Localisation and semiquantitative assessment of hepatic procollagen mRNA in primary biliary cirrhosis

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Abstract

Background—Chronic liver disease is characterised by excessive deposition of collagen and other extracellular matrix proteins, produced mainly, but not exclusively, by activated hepatic stellate cells in the perisinusoidal space. In primary biliary cirrhosis (PBC) fibrosis is concentrated mainly around the portal tracts.

Aims—To examine the hypothesis that, in addition to hepatic stellate cells, portal tract fibroblasts might play a significant role in the deposition of collagen in PBC.

Methods—Fifty liver biopsy specimens from patients with PBC were studied. An *in situ* hybridisation technique was adapted to localise and measure semiquantitatively type I procollagen mRNA in formalin fixed, paraffin wax embedded sections, using an ³⁵S labelled cRNA probe specific for the α_1 chain of rat type I procollagen. Hepatic fibrogenic activity was also assessed using serum type III procollagen peptide (PIIINP).

Results—In PBC, type I procollagen gene expression was significantly increased. Signal was localised mainly in and around inflamed portal tracts, to cells which had the appearances of portal fibroblasts. Signal activity in these cells correlated with the degree of portal fibrosis and inflammation and also with serum PIIINP concentrations.

Conclusions—Results are consistent with the hypothesis that the excessive extracellular matrix, deposited within the liver in PBC, is synthesised not only by hepatic stellate cells but also by portal tract fibroblasts. The semiquantitative assessment of procollagen mRNA in liver biopsy specimens may provide a useful method of evaluating the rate of synthesis of collagen and therefore disease activity in patients with PBC.

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Chronic liver disease is characterised by the excessive deposition of collagen and other extracellular matrix (ECM) proteins within the liver, the clinical consequences of which are hepatocellular dysfunction and portal hypertension. It is thought that activated hepatic stellate cells (also known as

myofibroblast-like cells, lipocytes, or Ito cells) in the perisinusoidal space are the main contributors to the fibrotic process. However, many hepatic cell types have the ability to produce ECM proteins *in vitro* and little data exist regarding the relevance of these findings to human disease.^{1 2}

Primary biliary cirrhosis (PBC) is a disease of unknown aetiology which is characterised by an abnormal immune response leading to destruction of the interlobular bile ducts and cholestasis. The disease insult and resulting fibrosis is concentrated mainly around the portal tracts.³ Our hypothesis was that in addition to production by hepatic stellate cells (HSC), fibroblasts from the portal tracts might play a significant role in the deposition of collagen in PBC, although the distinction between HSC in acinar zone 1 and portal fibroblasts may be difficult. This subtle distinction between the cell types responsible for the deposition of ECM proteins during hepatic fibrosis may have important implications for the treatment of this disease, with the likely future development of antifibrotic drugs which modulate HSC activation.

Examination of liver biopsy specimens using matrix specific stains permits a semiquantitative estimation of previous collagen accumulation in the liver, but comparison of sequential biopsy specimens has been the only method available in the clinical setting to assess the rate of collagen synthesis and therefore the activity of the collagen biosynthetic pathway. More recently, serum concentrations of procollagen peptides, especially type III procollagen peptide (PIIINP), have proved to be a useful, non-invasive measure of the activity of this pathway at a single time point and have been shown to reflect prognosis and response to treatment in patients with a variety of chronic liver diseases.⁴⁻⁶ Unfortunately, serum concentrations of these peptides are subject to many influences, including hepatic and renal clearance and fibrosis in other parts of the body; this makes interpretation of results difficult.⁴ Many of the techniques used experimentally to assess the activity of the fibrotic process, such as quantitation of the tissue activities of prolyl and lysyl hydroxylase, key enzymes in the post-translational modification of procollagen, are unsuitable for routine clinical use as they require large amounts of tissue.

Clearly a different method of assessing the activity of the fibrotic process is needed. The presence of a specific mRNA within cells

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reflects potential synthesis of the relevant protein⁷ and in the liver, the majority of evidence points towards transcription of the procollagen gene to procollagen mRNA as the rate limiting step in the synthesis of collagen.⁸⁻¹² Therefore, localisation and quantification of procollagen mRNA in liver tissue has the potential to answer two questions at the same time: firstly, it can identify which cells within the liver are actively producing collagen; and secondly, it can also quantitate the activity of the collagen biosynthetic pathway in a specific area of the liver at the time of the biopsy. Northern blotting techniques allow quantification of tissue levels of mRNA with a reasonable degree of accuracy, but require large volumes of tissue and utilise homogenates, making localisation of mRNA impossible. In situ hybridisation (ISH), which allows the identification of specific mRNAs in tissue sections, has previously been used on frozen liver biopsy specimens,¹³⁻¹⁷ but freezing tissue generally leads to a deterioration in cell morphology and also severely limits the amount of material available for research. Until now, ISH experiments on routinely fixed liver tissue have produced contradictory results.¹⁸⁻²⁰ In the present study, we have examined the possibility of adapting the technique of ISH for use on formalin fixed, paraffin wax embedded, needle liver biopsy material, not only to localise cells producing procollagen in PBC biopsy specimens, but also as a potential method of semiquantifying the rate of collagen synthesis in the specimen. The aim of this study was to develop a reliable and reproducible technique of in situ hybridisation for type I procollagen mRNA in formalin fixed liver biopsy specimens; to use the technique to examine stored specimens from patients with primary biliary cirrhosis; and to correlate results with other indicators of extracellular matrix metabolism.

Patients and methods

Fifty unselected biopsy specimens from patients with all stages of PBC were used. Biopsy specimens were taken after informed consent using a Tru-Cut needle, providing needle cores of between 0.1 and 0.2 cm in diameter and 1.5 to 2.0 cm in length; each contained between eight and 20 portal tracts for evaluation. All had been routinely fixed in 10% buffered formalin overnight and subsequently embedded in paraffin wax. Tissue blocks had been stored for between six and 36 months. In addition to assessment of Ludwig's histological stage,²¹ the degree of portal and parenchymal inflammation and fibrosis was semiquantitatively assessed on a scale of 0 to 3 by a blinded, independent histopathologist (MS). At the time of each biopsy, serum was stored at -20°C for later determination of serum PIIINP concentrations using two different commercially available assays: the Behring 226 assay, measuring both col 1 and col 1-3 (synthesis and degradation of type III collagen) and the Behring 238 assay, measuring only col 1-3 (synthesis of type III collagen only).

Sections from a Pagetic bone biopsy, which expresses large quantities of type I procollagen

mRNA, were used as positive controls.²² Two sections of each liver biopsy specimen were used: the test section was hybridised with the antisense probe; and the control section was hybridised with the sense probe. In addition, five biopsy specimens, reported as normal, were also included. These were from patients investigated for mildly abnormal liver function tests.

All glassware was pretreated with diethylpyrocarbonate (DEPC) and sterilised. Gloves were worn at all times. Sections (7 µm) of each biopsy specimen were cut, mounted on 3-aminopropyltriethoxysilane coated slides and dried overnight at 60°C. Sections were deparaffinised in xylene and then rehydrated. After immersion in 0.2 N HCl for 20 minutes followed by 0.05 M Tris HCl (pH 7.4) for two minutes at 37°C, the sections were incubated with 5 µg/ml proteinase K in 0.05 M Tris HCl (pH 7.4) for one hour at 37°C. Subsequently, the slides were sequentially immersed in phosphate buffered saline (PBS) for three minutes; 0.2% glycine in PBS for three minutes; 4% paraformaldehyde for four minutes; 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 minutes; and then air dried.

Sense and antisense probes to rat α_1 procollagen were generated by in vitro transcription using T3 and T7 RNA polymerase promoters (kindly donated to us by Hoechst AG, Frankfurt; data on file). For the antisense probe, the plasmid was linearised by incubating with HindIII in buffer B (Boehringer Mannheim) for two hours at 37°C and for the sense probe the restriction enzyme EcoRI in buffer H (Boehringer Mannheim) was used. The sense and antisense probes were transcribed and labelled using the Boehringer Mannheim RNA transcription kit and ³⁵S UTP. Plasmid was removed by the addition of DNase in the presence of RNase inhibitor.

The probes were subjected to limited alkaline hydrolysis to produce probes of 500 bases by addition of 160 µl DEPC water, 20 µl 0.4 M NaHCO₃, and 20 µl 0.6 M Na₂CO₃ (pH 10.0), and incubation at 60°C for 39 minutes. The reactions were stopped by addition of 6.6 µl 3 M sodium acetate and 1.3 µl glacial acetic acid. Nucleic acid was precipitated with 500 µl ethanol and 2 µl tRNA and stored at -70°C overnight. After centrifugation and decantation, the remaining pellet was dried and resuspended in 19 µl 100 mM dithiothreitol (DTT) with RNase inhibitor.

Labelled probe was added to hybridisation buffer, which consisted of 0.05 g dextran sulphate, 0.3 M NaCl, 0.02 M Tris HCl (pH 8), 0.005 M EDTA (pH 8), 0.01 M DTT, 1× Denhart's solution, 1 mg/ml *Escherichia coli* tRNA, and 50% deionised formamide. Both the sense and antisense mixtures were then incubated at 60°C for 10 minutes before being cooled on ice. Hybridisation was performed under sealed cover slips overnight at 50°C.

Post-hybridisation, the cover slips were removed in 4× saline sodium citrate (SSC). The slides were then sequentially washed in: 2× SSC with 10 mM DTT for five minutes; 2× SSC for 60 minutes; wash buffer (50% deion-

Figure 1 In situ hybridisation to identify type I procollagen mRNA, using an ^{35}S labelled RNA probe on formalin fixed, paraffin embedded liver biopsy material from a patient with PBC. Signal detected by autoradiography. Background stain: haematoxylin and eosin (Fujichrome "Velvia" ASA 50). (A) Sense: low power view of portal area and adjacent parenchyma showing inflammatory infiltration, bile duct damage, and interface hepatitis. Note low level of grain deposition in portal venous channels (original magnification $\times 160$). (B) Antisense: similar area to (A) showing intense, specific reactivity in portal fibroblasts and in cells in acinar zone 1 (original magnification $\times 160$). (C) Sense: bile duct destruction within an inflamed portal tract. Inflammatory cells spill out from the portal tract into the parenchyma with hepatocyte destruction at the limiting plate indicating interface hepatitis. There is low background signal level with no specific localisation (original magnification $\times 400$). (D) Antisense: signal is localised to cells within the inflamed portal tract and strongly to cells around the edges of the portal tract which have the appearances of fibroblasts (original magnification $\times 600$). (E) Same field as in (D) but with dark field illumination, highlighting intensity of grains within portal fibroblasts and fibroblasts within acinar zone 1 (original magnification $\times 600$).

ised formamide, 0.3 M NaCl, 0.02 M Tris HCl (pH 8), 0.001 M EDTA (pH 8), 0.01 M DTT and $1\times$ Denhart's solution) for four hours at 50°C ; and NTE buffer (0.5 M NaCl, 10 mM Tris HCl (pH 8), 1 mM EDTA) for three minutes at 37°C . Non-specifically bound probe was then removed by incubating the slides with a solution of 20 $\mu\text{l}/\text{ml}$ RNase A and 100 U/ml RNase T1 in NTE buffer for 30 minutes at 37°C . Final washes included: NTE buffer for 30 minutes at 37°C ; wash buffer overnight at 50°C ; $2\times$ SSC for 30 minutes; and $0.5\times$ SSC for 30 minutes. Finally, the slides were dried, coated in photographic emulsion (Ilford K5), and left to expose for 10–14 days. After developing and fixing, the slides were counterstained with haematoxylin and eosin.

After systematically viewing the whole biopsy specimen, signal expression from three sources—hepatocytes, cells in and around portal tracts which had the morphology of fibro-

blasts, and perisinusoidal cells in the parenchyma which had the morphology of hepatic stellate cells—was semiquantitatively assessed in each liver tissue section by three investigators independently (CJRG, AJF, and RFTM). The scoring system used was as follows: 0 = no localisation of signal; 1 = probable, but not definite localisation of signal; 2 = definite localisation of signal, but few cells expressing signal; and 3 = definite localisation of signal, with many cells expressing signal.

Results

Slides hybridised with the sense probe showed a variable degree of uniform background signal, with no localisation to any individual cells (fig 1A,C). Those hybridised with antisense probe again showed variable background signal, but also showed localisation of signal to individual cells. Signal strongly localised to osteoblasts in Pagetic bone biopsy specimens

Figure 2 Correlation of total ISH activity score (sum of activities seen over hepatocytes, hepatic stellate cells, and fibroblasts) assessed by independent observers.

hybridised with antisense probe. Most of the cells expressing type I procollagen mRNA in the liver biopsy specimens had the morphology of portal fibroblasts and were located in and around inflamed portal tracts and along the edges of fibrous septa radiating into the parenchyma from portal areas. In only a minority of sections was significant localisation of signal seen over cells lining sinusoids, in zones 2 and 3 of the hepatic acinus, which had the characteristics of hepatic stellate cells. No convincing localisation of signal was seen over hepatocytes in any of the biopsy specimens examined. Within each specimen, there was little variation

Figure 3 Correlation of fibroblast ISH activity score plotted with (A) histologically assessed portal fibrosis and (B) portal inflammation. Bars represent medians of groups. Groups were compared using the Mann-Whitney test, with a two tailed significance level of <0.05 .

in the intensity of signal over cells expressing type I procollagen mRNA (fig 1B,D,E).

There was good correlation in scoring of total ISH activity (hepatocytes + hepatic stellate cells + fibroblasts) between the two initial independent observers (CJRG and RFTM) ($p < 0.005$, $r = 0.63$; fig 2), which was subsequently confirmed by a third independent observer (AJF) in comparison with one of the original pair (RFTM) ($p < 0.005$, $r = 0.67$). For the remainder of the results therefore, the mean

Figure 4 Correlation of (A) total, (B) fibroblast, and (C) hepatic stellate cell ISH activity scores with histological stage, grouped as early (stages 1 and 2) and late (stages 3 and 4) disease. Bars represent medians of groups. Groups were compared using the Mann-Whitney test.

Figure 5 Correlation of fibroblast ISH activity scores (grouped as mild, 0-1; moderate, 1.5-2; or severe, 2.5-3) with serum PIIINP 226. Bars represent means of groups. Groups were compared using a *t* test.

of the two initial observers' ISH activity scores for each of the parameters measured, is used.

Significantly lower total ISH activity scores were seen in the five "normal" liver biopsy specimens when compared with five randomly selected PBC biopsy specimens hybridised in the same experiment (*t* test: $p=0.005$). Inter-assay variation was assessed using five PBC biopsy specimens hybridised in two different experiments. Signal was reproducibly localised to similar areas in different experiments and a significant correlation between total ISH scores in repeat experiments was obtained (Spearman's $r=0.41$, $p<0.005$).

When histologically assessed parameters were compared with ISH activity scores, the strongest correlations were observed between portal inflammation, portal fibrosis, and fibroblast ISH activity scores. This observation is consistent with the underlying pathophysiology of PBC, where the brunt of the inflammatory and fibrotic processes are concentrated around the portal tracts. The fibroblast ISH activity score increased significantly with both increasing portal fibrosis and portal inflammation (difference between groups graded ++ and +++ for portal fibrosis and portal inflammation: $p=0.003$ and $p=0.009$ respectively, Mann-Whitney; fig 3). Early stage disease

Figure 7 Correlation of serum PIIINP 238 concentrations with histological stage, grouped as early (stages 1 and 2) and late (stages 3 and 4) disease. Bars represent means of groups. Groups were compared using a *t* test.

(Ludwig's stage 1+2) tended to have lower total and fibroblast ISH activity scores when compared with late stage disease (Ludwig's stage 3+4) ($p=0.056$ and $p=0.067$ respectively, Mann-Whitney; fig 4), although this just failed to achieve statistical significance.

Total ISH activity scores were grouped as mild (0-2), moderate (2.5-4), or severe (4.5-6) and correlated with serum PIIINP concentrations measured by the 226 and 238 assays. With both assays, mean serum PIIINP concentrations increased with increasing total ISH activity scores, but differences did not reach statistical significance. No correlations were observed between hepatic stellate cell activity scores and serum PIIINP concentrations, but fibroblast ISH activity scores (grouped as mild, 0-1; moderate, 1.5-2; and severe, 2.5-3) increased with rising serum PIIINP 226 concentrations ($p=0.039$, *t* test; fig 5). No significant rise in serum PIIINP 238 concentrations was seen with increasing fibroblast ISH activity scores.

No significant correlations were observed between serum PIIINP concentrations and total inflammation, total fibrosis, acinar zones 2 and 3 inflammation, or fibrosis and portal inflammation. However, serum PIIINP 226

Figure 6 Correlation of serum PIIINP concentrations measured by the different assays: (A) 226; and (B) 238, with semiquantitatively assessed portal fibrosis. Bars represent means of groups. Groups were compared using a *t* test.

but not 238 concentrations increased significantly with worsening portal fibrosis (difference between portal fibrosis graded + and +++, $p < 0.001$, t test; fig 6). Serum PIIINP concentrations measured by the 238 assay, but not by the 226 assay, increased significantly with advancing histological stage (grouped as: early, 1+2; and late, 2+3) of disease ($p = 0.009$, t test; fig 7).

Discussion

This study describes the technique of ISH, which has been successfully adapted for the identification of procollagen mRNA in formalin fixed, paraffin wax embedded liver biopsy material from patients with PBC. Using our method, cells expressing procollagen mRNA could be localised in tissue sections and morphology was excellent. The technique was robust and gave reproducible results. In addition, procollagen mRNA did not appear to deteriorate in tissue sections with prolonged storage time, although this cannot be completely excluded. The identification of mRNAs in routinely processed biopsy material now provides a potent tool for research into hepatic fibrosis.

Assessment of fibrogenic activity in patients with chronic liver disease remains extremely difficult and novel techniques are needed before disease activity and response to treatment can be adequately assessed.²³ Experimental evidence suggests that the rate of collagen synthesis is under transcriptional control⁸⁻¹² and that during the induction of cirrhosis, hepatic procollagen mRNA levels increase either prior to, or at the same time as, an increase in collagen production, then decrease again once cirrhosis is established.⁸⁻¹² Hence, a technique which measures hepatic procollagen mRNA levels offers a potential way of assessing the activity of the collagen biosynthetic pathway. Needle biopsy of the liver is known to be associated with a large sampling error^{24, 25} and synthesis of collagen within the liver may be subject to the same spatial variability, but identification of procollagen mRNA in liver sections may provide more information than standard histology alone.

The localisation of type I procollagen mRNA to cells mainly within the inflamed portal tracts is consistent with the known pathophysiology of PBC and supports our hypothesis that fibroblasts from this area contribute significantly to the deposition of collagen in this disease. The presence of cells expressing procollagen mRNA around the edges of the portal tracts can be explained in two ways. Firstly, active portal fibroblasts may migrate into the parenchyma; or secondly, cytokines from the advancing inflammatory cells may activate resting hepatic stellate cells directly ahead of them.¹ It may well be impossible to differentiate between these two processes, although experimental work in a rat bile duct ligation model by Tuchweber *et al*²⁶ and Desmouliere *et al*²⁷ suggests a role for portal tract derived fibroblasts. Immunohistochemical studies to identify the smooth muscle protein, α_1 actin, are unlikely to resolve this problem, as both activated hepatic stellate cells and fibroblasts

have both been shown to express similar cytoplasmic intermediate filament proteins.²⁸ In our hands, cells having the morphological appearances of portal fibroblasts and hepatic stellate cells both expressed the α_1 smooth muscle actin and desmin proteins immunohistochemically (data not shown). The question is, however, of great importance, as drugs designed to prevent activation of hepatic stellate cells alone would be unlikely to prevent completely the accumulation of collagen seen in PBC.

Previously, there has been conflicting evidence from experimental models concerning the role of hepatocytes in the fibrotic process.^{14, 15, 29, 30} Although most studies indicate that hepatic stellate cells synthesise the majority of collagen in chronic liver disease, hepatocytes can be induced to produce collagen in vitro.^{31, 32} The relevance of this finding, however, to human disease is unknown. The lack of any signal localisation over hepatocytes in our study, indicates that these cells make little or no contribution to the deposition of collagen in PBC.

The expression of procollagen mRNA was limited to only a minority of cells, even in the most active biopsy specimens. Therefore, counting grains over a large number of cells of a certain type in the biopsy specimen and calculating the mean number of grains per cell would be an inaccurate method of quantifying the signal. An alternative method calculates the mean number of cells expressing the gene in several high powered fields of the microscope. However, this latter technique is unsuitable in that the changes seen were patchy and an impartial selection of which area of the slide to move into view would have been impossible. It was therefore decided to adopt the scoring system where the whole biopsy section was scanned before allocating a semiquantified score of procollagen mRNA expression, as described in the Methods section. The ISH activity scores obtained using this method were highly reproducible, with good correlation shown between independent observers and excellent intraobserver correlation.

In PBC, the disease insult and resultant fibrosis is concentrated around the portal tracts. It was no surprise therefore that the ISH activity scores of cells in, and immediately around the portal tracts showed the best correlations with standard histological assessments of fibrosis and inflammation, histological stage, and with serum markers of collagen turnover. ISH activity scores from cells with the appearances of activated hepatic stellate cells in zones 2 and 3 of the hepatic acinus showed no correlation with these parameters. Portal fibroblast ISH activity scores correlated best with assessments of portal fibrosis, but also with portal inflammation. This observation was consistent with the trend observed between increasing fibroblast ISH activity score and higher Ludwig histological stage (1+2 versus 3+4), the latter being largely dependent on the assessment of the degree of hepatic fibrosis. Our results therefore indicate that the expression of procollagen mRNA might be increased with

advancing histological stage. An acceleration in the synthesis of collagen in Ludwig's stages 3 and 4 of PBC may play a role in the rapid deterioration in hepatic function which is often seen in the later stages of the disease.³³

Technical constraints determined that we identified type I procollagen mRNA in the liver biopsy specimens and type III procollagen peptides in the serum. There is however good evidence that these two fibrillar collagens are synthesised in roughly similar proportions in hepatic fibrosis.³⁴ In animal models, the final amount of collagen deposited correlates with the area under the curve of serum PIIINP measurements during the induction of cirrhosis.³⁵ In addition, increases in serum PIIINP have been shown to correlate with hepatic type III procollagen mRNA levels.⁹ The PIIINP 238 assay measures only the larger col 1-3 peptide which is cleaved from the procollagen molecule immediately prior to its inclusion in a collagen fibril, whereas the PIIINP 226 assay also measures col 1, which is released during the degradation of collagen fibrils.⁴ The numerical difference between these two assays may therefore reflect the rate of collagen degradation.⁴ There was a significant increase in serum PIIINP 226 concentrations with increasing fibroblast ISH activity scores and also a trend towards an increase in serum PIIINP 238 concentrations. It is known that during the induction of experimental fibrosis the col 1-3 peptide predominates in the serum, but once cirrhosis is established, the proportion of the col 1 fraction increases.³⁶ Collagen fibrils are degraded as part of a remodelling process in liver fibrosis¹ and it is possible that, as collagen synthesis increases, collagen degradation also increases and that this is reflected in the better correlation of fibroblast ISH activity scores with the assay which measures both synthesis and degradation. A changing (increasing) ratio of type I:type III collagen in the later stages of cirrhosis may also affect observed correlations between ISH for type I collagen and serum assays reflecting synthesis/turnover of type III collagen. We propose to extend the current study to identify type III procollagen mRNA in liver biopsy specimens from patients with PBC and to correlate these results with serum markers of type III collagen turnover.

ISH provides a novel method in patients with PBC for direct assessment of the rate of collagen synthesis using a single biopsy sample, although such a method has been described in patients with chronic viral hepatitis,³⁷ and for quantitation of the activity of the disease process at the time of the biopsy. Long term follow up studies are now needed to assess the value of semiquantitative assessment of procollagen mRNA in liver biopsy sections and whether this technique can provide additional prognostic information compared with standard histological examination.

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