uniformity more quantitatively. A nonuniform tissue sample would result in portions of the sample contacting the upper plate before others, causing the normal force to be relatively high once the entire tissue was in contact. Nonuniform tissue samples were discarded, and additional samples from the same tissue were dissected. G′ values were measured within 30 min of the cylindrical tissue cores being cut. Control experiments demonstrated that G′ measurements were unchanged over several hours of liver storage in

<table>
<thead>
<tr>
<th>Days of Rx</th>
<th>Inflammation: Portal (0–4)</th>
<th>Inflammation: Lobular (0–4)</th>
<th>Apoptotic Cells per 10 hpf</th>
<th>Mitoses per 10 hpf</th>
<th>Hepatocyte Ballooning (0–1)</th>
<th>Hepatocyte Steatosis (%)</th>
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<td>No Rx</td>
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<td>3.6 (1–7)</td>
<td>9.2 (4–15)</td>
<td>0.6 (0–1)</td>
<td>35 (0–75)</td>
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Values shown are means with ranges in parentheses. Rx, treatment; hpf, high-power fields.

Fig. 1. Liver stiffness increases with time of injury. The average G′ value (±SE) for livers at each time point from carbon tetrachloride (CCl₄)-injected (●) and vehicle-injected (■) animals was plotted as a function of time of treatment. The 0 time point used for both curves is the average value for livers from uninjected animals. At all times from day 7 onward, with the exception of day 28, there is a significant (P < 0.005) difference between CCl₄-injected and control animals. Note the rapid increase in G′ values after the initiation of injury as well as the leveling off after 28 days (*P < 0.05, **P < 0.005).

Fig. 2. α-Smooth muscle actin (α-SMA) demonstrates a rapid initial increase. Livers were stained with an antibody against α-SMA, which was digitally quantified and plotted with G′ (same G′ values as in Fig. 1). All values are means ± SE and are shown for CCl₄-injected animals only; 0 time point values are from uninjected animals. Significance is expressed compared with the 0 time point (*P < 0.05, **P < 0.005).
HBSS and did not vary with the plane of dissection of the disc. Perfusion of livers with heparin before rheometry did not alter $G'$ in control experiments, so the livers described here were used without prior perfusion. In individual livers, there was minimal variability in $G'$ from one lobe to another.

The shear storage modulus $G'$, a measure of liver stiffness and compliance, was measured using a Rheometrics RFS-3 controlled strain rheometer and is reported in pascals (Pa). $G'$, measured by oscillatory deformations, depends in general on the frequency of oscillation and on the magnitude of deformation (strain). Liver samples were only weakly dependent on the frequency of deformation, but $G'$ decreased with increasing strain. $G'$ was measured at a frequency of 1 rad/s over a range of strain values. The $G'$ vs. strain plot was linear to 2% strain, and strain softening was minimal over this range. Reported values are for 2% strain. A moisture trap enclosed the testing area, and total test time was under 3 min to minimize drying or aging of the samples.

**Sirius red and α-SMA staining and quantification.** Samples were stained for the presence of collagen by sirius red, which detects primarily type I collagen, as described (5, 26). Four random fields from each sample were selected, and staining was quantified using IP Lab digital analysis software (Scanalytics, Rockville, MD). Data are expressed as percent staining, indicating the percentage of the area of each microscope field stained.

**Histological staining and grading.** Slides were stained using standard methods with hematoxylin and eosin (H&E), trichrome, and reticulin stains. They were graded in a blinded fashion by two experienced pathologists (E. E. Furth and Z. Gombos) according to the following criteria: percentage steatotic hepatocytes; inflammation, 0–3 (0 = none, graded independently for portal and lobular regions); apoptotic and mitotic hepatocytes (number per high-power field); fibrosis by H&E and trichrome, 0–4 (normal, mild expansion, septae, bridging fibrosis, and cirrhosis); ballooning hepatocellular degeneration, 0 = absent, 1 = present; and reticulin, 0–4 (portal normal = 0, lobular normal = 4). These reticulin scores reflect the distribution of reticulin staining in the normal liver, where the sinusoids are lined by a rich reticulin framework, whereas periportal reticulin is discrete without projections into the parenchyma. Each pathologist initially reviewed all slides independently; scores were agreed on by both.

**Statistical analysis.** Mean ($\pm$SE) values were calculated for each variable, and correlations with time were calculated using Spearman’s rho. Linear regression analysis was employed to determine whether trends in $G'$ were correlated with trends in fibrosis measures at early (days 3–10) and later (days 14–70) time points. The sample size was adequate for detection of correlation, $r$ values of 0.50 and higher with $>90\%$ power at two-sided 5% significance. Significance values were calculated by students $t$-test for parametric and by the Mann-Whitney
rank sum test for nonparametric variables. Definitions of statistical significance are provided in the text and figure legends; inflated type I error due to the multiple comparisons carried out cannot be ruled out but is unlikely, given that $P < 0.005$ for most measures.

RESULTS

Study design and histology. To study the temporal relationship between liver stiffness and the development of fibrosis, we induced fibrosis in rat livers by a standard protocol of twice weekly intraperitoneal injections of CCl$_4$. Animals were killed in groups and livers removed for stiffness measurements at times ranging from 3 to 70 days after the initiation of treatment. Liver inflammation at the time of death (3–4 days after injection) was minimal, as shown in other studies and confirmed here (Table 1), and was therefore unlikely to contribute to stiffness values (24). Although mitoses, apoptosis, hepatocyte ballooning, and steatosis generally increased with time in the treated group, the increases were not linear, and there was significant variability from animal to animal. There was no direct correlation between any of these parameters and liver stiffness or fibrosis.

Early increase in liver stiffness. The left lobe (28) was removed from each liver, and shear storage modulus ($G'$, a measure of stiffness) was determined on the fresh tissue. Because the livers were excised before measurement, $G'$ values reflect the fixed mechanical properties of the liver and do not incorporate blood flow-dependent mechanical changes. Baseline $G'$ for uninjected rats was 374.1 ± 49.7 Pa ($n = 6$). Vehicle-injected rats killed at later time points ($n = 2$ per time point, total $n = 16$) showed similar mean $G'$ values of 505.8 ± 22.4 Pa. Whole liver $G'$ was observed to increase within 3 days of the first injection and was significantly greater than $G'$ of control livers at the 7-day time point (763 ± 84.2 Pa, $P < 0.005, n = 6$). $G'$ continued to increase through day 28 and then stabilized, although there was significant variability in $G'$ values at later time points ($n = 5$ or 6 at each time point; Fig. 1). Differences between the $G'$ of CCl$_4$-treated and veh-
cle-injected rats were significant at every time point from 7 days forward ($P < 0.01$). $G'$, the shear loss modulus, changed similarly to $G'$ (data not shown).

**α-SMA increased early after injury.** To determine the course of myofibroblast activation compared with increases in $G'$, liver sections were stained with antibodies against α-SMA, and staining was quantified digitally (Fig. 2). There were significant (albeit modest) increases in α-SMA expression even after 3 days, although the majority of the increase occurred at later time points. Significant increases in α-SMA preceded increases in all of the measures of fibrosis employed, including sirius red, trichrome, and reticulin staining. Increases in α-SMA were almost exclusively in the portal tract at early time points; by late time points, α-SMA staining extended further into the parenchyma, paralleling the pattern of fibrosis observed by trichrome and sirius red staining (Fig. 3) and consistent with patterns of fibrosis reported in early $CCl_4$ intoxication (40).

**Early changes in reticulin staining.** Fixed tissue was stained with standard reticulin stains and graded separately for portal and parenchymal regions. Interestingly, portal reticulin increased shortly after injury, demonstrating a statistically significant difference with controls by day 7, whereas trichrome staining lagged behind, not achieving significance until day 10 (Fig. 4A). This result suggests that ECM deposition begins in the portal region in this model of fibrosis (40) and, more specifically, begins with type III collagen, as has been shown by other methods (27). Of note, portal and parenchymal reticulin demonstrated opposing changes: portal reticulin increased significantly with time, whereas parenchymal reticulin decreased, the decrease in parenchymal reticulin reaching statistical significance by day 14 (Fig. 4B). The reticulin stain is believed to react with glycosaminoglycans associated with type III collagen (36); thus, the prominent decrease in parenchymal reticulin staining could indicate a loss of either type III collagen or sinusoidal proteoglycans. The changes in portal and parenchymal reticulin staining highlight the fact that the quality of the ECM changes in fibrosis, with a decrease in some matrix components and uneven increase in others.

**Lack of correlation between fibrosis and stiffness.** Fibrosis was assessed in three ways: sirius red staining (with digital quantification), trichrome, and H&E staining. It was observed initially as portal tract expansion followed by portal bridging without zonal injury. There was a significant correlation among the three measures of fibrosis (Fig. 5A) and with all measures and time ($P < 0.001$; Table 2). There was, however, no statistically significant correlation between trends in increasing $G'$ and trends in fibrosis measures. In particular, $G'$ increased significantly by days 7 and 10, although there were only slight increases in the different fibrosis measures. By linear regression analysis, no fibrosis measure was statistically correlated with changes in $G'$ at early time points (days 3–10), whereas only trichrome was significantly correlated ($P = 0.03$) with later $G'$ (days 14–70), with a correlation value of $r = 0.62$ (Table 3). This implies the existence of one or more unidentified variables that contribute to stiffness. In particular, there is a clear increase in $G'$ at early time points, before significant fibrosis, that cannot be explained by early matrix deposition (Fig. 5B).

**BAPN treatment blunts early changes in stiffness during $CCl_4$ treatment.** We hypothesized that early changes in $G'$ resulted from increased collagen cross-linking, which has been shown in vitro as well as in vivo studies to increase the $G'$ of collagen in the absence of increased deposition. Lysyl oxidase (LOX)-mediated cross-linking activity in particular increases collagen stiffness and is known to be upregulated early after liver injury (4, 12, 22, 30, 37). We therefore treated a second cohort of rats with the LOX inhibitor BAPN over a period of 2 wk. $CCl_4$-injected animals again demonstrated an increase in stiffness compared with normal and vehicle-injected control animals; this was statistically significant at all time points (Fig. 6A). There was a statistically significant decrease in $G'$ at day 10 (similar to the decrease observed in the first rat cohort at day 14; $P = 0.03$).

BAPN alone did not alter the stiffness of the liver over 2 wk (Fig. 6A), and it had no effect on liver histology (data not shown). There was a marked blunting of the early increase in liver stiffness, however, which was statistically significant at 3 and 14 days and near significance at 10 days ($P = 0.07$). There was also a significant decrease in the area of the liver staining positive for α-SMA in the $CCl_4$/BAPN group compared with the $CCl_4$ group at days 3, 6, and 14, although there was no significant difference in matrix deposition, as assessed by trichrome and sirius red staining (Fig. 6B and data not shown).

**DISCUSSION**

We have demonstrated the following in the rat $CCl_4$ model of fibrosis: 1) liver stiffness ($G'$) increases progressively in the setting of chronic liver injury over 10 wk; 2) increases in $G'$ precede abnormal matrix deposition; 3) the relationship between fibrosis and $G'$ is not linear; and 4) early increases in $G'$ can be blunted by inhibition of collagen cross-linking enzymes.

**Table 2. Significance of association with time**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Stiffness (G')</th>
<th>% Sirius Red</th>
<th>Trichrome</th>
<th>Fibrosis by H&amp;E</th>
<th>Reticulin Portal</th>
<th>Reticulin Parenchymal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spearman’s rho</td>
<td>0.80</td>
<td>0.87</td>
<td>0.90</td>
<td>0.79</td>
<td>0.90</td>
<td>−0.77</td>
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Values shown are correlation coefficients. H&E, hematoxylin and eosin.
in the LOX family. Statistical analysis suggests that liver G' is determined by one or more variables in addition to the quantity of ECM. Our findings are consistent with the hypothesis that mechanical alterations including collagen cross-linking precede fibrosis and suggest the possibility that these mechanical changes induce myofibroblast differentiation in early liver disease.

Most papers on the subject report a correlation between liver stiffness and the degree of fibrosis. The most extensive data are from the recent literature on the Fibroscan apparatus and suggest a linear correlation between fibrosis and stiffness. Several papers have recently been published on MRE in the liver and reach similar conclusions (23, 39, 46). None of the published studies, however, cover the range of disease we report here, and with the exception of one MRE study, in which the single patient with hepatitis C virus (HCV) but without detectable fibrosis had a stiffer liver than any of 12 normal control patients (39), none compare normal liver to liver soon after the onset of injury. Our work, which specifically included animals with early fibrosis, demonstrated a surprising early rise in liver stiffness and a lack of direct relationship between fibrosis and G'. It is possible that the relationship between fibrosis and stiffness is linear at later time points than examined here. The rats we studied (even at day 70) are still within the window where complete recovery from fibrosis can occur (24); G' might increase at later time points, as remodeling decreases and collagen cross-linking becomes more entrenched, and might track more closely with ECM deposition (25).

Our in vivo data are consistent with a role for mechanical changes in fibrosis and, potentially, myofibroblast activation. We have demonstrated in vitro that both HSC and portal fibroblasts activate only when their underlying supports are stiff (31; Chan EP, Gaça MDA, Georges PC, Uemura M, Janmey PA, Wells RG, unpublished observations); it therefore makes sense that early changes in mechanical stiffness of the whole liver (and microregions within the liver) could result in myofibroblast activation. At later time points, when matrix deposition could reasonably be expected to alter liver mechanics by increasing stiffness, there may be an accelerating cycle of matrix deposition and myofibroblast activation.

The cause of the early increase in liver G' is not known. One potential mechanism of increased stiffness is hepatocyte necrosis with collapse of the liver reticulin meshwork. We believe this is unlikely. Although CCl4 intoxication results in cirrhotic necrosis particularly early in treatment, recovery occurs quickly in this model; necrosis at the time of death (3–4 days after an injection) was minimal (Table 1) (24). Similarly, inflammatory cell infiltration, another possible explanation for liver stiffening, was not significant (Table 1). Increases in collagen-associated glycosaminoglycans, as reflected in reticulin staining, could result in increased water content (edema), which, in turn, might have mechanical implications; again, however, this was not seen. Our stiffness measurements were all taken ex vivo so that hemodynamic alterations in early fibrosis were not a factor, although they may well be important in vivo.

Data from BAPN-treated animals suggest that increased ECM cross-linking by LOX family members is at least partially the cause of early increases in G'. This is not surprising given in vitro data that LOX treatment of isolated collagen gels resulted in a significant increase in G' (12, 22). In vivo studies of rat heart and aorta also showed that increased stiffness was secondary to cross-linking by members of the LOX family rather than to increased collagen deposition (4, 30, 37).

Although the role of LOX in mediating liver stiffness has not been previously studied, a BAPN analog was shown many years ago to decrease fibrosis and fibroblast proliferation in the CCl4 model (14, 15). Desmouliere et al. (10) demonstrated that LOX family members increase early in disease, preceding the appearance of α-SMA-expressing myofibroblasts. These authors concluded that ECM changes precede and may mediate myofibroblast differentiation, a statement consistent with the findings we report here. We did not observe changes in fibrosis in BAPN-treated vs. untreated animals, although there were significant changes in myofibroblast activation. This may be due to the fact that ECM deposition lags behind the appearance of myofibroblasts, in which case, changes would be expected at
later time points. Alternatively, the earliest ECM deposition may be from sources other than myofibroblasts.

This work has significant implications for the diagnosis and treatment of fibrosis. If increased liver stiffness, independent of cause, precedes early fibrosis in human chronic liver disease, then noninvasive measurements of stiffness (transient elastography or MRE) could be used to identify individuals, for example, those infected with hepatitis C, at risk for the development of fibrosis. Likewise, if causes of the increased liver stiffness are identified, new therapies could be targeted specifically to early stages of disease.

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