CYP11B2 expression in HSCs and its effect on hepatic fibrogenesis

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INTRODUCTION
It has been reported that renin-angiotensin system exists in tissue¹ and aldosterone can be synthesized in extra-adrenal tissue including heart, blood vessels² and brain³. Recent studies have brought rich evidences in favour of aldosterone as a strong stimulator of fibrogenesis and mitogenesis⁴⁻⁹. Recently Wu PS reported that aldosterone synthase gene -CYP11B2 can be expressed in hepatic stellate cells (HSCs) of liver¹⁰. As the activation of HSCs is the central event in fibrogenesis of liver¹¹⁻²⁶, we undertook the present study to investigate the relationship between hepatic fibrogenesis and locally produced aldosterone in liver.

MATERIALS AND METHODS

The establishment of animal model
Male Wistar rats (250g-280g, n=48, purchased from Animal Center of the First Military Medical University) were divided into 2 groups randomly. Model group (n=24): the rats were injected with 40% CCl₄ (the mixture of CCl₄ and olive oil) 0.25mL/100g subcutaneously three times a week. Control group (n=24): the rats were injected with olive oil only.

Histology
At the end of the week 4, 6, 8, and 10, 6 rats in each group were sacrificed. The rat liver was regularly fixed, embedded, sliced and stained with VG and HE. Cryosections of liver tissue were rinsed in 0.2g/L gold chloride solution for 6h at room temperature, then the sections were placed in 50g/L sodium thiosulphate for 5min and mounted with resin.

Extraction of total RNA
The tissue of liver was promptly frozen in liquid nitrogen and stored at -70°C prior to use. Total RNA was extracted with GTC solution (6M guanidinium thiocyanate, 5mM sodium citrate, 5g/L sodium sarcosyl, 0.1M beta-mercaptoethanol). The amount of total RNA was measured at 260nm using an ultraviolet spectrophotometer.

RT-PCR for CYP11B2 mRNA
One microgram of total RNA was incubated at 25°C for 30min in 20µL reverse-transcriptase buffer containing 20u AMV reverse trans criptase and 2µL random primer p(dN)₆(Boehringer Mannheim, Germany). Reverse transcription was terminated by heating at 99°C for 5min. The polymerase chain reactions were performed in 50µL PCR buffer containing 0.32µg of each primer and 2.5 u Taq DNA polymerase (Promega). Samples were subjected to 30 cycles of PCR amp lification. Each cycle includes denaturation at 94°C for 1min, annealing at 56°C for 1min, and primer extension at 72°C for 2min. The rat CYP11B2 PCR primers were 5’-ACCATGGATGTCAGAA-3’ and 5’-GAGAGCCTGACGAGTCA-3’, synthesized by Shanghai Cell Institute according to the published sequences of Oaks and Raft²⁷, corresponding to positions 657-954 of the gene that does not cross-react with the CYP11B1 gene. As control, β-actin primers were used and had the following sequences: 5’-TTTCTGCAAGTTGTTGATTGTCT-3’ and 5’- CCTAGACCATAGATCTC-3’[28]. Each 6µL amplific ation mixture was subjected to electrophoresis on 15g/L agarose gel, and DNA was visualized by ethidium bromide staining. The signal intensity was quantified by a computerized medical image-processing system (GDS-7500, UVP, England). Six separate gels were run for the enzyme, and the data were then averaged. The ratio of CYP11B2 to β-actin was used to express relative mRNA levels.
In situ hybridization

The Wistar rats (model group, 6th week, n=6; control group, n=6) were anesthetized by intraperitoneal injection of pentobarbital, and perfused with 9g/L NaCl and 40g/L paraformaldehyde solution buffer at pH 7.4 in 0.1M phosphate buffered saline (PBS). Each specimen was divided into halves, immersed in the same fixative solution for 4h at 4°C. After the tissue samples were rinsed in PBS containing 300g/L sucrose, cryosections 10μm thick were cut and amounted on polylysine-coated slides. In situ hybridization[29], slides were washed in 0.1M PBS, treated with 3g/L triton X-100 for 20min, washed in 0.1M glycine for 5min; and incubated in 2µg/mL proteinase K at 37°C for 30min, fixed in buffered 40g/L paraformaldehyde at RT for 10min, washed in PBS for 20 min; immersed in 2.5g/L acetic anhydride in 0.1M triethanolamine solution at RT for 10min, washed in 2×SSC for 15min; incubated in a prehybridization mixture at RT for 8h. After blotting the solution, the slides were incubated in a hybridization solution consisting of 500g/L formamide, 5×SSC, 20g/L SDS, 1×Denhardt’s solution, 100mg/L salmon sperm DNA, 100g/L dextran sulfate, 2mg/L Digoxigenin labeling probe (Boehringer Mannheim, Germany) at 42°C for 24h. After hybridization, the slides were washed in 4×SSC, 2×SSC, 1×SSC, 0.5×SSC and 0.05M PBS at 37°C for 20min respectively. The detection procedure was performed according to the maneuver of DIG DNA labeling and detection kit (Boehringer Mannheim, Germany). The slide exposure duration was 12 hours at 4°C. The slides were dehydrated in ethanol and mounted with resin. A negative control was prepared for each sample using a hybridization solution without CYP11B2 probe. We selected 7 high power fields randomly and counted the positive cells per high power field.

Statistics

Analysis of data was performed with one way ANOVA (SPSS 7.5) and rank sum test. Results were expressed as mean ± SD, a value of P<0.05 was regarded as statistical significance.

RESULTS

Morphological changes

Observed with naked eyes, at the end of week 4, the liver surface of model group presented tiny particle-like changes. After that, the tissue became hard and shrank progressively. Microscopically, at the end of week 4, fibroblasts proliferated obviously in the portal tracts of model group, and collagen invaded into the hepatic lobules along with the injured limiting laminae, but the collagen was not completely connected with each other. At the end of week 6, most rats developed cirrhosis in model group.

RT-PCR

The expressions of CYP11B2 mRNA in model group were significantly up-regulated compared to those in control group (P<0.01). The levels of CYP11B2 mRNA in model group (week 8) were higher than those in model group (week 4) (P<0.05), without significant difference between the two groups (P>0.05) (Figures 1,2).

In situ hybridization

Duck purple precipitate was localized in the endoplasm of HSCs corresponding to the gold chloride stained section. The number of positive cells per high power field in model group (4.5) was higher than that in control group (0.55) (P<0.05).

![Figure 1](PDF) The ratio of CYP11B2/β-action presents the relative level of CYP11B2.

![Figure 2](PDF) Lanes 1: model group (week 4) β-actin; Lanes 9: model group (week 4) CYP11B2; Lanes 2: model group (week 8) β-actin; Lanes 8: model group (week 8) CYP11B2; Lanes 7: model group (week 10) CYP11B2; Lanes 4: control group β-actin; Lanes 6: control group CYP11B2; Lanes 5: Makers.
DISCUSSION

It has been reported that renin-angiotensin system exists in tissues. Aldosterone can be synthesized in extra-adrenal tissue including blood vessels and brain. Aldosterone is a strong stimulator of fibrogenesis and mitogenesis which exerts a marked effect on stimulating the proliferation of myofibroblasts and collagen production.

There are two genes encoding the enzymes of aldosterone biosynthesis\[30,31\]. One is P450 11\(\beta\) (CYP11B1) that is responsible for the early steps of aldosterone biosynthesis. The other is P450 ald (CYP11B2) that encodes the key enzyme for the final steps of its biosynthesis.

HSCs are mesenchymal cells located in the space of Disse. They demonstrated synthetic activity of collagen and other extracellular matrix proteins involved in hepatic fibrosis. As a source of cytokines, prostaglandins and other bioactive substances, they play a crucial role in the mechanisms of liver injury, regene ration and fibrosis. Now, fat-storing cell function is expanding from a retinol (fat)-storing site to a center of extracellular matrix metabolism and mediator production in the liver. Transition of the stellate cells from the vitamin A-storing phenotype to “activated” or “myofibroblastic” cells is the central pathobiochemical event for liver fibrosis\[32-36\].

Using RT-PCR, we clearly provided direct evidence for the expression of CYP11 B2 mRNA in liver. The expression was up-regulated when fibrogenesis occurred. In situ hybridization further revealed that the expression of CYP11B2 mRNA located in the endoplasm of HSCs increased in fibrotic liver.

In conclusion, based on present study, we first proposed that there is a positive acting relationship between the expression of CYP11B2 in HSCs and fibrogenesis of liver. Locally produced aldosterone in liver is likely to promote the process of fibrogenesis. The present study provides a new way to investigate the mechanism and treatment of hepatic fibrosis.

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