EXPRESSION OF TYPE IV PROCOLLAGEN AND PROLYL 4-HYDROXYLASE mRNA IN CARBON TETRACHLORIDE-INDUCED LIVER FIBROSIS STUDIED BY IN SITU HYBRIDIZATION

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An experimental model of liver fibrosis was used to study the expression of type IV procollagen and prolyl 4-hydroxylase mRNAs by in situ hybridization. Paraffin sections of rat liver tissue were examined using non-radioactive digoxigenin-labeled cDNA probes and in situ hybridization employing mouse α1 (IV) procollagen cDNA and chicken prolyl 4-hydroxylase β-subunit cDNA in order to identify cells responsible for the production of type IV procollagen and prolyl 4-hydroxylase mRNAs after administration of carbon tetrachloride (CCl₄). Localization of type IV procollagen and prolyl 4-hydroxylase mRNAs was demonstrated in the cytoplasm of mesenchymal cells after six weeks of CCl₄ administration. Type IV procollagen and prolyl 4-hydroxylase mRNAs were noted in hepatocytes after eight and ten weeks respectively. The expression of type IV procollagen mRNA was found in a large number of hepatocytes in proportion to the extent of fibrosis. These results suggest that in addition to the mesenchymal cells, hepatocytes also play an important role in fibrogenesis of the liver.

Prolyl 4-hydroxylase is a key enzyme in collagen biosynthesis, in which it catalyzes the conversion of peptidyl proline to peptidyl hydroxyproline in collagenous polypeptides (11). It has been reported that an increase in collagen synthesis is usually accompanied by an increase in prolyl 4-hydroxylase (1) and this has also been found to be the case in liver fibrosis (18).

Collagen type IV is a nonfibrillar collagen (23), the amount of which in the liver contributes to the rearrangement of the lobular architecture, in turn possibly modulating hepatocellular function and being involved in portal hypertension (13). Some reports have suggested that this collagen in liver is synthesized mainly by fibroblasts or the precursor cells (4, 14). On the other hand, it has been reported that hepatocytes are able to produce several types of collagen and other extracellular matrix components in vitro (6, 20, 24).

Molecular biological studies using slot-blot hybridization have revealed that procollagen mRNAs are present in fibrotic lesions in liver tissue. However, since the individual cells containing procollagen mRNA cannot be recognized by the filter hybridization technique (16), it is unclear which types of cell contribute to the production of collagen in liver fibrosis. Using the in situ hybridization technique, the cells...
that contribute to the synthesis of collagen could be identified. In this study, the hepatic localization of type IV procollagen mRNA and prolyl 4-hydroxylase mRNA was examined by in situ hybridization at various stages after administration of carbon tetrachloride (CCL$_4$) to rats.

**MATERIALS AND METHODS**

Fifty percent CCL$_4$ in olive oil (2 ml/kg body weight) was given to male Wistar rats (initial weight, 100-120 g) by intraperitoneal injection twice a week for 10 weeks. Five rats were killed in each group after 1, 2, 4, 6, 8 and 10 weeks of treatment. The control rats were treated with normal saline.

For histological observation, the liver specimens were fixed in 10% formalin, embedded in paraffin, sectioned and stained with H & E and Azan stains.

For in situ hybridization, mouse α1 (IV) procollagen C87 clone cDNA (1.3 k bp, 7S region) in plasmid pBR 322 was generously provided by Dr. Linda Wood (26), and chicken prolyl 4-hydroxylase β-subunit 9-10B cDNA (700 bp, N-terminal region) in plasmid Puc 9 (10) and the Pst-EcoR1 fragment of pBR 322 (750 bp) were used as controls (Fig. 1). The cDNA probes were labeled with digoxigenin-dUTP by the random priming method using a DNA labeling and detection kit (Boehringer Mannheim GmbH). Most of the digoxigenin-labeled cDNA probes were detected in the 250-400 bp nucleotide region by ethidium bromide staining in 1% agar gel.

The modified Hayashi method was used for the preparation of paraffin sections of liver tissue (7). The sections were rehydrated with Dulbecco’s phosphate-buffered saline (PBS) and incubated with proteinase K (10 µg/ml Sigma Chemical Co.) in Dulbecco’s PBS for 10 min at 37°C. Then, the sections were post-fixed in 4% paraformaldehyde/PBS for 5 min, and quenched twice with glycine (2 mg/ml) in Dulbecco’s PBS. The sections were kept in 40% (v/v) deionized formamide/2x SSC. The hybridization buffer contained 0.6 M NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.6), 120 µg/ml herring sperm DNA (Boehringer Mannheim GmbH), 200 µg/ml yeast RNA (Boehringer Mannheim GmbH), 1x Denhart’s solution, 10% (v/v) dextran sulfate (Sigma Chemical Co.), 40% deionized formamide and 300-500 ng/ml labeled DNA probe (denatured). Fifty microliters of the hybridization buffer were applied to each section, and the sections were incubated in a moist chamber for 14-16 hr at 40°C, then washed three times with 50% formamide/2x SSC for one hr at room temperature (RT) and washed twice with 2x SSC for 15 min. For immunological detection, the same kit for DNA labeling was used. The sections were washed briefly with buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.6) solution, and incubated with 0.5% (w/v) blocking reagent in buffer 1 solution for 30 min at RT. After washing again briefly with buffer 1 solution, the sections were incubated with a 1/5000 dilution of polyclonal sheep anti-digoxigenin Fab fragment conjugated with alkaline phosphatase in buffer 1 solution for 30 min at RT. The sections were washed twice with buffer 1 solution for 15 min at RT and equilibrated with the buffer 2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl$_2$, pH 9.5) solution for 10 min at RT. Then, the sections were incubated with coloring solution including NBT and BCIP in a dark box for 30 min and the reaction was stopped with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The sections were mounted with aqueous mounting medium (Daido Sangyo Co.) and observed by light microscopy.
RESULTS

Morphological findings:

After four weeks of CC14 administration, lipid droplets were demonstrated in the hepatic intracellular regions of the central zones and in the parenchymal areas. After six weeks of CC14 administration, acidophilic and necrotic hepatocytes were observed along the septal area and cell regeneration had become prominent. Nodules of regenerating hepatocytes were completely surrounded by fibrous septa including proliferating bile ducts, arterioles and venules, followed by cirrhotic change.

Localization of type IV procollagen and prolyl 4-hydroxylase mRNAs:

Control and experimental liver sections showed no hybridization with the labeled Pst-EcoRI fragment of the plasmid PBR 322 probe (data not shown). Type IV procollagen mRNA and prolyl 4-hydroxylase mRNA were not detected until four weeks after administration of CC14. However, both mRNA were observed in the mesenchymal cells in the portal area and the surrounding zone of central veins at six weeks (Fig. 2). After eight weeks, type IV procollagen mRNA was observed not only in the mesenchymal cells but also in some hepatocytes located close to central veins and in the fibrous septa (Fig. 3). Type IV procollagen mRNA was observed much more clearly in the mesenchymal cells after ten weeks than at eight weeks. Prolyl 4-hydroxylase mRNA was also observed in the mesenchymal cells in the fibrous septa and hepatocytes (Fig. 4).

Fig. 1. Structures of prolyl 4-hydroxylase (9–10 B) and type IV procollagen (C 87) cDNAs used for in situ hybridization.
DISCUSSION

Collagen fibers are a major structural component for the maintenance of architectural integrity in the liver (21). The toxic effect of CCl₄ is due to its conversion of CCl₄ to the highly reactive free radical CCl₃ (CCl₄ + e⁻ → CCl₃ + Cl⁻) in the smooth endoplasmic reticulum of the liver cells (8). Free radicals may cause peroxidation of lipids in cellular and organellar membranes (9). A single dose of CCl₄ leads to centrilobular necrosis and steatosis. The subsequent fibrosis is probably a complex inflammatory reaction that includes immune and repair responses (17).

After liver injury, the endothelia, hepatocytes and mesenchymal cells contribute to the production of extracellular matrix and neovascularization in Disse’s spaces (22). Hepatocytes as well as mesenchymal cells may contribute to collagen metabolism. Mature hepatocytes are capable of simultaneously synthesizing various types of collagen including types I, III and IV in vitro (3). Immunohistochemically, type I and IV collagens are detected in the fibrous septa in cirrhotic liver (2, 12). In situ hybridization can effectively demonstrate mRNAs in liver cells. In this study, type IV procollagen mRNA was found to be localized in the cytoplasm of mesenchymal cells and hepatocytes by in situ hybridization. An increase in α1 (I), α1 (III) and α1 (IV) procollagen mRNA levels has already been found in CCl₄ induced hepatic fibrosis (15, 16). Increased amounts of mRNAs for type IV collagen and prolyl 4-hydroxylase may be found at an early stage in the development of hepatic fibrosis (18). The increase in tissue collagen is correlated with an increase of procollagen and prolyl 4-hydroxylase activities in mouse teratocarcinoma cell lines F9 and P19 (19). However, the amounts of types I, III and IV procollagens do not increase simultaneously (15, 18).

The genes for the type IV procollagen α1 (IV) and α2 (IV) chains have been found on chromosome 13, being located on opposite strands and sharing a 120-bp promoter region (5). In mouse F9 cells, an increase has been found in the type IV procollagen gene, which is transcriptionally regulated by retinoic acid and dibutyril cyclic AMP (25). The regulation of type IV procollagen at the transcriptional level may be different from that of other types of collagen in fibrotic liver. From the results of our present study, it is concluded that hepatocytes as well as mesenchymal cells are responsible for the production of collagen after hepatocellular injury.

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Figs. 2a, b. Type IV procollagen (a) and prolyl 4-hydroxylase mRNAs (b) are detected in the mesenchymal cells of portal area and surrounding central veins in the paraffin section of rat liver tissue after six weeks of CCl₄ administration. ×100
Figs. 3a–c. Type IV procollagen mRNA is detected in the hepatocytes (H) and endothelial cells (E) close to a central vein (a) and in fibrous septa (b, c) after eight weeks. ×100
Figs. 4a–c. After ten weeks, a large number of mesenchymal cells are noted in the fibrous periporal zone (a, H & E). Type IV procollagen mRNA is clearly detected in the hepatocytes (H), endothelial cells (E) and mesenchymal cells of periporal zone (b). Prolyl 4-hydroxylase mRNA is detected in the mesenchymal cells and hepatocytes of periportal zone (c). ×100
REFERENCES


