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Altered Proteoglycan Gene Expression in Human Biliary Cirrhosis⁺

Ilona KOVALSZKY,¹ Julia O NAGY,¹ Mónika GALLAI,¹ Anna SEBESTYÉN,¹
Zsuzsa SCHAFF,¹ Sándor PAKU,¹ András JENEY¹ and Renato V. IOZZO²

¹Semmelweis University of Medicine, First Institute of Pathology and Experimental Cancer Research; ²Thomas Jefferson University, Philadelphia, USA

Proteoglycans play key roles in the physiological assembly of extracellular matrices and in the modulation of growth factor activities. During liver regeneration there is a profound remodelling of the connective tissue network with a concurrent alteration in proteoglycan gene expression. In the present study we have analyzed in detail the biochemical and molecular properties of the proteoglycans associated with biliary cirrhosis. The three major proteoglycans of human liver, namely decorin, syndecan and perlecan, were markedly elevated in the cirrhotic parenchyma as compared to normal liver tissue. Particularly elevated (eight

fold) was the perlecan. This proteoglycan had not only heparan sulfate but also chondroitin and dermatan sulfate. Reverse transcriptase PCR revealed a marked enhancement of decorin and syndecan expression and detectable message for perlecan was found only in the cirrhotic liver. These results indicate that significant proteoglycan alterations are associated with the development of biliary cirrhosis and provide basis for future studies aimed at the characterization of the molecular events involved in the regulation of extracellular matrix deposition in this common human disease. (Pathology Oncology Research Vol 3, No 1, 51–58, 1997)

Key words: proteoglycan, biliary cirrhosis, decorin, perlecan, syndecan, GAG

Introduction

Biliary cirrhosis, one of the most life-threatening diseases of the liver, is associated with a marked proliferation of connective tissue cells and a striking deposition of extra-

cellular matrix products.^{41,42,48,49} A large body of evidence has provided the theoretical basis for explaining the main pathogenetic mechanisms governing the induction, maintenance, and progression of this fibro-regenerative process. Recent findings indicate that nonparenchymal liver cells, particularly the perisinusoidal fat storing cells, are the main source of extracellular matrix protein synthesis.^{3,32,53} The activation of these cells which is induced by several growth factors and cytokines, results in an imbalance of matrix-synthesizing abilities and degrading processes.^{1,8,12,18,50,56} In contrast, endothelial cells and hepatocytes promote the deposition of the matrix proteins, presumably by a direct activation of the cell-surface transducing mechanisms or via their cell surface matrix receptors.^{11,22}

Proteoglycans are intrinsic components of this altered stroma and have been implicated in the pathogenesis of liver fibrogenesis. Both human and animal studies have demonstrated that the glycosaminoglycan constituents of the proteoglycans are markedly elevated in amounts and exhibit qualitative changes in cirrhotic liver parenchyma as compared to normal liver.^{2,7,24,25,44,58} A constant finding amongst these studies is a relative increase in chondroitin and dermatan sulfate glycosaminoglycans, two linear sulfated carbohydrates that are primarily found in extracellu-

Received: March 22, 1997; accepted: April 15, 1997

+This work was supported in part by grants ETT M59 from the Hungarian Ministry of Health and T 017085 from the National Scientific Research Foundation. Dr. Iozzo was supported by NIH grants CA-39481 and CA-47282 and is the recipient of a Faculty Research Award (FRA-376) from the American Cancer Society.

Correspondence: Dr. Ilona KOVALSZKY, First Institute of Pathology and Experimental Cancer Research, Semmelweis University of Medicine; Üllői út 26, H-1085 Budapest, Hungary. Tel: (36)(1) 266-1638/4449

Abbreviations: bFGF – basic fibroblast growth factor; BM – basal membrane; CS – chondroitin sulfate; CSPG – chondroitin sulfate proteoglycan; DEPC – diethyl pyrocarbonate; DS – dermatan sulfate; ECM – extracellular matrix; EDTA – ethylenediaminetetraacetic acid; EHS – Engelberth-Holm-Swarm; GAG – glycosaminoglycan; HPLC – high performance liquid chromatography; HS – heparan sulfate; HSPG – heparan sulfate proteoglycan; OD – optical density; PG – proteoglycan; RNA – ribonucleic acid; TCA – trichloroacetic acid; TFMS – trifluoromethanesulfonic acid; TGF- β – transforming growth factor beta-1

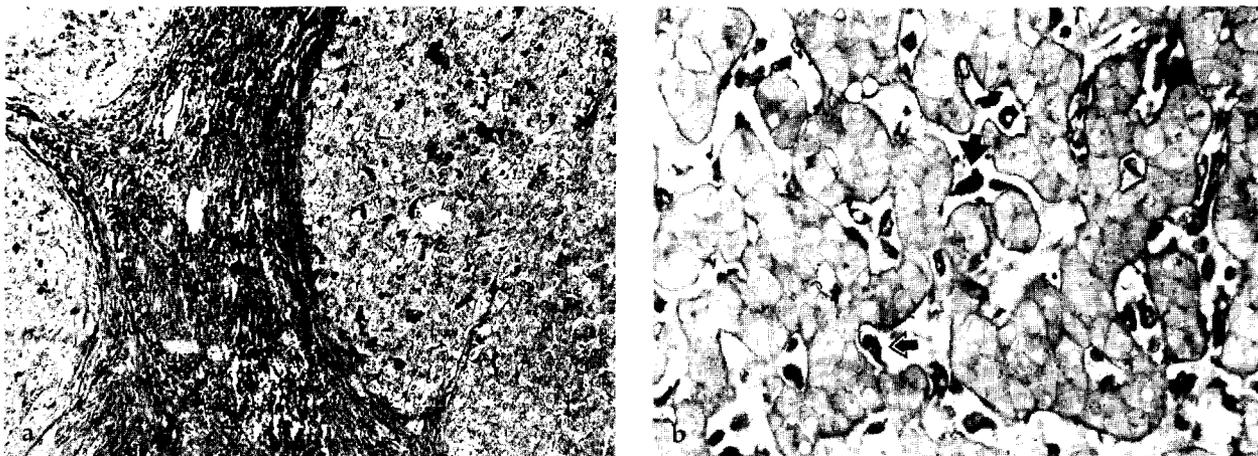


Figure 1. Histology of cirrhotic liver. (A) Cirrhotic liver stained with Mallory's trichrome for connective tissue. (B) Higher magnification of hepatic cells stained with colloidal iron for sulfated proteoglycans. Note the presence of intracytoplasmic deposits of proteoglycans (A= X 100; B= X 400).

lar proteoglycans. The recent cDNA cloning of various proteoglycan protein cores^{36,37,40,45,47} provides the opportunity to study in more detail and with more specific probes the molecular nature of the abnormal proteoglycan gene expression associated with biliary cirrhosis.

Because very little is known about the proteoglycan makeup of human hepatic cirrhosis, we decided to investigate in detail the biochemical and molecular biological nature of the proteoglycans associated with biliary cirrhosis and to compare to that of normal human liver. The results show a profound change in proteoglycan pattern, and further stress a role for these important macromolecules in this severe human disease.

Materials and Methods

Tissue processing and morphological studies

Liver specimens were obtained from the 2nd Pediatric Department of Semmelweis University of Medicine. The liver of a 10 years old girl was removed because of congenital biliary atresia and secondary biliary cirrhosis. This specimen and part of the adult donor liver, not utilized for transplantation, were subjected to routine histological, biochemical and molecular biological studies. Sections were stained with hematoxylin-eosin; connective tissue and glycosaminoglycans were detected with Mallory staining and with colloidal iron binding,⁵⁵ respectively.

Purification and characterization of proteoglycans

Liver tissue was homogenized under liquid nitrogen, then extracted with 4 M guanidin HCl, 1% Triton X-100, 50mM sodium acetate, 10 mM EDTA, 5 mM N-ethylmaleimid, 0.5 mM phenylmethanesulphonyl fluoride, 10

ug/ml Soy bean trypsin inhibitor, pH 5.0, at 4°C for 24 hours. Cell debris was removed by centrifugation. TCA precipitation of supernatant was carried out³⁹ to remove nucleic acids and the majority of liver proteins other than PGs. The supernatant was dialyzed against 7 M urea, 50 mM Tris-HCl, pH 7.0, containing protease inhibitors as above. Dialyzed material was loaded onto 1 ml DEAE-52 (Whatman, USA) columns equilibrated with 7 M urea, 50 mM Tris-HCl, pH 7.0. The resin was washed with 10 volume of the same buffer containing 0.1 and 0.2 M NaCl, and PGs were eluted with 1 M NaCl, or step-wise with increasing concentration of NaCl at pH 7.0. 3-11% polyacrylamide gradient gels³⁸ were run overnight (0.8 mA/lane), fixed and stained with Coomassie brilliant blue for proteins or with Alcian blue 8GX for PGs. For Western blotting, PGs were separated as described above and electrotransferred to Schleicher-Schüll BA-S 83 reinforced cellulose nitrate membrane.

After transfer, blots were dried and blocked with 1% BSA in TBS buffer (20mM Tris-HCl, pH 7.5, 150 mM NaCl) overnight. Filters were washed three times with TBST (TBS+0.05% Tween 20) then reacted with 1:100 diluted polyclonal antibodies against EHS proteoglycan and decorin. Peroxidase or alkaline phosphatase conjugated goat anti rabbit serum was used as secondary Ab. Reaction was detected with DAB for peroxidase and with NBT and BCIP for alkaline phosphatase. For HPLC, 100 of isolated PGs, corresponding to PG content of 0.1 g liver, was loaded onto 7.5x600 mm Spherogel TSK SW (Beckman, USA) column for size exclusion chromatography. Samples were eluted using 0.01 M Na phosphate, 0.15 M NaCl, pH 6.5, at 2 ml/min. flow rate. The approximate molecular size of the peaks were estimated by comparing their retention time with that of the molecular size standards.

Characterization of glycosaminoglycans and TFMS deglycosylation.

Isolated PGs or fractions eluted from the HPLC columns were used. Buffer was exchanged for H₂O by washing the samples in Millipore (USA) UFP2 LGC 24 disposable filter units. NaOH in a final concentration of 0.5 M was added and samples were incubated at room temperature for 4 hours. After neutralization with HCl, the remaining peptides were removed by 10% TCA precipitation. Samples were clarified by centrifugation (2000xg for 20 min.), and GAGs were precipitated with 96% ethanol, 1% acetic acid, 1% potassium acetate overnight at 4°C. Samples were run on cellulose acetate strips in 0.1 M Ca acetate buffer, pH 5.6, with 3 mV and 0.1 mA per lane for 2 hours. Filters were stained with 0.1% Alcian blue 8GX in phosphate buffer. To identify them, the migration of isolated GAGs were compared with those of commercial GAG standards. About 10 µg GAG were digested with 0.2 U/ml Chondroitinase ABC (Seikagaku, Japan) in 0.1 M Tris-HCl, pH 8.0, for two hours at 37°C.

For deglycosylation, 2 mg of PG was treated with 1:2 (v/v) of anisol:TFMS (Fluka, Switzerland) under nitrogen for 6 hours at 4°C, with continuous shaking. Subsequently, fifty volume of n-hexane with 10% ether (v/v) was added, and the precipitation was induced with 1 drop of pyridine. Samples were placed to -70°C for 1 hour, then centrifuged at 10,000xg for 30 min. Pellet was washed with ether, then dissolved in 96% ethanol. Insoluble protein was further pelleted at 10,000xg for 1 hour. Pellets were dried and dissolved in 50 µl sample buffer, boiled for 5 min., and run on polyacrylamide gels.¹⁶

Zymography for gelatinase activity

Isolated PGs were separated on SDS-polyacrylamide gel, which contained 1 mg/ml gelatin. After electrophoresis the gels were washed in 50 mM Tris-HCl, 2.5% Triton X-100, pH 7.5, at room temperature for two hours, and rinsed with 50 mM Tris-HCl buffer, pH 7.5. Subsequently, parallel samples were incubated in 50 mM Tris-HCl, 10 mM CaCl₂, pH 7.5 or in 50 mM Tris-HCl, 10 mM EDTA, pH 7.5, for 18 hours. Gels were stained with Coomassie Brilliant blue.⁹

Isolation of RNA and reverse transcriptase PCR

Total RNA was isolated by the method of Chomczynsky and Sacchi¹⁰ with 10 mM vanadyl ribonucleoside as RNase inhibitor. For RT-PCR, 1 µg DNase-treated RNA was used. First strand synthesis was made using MMLV reverse transcriptase (BRL, USA) and 20 pmol downstream primer, 50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl₂, pH 8.3, with 1 mM dNTPs, 20 U RNasin (Promega, USA) in 20 µl final volume. Samples were incubated for 30 minutes at 42°C. Reverse transcriptase was then denatured at 95°C for 5 min-

utes, and the amplification was carried out with 50 pmols of primers, 1x PCR buffer, 2 U Taq polymerase (Perkin-Elmer, USA) in 100 µl final volume for each samples. Thirty cycles were as follows: 94°C 1 min., 92°C 30 sec., 60°C 30 sec., 75°C 1.5 min.

The specificity of the products were confirmed by hybridization with the corresponding cDNA probes. Ten µl of the PCR products were separated by agarose gel and processed for Southern blotting. Human cDNAs for decorin,³⁶ syndecan,⁴⁰ or perlecan¹⁵ were individually labeled by random priming (Boehringer, Germany) to high specific activity (~10⁹ cpm/µg DNA). Filters were hybridized at 42°C in a solution containing 50% formamide, 5xSSC, 25 mM Na phosphate buffer, pH 6.8, 2x Denhardt's solution, 25 µg/ml salmon sperm DNA, 0.01% SDS and 5% dextran sulfate.

	Primers	Position in cDNA
Decorin		
sense	5'-GAT GAG GCT TCT GGG ATA-3'	172-189
antisense	5'-CAT CTG GTT CAG TCC ATC-3'	591-609
Perlecan		
sense	5'-TCC CTG GAC ACA GAT GGC-3'	856-873
antisense	5'-ACC CAT GCA GAA ACA GGG-3'	1152-1170
Syndecan		
sense	5'-ATG TCG ACG GAG GTG GAG-3'	247-267
antisense	5'-GCT GAG CCT GCA GCT GGC-3'	460-477

Other assays

Uronic acid was determined by using β-D-glucuronic acid as a standard.⁶ Proteins were quantitated by the method of Hartree.²⁸ Polyclonal antibody against decorin was obtained from Telios Pharmaceuticals (La Jolla, USA). Antibody against EHS HSPG was raised by repeated s.c. administration of 500 µg purified PG with 0.5 ml Freund adjuvant to New Zealand rabbits. After reaching the optimal titer, animals were exsanguinated in anaesthesia. The polyclonal Ab did not cross-react with laminin or fibronectin.

Results

Histology

The donor liver showed no morphological alteration. In the liver with advanced cirrhosis pseudolobuli of various size were surrounded by abundant connective tissue which was positive with the Mallory trichrome stain (*Fig. 1A*). Alongside the sinusoids, especially adjacent to fibrous septa, oval or spindle shape cells could be recognized even with HE staining, in quite large number. In addition, the cytoplasm of these cells was positive for proteoglycans (*Fig. 1B*). These results suggest that proteoglycan synthesis occurs primarily in nonparenchymal liver cells.

Table 1. Glycosaminoglycan and protein content of PGs isolated from normal and cirrhotic liver

	Normal liver	Cirrhotic liver
Uronic acid (mg/g wet weight)	58.3 ± 3.5	153.0 ± 8.1
Protein (mg/g wet weight)	108.9 ± 9.6	232.8 ± 17.5
Heparan Sulfate	60-65%	55-60%
Dermatan Sulfate	30-35%	30-35%
Chondroitin Sulfate	0-5%	15-20%

PGs were isolated from two gram liver samples. Uronic acid and protein content was determined by the method of Bitter et al,³⁴ and Hartree,³⁵ respectively. The same PGs were beta eliminated and GAGs separated by zone electrophoresis to determine the proportion of sugar components. Results for uronic acid and protein represent the mean ± SD of four independent determinations.

Biochemical properties of proteoglycans in biliary cirrhosis

Purified PGs isolated from 1 g of cirrhotic liver contained twice as much protein and three times more uronic acid than PGs from normal liver (Table 1). Interestingly, the uronic acid/protein ratio was also increased in the cirrhotic tissue (from 0.53 to 0.66), thus suggesting that the cirrhotic PGs were more glycosylated than the normal counterpart. The GAG pattern was also significantly changed. While the HS:DS ratio remained essentially the same (2:1) in both instances, the proportion of CS dramatically increased in the cirrhotic liver (Table 1).

Proteoglycans were purified and analyzed by size-exclusion HPLC on Spherogel TSK column (Fig 2). Four broad peaks, designated I-IV, appeared at retention time of 20, 25, 36, and 42 minutes, respectively. Peaks I-III corresponded

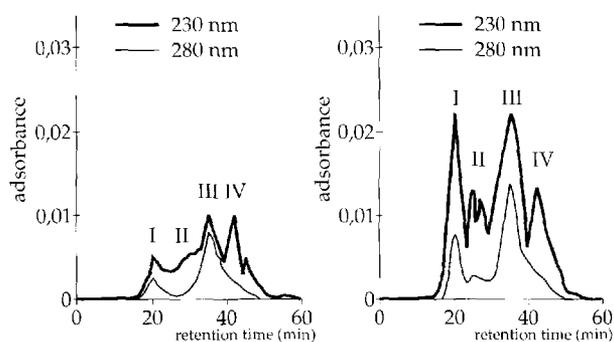


Figure 2. Separation of PGs on Spherogel TSK SW (Beckman) HPLC column, according to their molecular size. Mr of peak I-IV are as follows: >450, 200-250, 80-100, 30-50 kDa. Because equal amounts of extractable PGs were loaded on the columns, the individual peaks are comparable between normal (A) and cirrhotic (B) PG pools. For additional details, see Tables 1-3.

to intact PGs, while peak IV was composed of degraded PG. These peaks represented approximate molecular mass of >450, 200-250, 70-100, 30-50 kDa, respectively (Fig.2). Analysis of PGs isolated from comparable quantity of normal and cirrhotic liver tissue showed that all fractions increased in the latter. Of note, the higher absorption values at 230 nm indicate enhanced glycosylation of the cirrhotic PGs, in agreement with the data presented above. By means of HPLC and ion exchange chromatography the proportion of the three PG peaks and their degree of glycosylation were also determined (Table 2). The vast majority (90%) of normal liver PG occurred in the 80-100 kDa size range (peak III), while the higher molecular weight proteoglycans (peaks I and II) represented only a minor component (10%). The proteoglycan pool of peak III was glycosylated exclusively with heparan sulfate chains. In contrast, in the cirrhotic liver there was a marked increase of the high molecular weight fractions I and II, which together comprised 38% of total PG pool. In addition, pool I differed in other two ways: (i) it contained heparan sulfate and chondroitin sulfate, rather than heparan sulfate and dermatan sulfate as in normal liver (Table 2), and (ii) was less charged inasmuch as a larger proportion of it was recovered in the 0.3 M NaCl fraction (Table 3). Taken together, these results indicate that both quantitative and qualitative changes occur in human biliary cirrhosis and suggest that high molecular weight proteoglycans with both heparan and chondroitin sulfate chains are associated with this disease.

Western blotting of decorin and perlecan proteoglycan

Antibodies against decorin or perlecan were used on native or TFMS deglycosylated proteoglycans (Fig.3). Decorin Ab reacted with 2 bands of 45 kDa (Fig.3, lane 2), two species of decorin which are likely due to the presence of different degree of glycosylation. The reaction was clearly stronger in the cirrhotic PG pool in agreement with the biochemical data. Interestingly, native PG fractions from normal liver gave hardly any reaction with anti-perlecan antiserum, indicating low levels of expression of this proteoglycan under normal conditions. In contrast, the cirrhotic sample was strongly reactive with the anti-perlecan antiserum, with a broad band of high molecular weight typically seen in electrophoresis of this proteoglycan (Fig.3, lane 5). Following TFMS deglycosylation, two sets of proteins appeared in cirrhotic liver, a large molecular size band of ~400 kDa and smaller of ~68 kDa (Fig. 3, lane 6). Normal liver PG did not contain the large band and the smaller bands were hardly visible (Fig.3, lanes 3 and 4). These data indicate that the high molecular weight proteoglycan that is increased in cirrhotic liver is perlecan and suggest that the protein core is substituted with both heparan and chondroitin sulfate chains.

Table 2. Distribution of various PG fractions in normal and cirrhotic liver

HPLC fraction	Mr Da	Normal liver		Cirrhotic liver			
		GAG mg/g wet weight	protein %	GAG mg/g wet weight	protein %		
I	>450	HS-DS	9	8	HS-CS	71	31
II	200-250	HS-DS	2	2	HS-DS	16	7
III	80-100	HS	97	90	HS	145	62
Total			108		232		

Areas under 280 nm peaks of HPLC fractions were used to calculate the proportion of each PG fractions. Since for sufficient separation of fraction II the combination of ion exchange chromatography and size exclusion chromatography on HPLC was required; data presented implicate the results of combined separations as well. Data are representatives of three independent experiments. Standard deviation among the results of individual experiments were smaller than 15%.

Detection of decorin, syndecan and perlecan messages by reverse transcriptase PCR

To assess the expression of decorin, syndecan and perlecan in normal and cirrhotic liver, we employed RT-PCR and Southern blotting (Fig.4). To minimize quantitative errors, the same amount of total RNA and identical conditions were used in both the RT-PCR procedure and the subsequent hybridization.

The results showed a marked upregulation of decorin and syndecan in cirrhotic liver. In addition, perlecan message was detected only in cirrhotic RNA sample (Fig.4, lane 4), in agreement with the Western blot presented above. Taken together, these results support the biochemical data and further indicate that syndecan message is also increased in biliary cirrhosis.

Table 3. Charge distribution of PGs in various fractions of normal and cirrhotic liver

HPLC fractions	M NaCl	Normal liver			Cirrhotic liver		
		0.3	0.4	0.7	0.3	0.4	0.7
I	>450	0	91.3	8.6%	23.5	76.5	0%
II	200-250	100	0	0%	100	0	0%
III	80-100	46.6	4.2	49.2%	48.5	0	51.5%

Samples, separated stepwise on ion exchange chromatography, were further fractionated according to their size on HPLC. Material could be detected only in 0.3, 0.4, 0.7 M NaCl containing fractions. The data are representatives of three independent experiments. Standard deviation among the results of individual experiments were smaller than 15%.

Gelatinase activity in isolated proteoglycans from cirrhosis

During the several extractions of PGs, we noticed that the purified PG samples from the cirrhosis were more susceptible to degradation than the corresponding samples isolated from normal liver. For example, we often observed low Mr 10-15 kDa peaks in the cirrhosis, but we never observed them in the normal counterpart (data not shown). The fast degradation of cirrhotic PGs raised the possibility that proteolytic enzymes might have co-purified with the PGs. This presumption was confirmed by gelatinase zymography. When isolated PGs without TCA precipitation (TCA precipitates macromolecules other than PG from guanidine HCl) were loaded on gelatine containing polyacrylamide gel both normal and cirrhotic liver PG contained gelatinase activity at 92 and 300 kDa, respectively (Fig. 5). These activities, however, disappeared if guanidine HCl dissolved samples were precipitated with TCA. At the same time cirrhotic PG still contained activity at very high molecular size position, resistant to TCA precipitation, which was invisible before, due to the masking effect of Coomassie-blue stained proteins. Further characterization revealed that in contrast to the TCA precipitable proteases the large molecular size activity, observed in cirrhotic PG was resistant to EDTA. This activity, however, was abolished by reducing conditions (not shown). The independence from cations suggests that the cirrhosis-associated gelatinase is not a metalloproteinase.

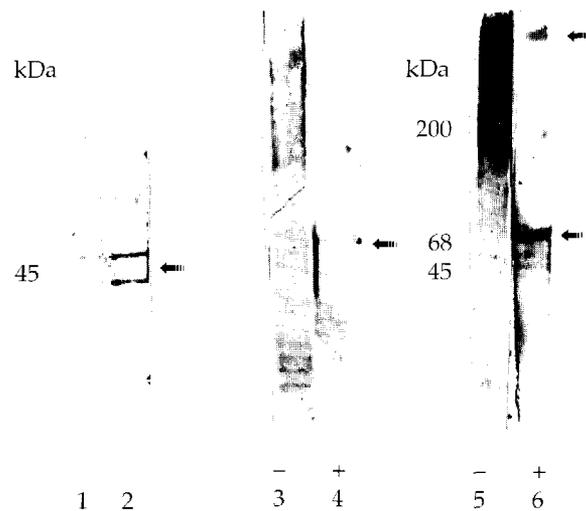


Figure 3. Western blot of normal (lanes 1, 3 and 5) or cirrhotic (lanes 2, 4 and 6) proteoglycans reacted with anti-decorin (lanes 1,2) and anti-perlecan (lanes 3-6) antibodies. Native (-) or TFMS deglycosylated (+) PG was separated on gradient PAGE and blotted to nitrocellulose membrane. Arrows indicate the position of protein cores reactive with the Abs. Reaction with native PG appeared as a smear in the high molecular size region. The intensity of the reactions in cirrhotic PG was much stronger than in normal liver.

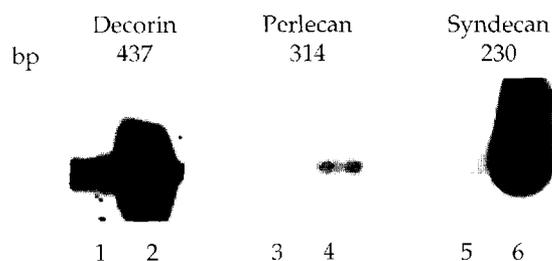


Figure 4. Detection of PG mRNA by reverse transcriptase PCR and Southern blotting. Lanes 1, 3 and 5 are from normal liver, while lanes 2, 4 and 6 are from cirrhotic liver. Primer sets specific for the indicated proteoglycans are described in "Material and Methods". The specificity of the reaction was determined by the expected size of the product and by hybridization with specific cDNA probes.

Discussion

The nature of the hepatic glycosaminoglycans have been known for some time. Specifically, normal hepatic parenchyma contains heparan sulfate as the major GAG with trace amounts of dermatan and chondroitin sulfate.³⁴ In contrast, in both experimentally-induced and human cirrhosis there is a marked increase in GAG content with an associated elevation of dermatan and chondroitin sulfate.^{2,7,21,44} Introduction of molecular cloning and the availability of specific antibodies has provided tools to determine the types and localization of PGs in the liver. Data from the last 2-3 years indicate that liver cells produce different PGs,^{4,23,27,52} with fibroglycan as the major HSPG of normal rat liver.⁴⁷ Although not directly proven, this heparan sulfate proteoglycan is produced by the hepatocytes, while the other extracellular PGs, such as decorin, perlecan, versican, and biglycan^{4,23,35,52} are synthesized by the nonparenchymal liver cells. Because decorin, biglycan and versican are DS- and CS-containing PGs, it is conceivable that the elevated DS and CS content, observed in the studies summarized above, reflects an increased deposition of these PGs in cirrhosis. Certainly, expression of decorin and biglycan increases in activated Ito cells *in vitro*,²⁷ while endothelial cells lining the sinusoids synthesize primarily perlecan.⁵² Also, relative to normal, nonparenchymal cells isolated from cirrhotic liver express more decorin, versican, and perlecan on mRNA level.⁴³ This suggests that nonparenchymal liver cells activated by chronic liver injury also produce extracellular PGs.

No data are available concerning the changes of fibroglycan and syndecan in cirrhotic liver. It was proposed that hepatocytes and Ito cells may be the source of syndecan.⁴ Our studies (unpublished data) indicate, that Kupffer cells are responsible for syndecan production. Probably hepatocytes respond to injury by elevated fibroglycan expression, but this presumption needs to be proven. Enhancement of ECM is considered a result of an imbalance between the synthesis and degradation of extracellular matrix con-

stituents, a process finely regulated by growth factors and cytokines.^{1,8,12,50} The significance of PG modulation is not yet completely understood. It was shown that highly specific function of hepatocytes required well defined matrix composition.^{5,19} PGs and GAGs are able to induce synthesis of liver specific proteins, and promote intercellular communication of hepatocytes via mechanisms that are not yet elucidated.²⁰ HS chains can inhibit hepatocyte proliferation,^{17,30} can bind several factors like b-FGF,^{51,60} hepatocyte growth factor (Lyon et al. Annual research report 1992; Christie Hospital p.104), and matrix proteins³³ that are implicated in the regulation of liver function and tissue integrity. The protein core of decorin, for example, binds and inactivates TGF- β 1, the major stimulating factor of matrix proliferation^{54,59} Synthesis of several PGs are also regulated by this cytokine and its effect on decorin expression may be cell-specific. Inhibition has been reported on fibroblast decorin mRNA level,³¹ while stimulation on that of the perisinusoidal lipocytes⁴³ was found. TGF- β 1 responsive silencing cis-acting elements on the decorin gene would explain the inhibitory effect of this growth factor.^{14,29} The conflicting data suggest the existence of various feed-back loops in different decorin producing cells. Decorin also regulates the assembly of collagen fibers⁵⁷ and thus is directly involved in the assembly of the matrix around the regenerating liver nodules. The present work shows significant biochemical alterations in the proteoglycan gene expression in human biliary cirrhosis and reports novel findings regarding the upregulation of perlecan in this process. The marked

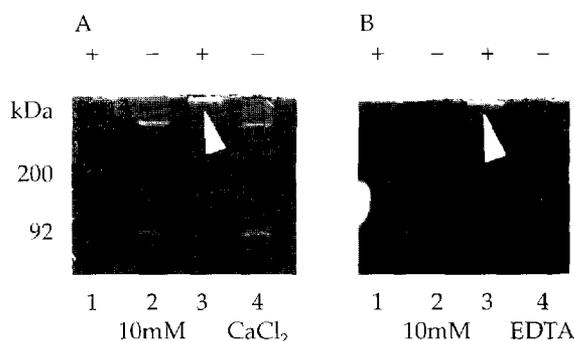


Figure 5. Gelatinase activity co-purifying with proteoglycan pools in the presence (A) or absence (B) of calcium. The plus and minus signs indicate TCA precipitation before the assay. In panel A, notice that in both normal (lane 2) and cirrhotic (lane 4) samples the two typical activities were found corresponding to the 92 kDa collagenase and the 300 kDa gelatinase. Interestingly, TCA precipitation removed both of these activities (lanes 1 and 3) but a new one appeared on the top of the gel in the cirrhotic liver (lane 3). (This band was masked by the proteins stained with Coomassie brilliant blue). Panel B shows that removal of calcium by EDTA abolishes the two lower-Mr activities but leaves intact the 300 kDa activity (lane 3), thus precluding the possibility that the enzyme is a metalloproteinase.

increase in the total glycosaminoglycan content correlated with increased glycosylation of the PGs in cirrhosis. The connective tissue of regenerating nodules contained more protein core and more glycosaminoglycans attached to it. Particularly elevated was the large perlecan proteoglycan, which we have recently cloned in the human⁴⁵ and assigned to the telomeric region of chromosome 1.¹⁵ The presence of perlecan has been demonstrated alongside the normal human liver sinusoids.⁴⁶ Since this PG is one of the major component of basement membrane its increased deposition presumably accompanies the sinusoidal capillarisation. Interestingly, the data indicated that the perlecan from cirrhotic liver contained both heparan and chondroitin sulfate chains. This is in agreement with the finding of a hybrid proteoglycan containing both chondroitin and heparan sulfate chains in a permanent cell line established from the murine EHS tumor.¹³ Collectively, these findings suggest that in human biliary cirrhosis the PG make up is similar to that of experimentally induced disease, in that only the extracellular matrix proteoglycans are markedly elevated. This concept was corroborated by the Western blotting and by the RT-PCR, where increased expression of decorin, perlecan and syndecan was observed.

Finally, our study has attempted to identify the cause of an apparent propensity of the cirrhotic proteoglycan to undergo proteolytic degradation during the extraction procedure, in spite of the presence of numerous protease inhibitors in the extracting buffer. We have identified at least three major proteolytic activities co-purifying with the proteoglycan. One of these, the 300 kDa fraction, was recovered exclusively in the cirrhotic sample, thus providing a plausible explanation for the enhanced PG degradation in this disease. Interestingly, this activity was not inhibited by EDTA indicating that the enzyme is not a metalloproteinase; however, its activity could be abrogated by reducing agents such as mercaptoethanol. The fact that this degradative activity could not be removed by TCA precipitation indicate a tight coupling with the cirrhotic PG and suggest that one of the possible function of extracellular matrix proteoglycans is the binding of proteases.

Acknowledgements

We thank Dr E Rouslahti and Dr M Jalkanen for providing the cDNAs for decorin and syndecan, respectively.

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