Retinoid metabolism during development of liver cirrhosis

Sathish Kumar Natarajan, Simmy Thomas, Anup Ramachandran, Anna B. Pulimood, Kunnissery A. Balasubramanian *

The Wellcome Trust Research Laboratory, Department of Gastrointestinal Sciences, Christian Medical College, Ida Scudder Road, Vellore 632004, India

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Abstract

The changes in retinoid metabolism have been documented in liver cirrhosis. However, the dynamic alterations in levels of this vitamin between circulation and liver during development of the liver cirrhosis are not well understood. The aim of this study was to measure retinoids in the liver and circulation in parallel, during and after development of cirrhosis induced by carbon tetrachloride and thioacetamide. Retinoid levels were measured by HPLC. A decrease in retinaldehyde and total retinol, together with an increase in retinoic acid was evident in liver from both carbon tetrachloride or thioacetamide treated rats within a month after initiation of treatment. Activity of enzymes involved in retinoid metabolism such as retinaldehyde oxidase, retinaldehyde dehydrogenase, and retinaldehyde reductase were decreased in the liver. In parallel, levels of retinol and retinaldehyde in the serum were increased while retinoic acid was decreased. This study indicates that during development of cirrhosis, there is reciprocal transfer of retinoid metabolites between the circulation and the liver.

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Retinoids (vitamin A and its derivatives) have profound effect on morphogenesis and are essential for vision, reproduction, growth, differentiation, and maintenance of the health of organisms [1,2]. Retinoid homeostasis is maintained by a number of interconnected systems, of which the liver is a critical player, involved in homeostasis, metabolism, and storage of retinoids [3]. Vitamin A is absorbed from the diet in the intestine as α-carotene, β-carotene, and β-erythroxyanxin. These carotenoids are cleaved by the carotene cleavage enzyme to retinaldehyde, which is then metabolized to either retinol or retinoic acid by retinaldehyde reductase or retinaldehyde dehydrogenase, respectively. The retinol formed in the enterocytes is esterified with fatty acids by lecithin: retinol acyl transferase to form retinyl esters which are transported as chylomicron retinyl esters through circulation, which is then cleared by the liver [4,5]. In addition to the liver, 25–30% of chylomicron retinoids are cleared by extra hepatic tissues [5]. From the liver, retinol is transported to other tissues as a retinol–retinol binding protein (RBP)1 complex, which forms protein–protein interactions with transthyretin (TTR) [6]. In addition, the circulation also contains all-trans-retinoic acid bound to albumin and soluble retinoyl-glucorono- nides.

In liver cells, retinoic acid taken from the circulation is bound to cellular retinoic acid binding protein (CRABP) I and...
or II for transcriptional activation of vitamin A-responsive genes [4]. Alterations in retinoic acid metabolism have been noted in cirrhosis, [2,7,8] and decreased levels of retinol, RBPs, TTR, and β-carotene in the serum in patients with liver cirrhosis has been demonstrated [9–11]. We hypothesized that liver cirrhosis would result in a significant alteration in retinoid metabolism during the course of development of disease; with early changes progressively leading to profound alterations by the time cirrhosis is evident histologically. To understand these processes, we have followed retinoid levels in the liver during development of liver cirrhosis using two different animal models, in an effort to understand mechanisms involved in these changes during the disease.

Materials and methods

Tris (hydroxymethyl) aminomethane (Tris), N-[2-hydroxyethyl] piperazine-n'-[2-ethanesulfonic acid] (Hepes), bovine serum albumin (BSA), nicotinamide adenine dinucleotide (NAD), its reduced form (NADH), all-(Hepes), bovine serum albumin (BSA), nicotinamide adenine dinucleotide (NAD), its reduced form (NADH), all-

Alcohol and benzene were used for this study. Four groups of animals were used (Control, phenobarbitone control, TAA, and CCl4 treatment). Each group comprised of six animals. This study was approved by Institutional Animal Ethics Committee (IAEC).

Induction of liver cirrhosis in rats

Cirrhosis was induced by administering carbon tetra chloride (CCl4) or thioacetamide (TAA). For CCl4-induced liver cirrhosis, rats were treated with phenobarbitone (35 mg/dl) in tap water, which was the source of drinking water for 14 days before initiation of the experiment. Intragastric instillation of CCl4 in coconut oil was given using a 2.5 ml syringe attached to 2 mm-diameter tygon tubing twice a week under light halothane anesthesia. The initial dose of CCl4 was 40 μl/rat. Subsequent doses were adjusted based on the change in body weight. Control animals received phenobarbitone, along with coconut oil alone without CCl4 [12]. Treatment of phenobarbitone and CCl4 was stopped after each time point during the experiment, namely 1, 2, 3, 4, and 5 months and the animals were sacrificed by decapitation after 10 days. For thioacetamide-induced liver cirrhosis, rats were administered intraperitoneal injection of TAA (200 mg/kg, ip) in saline twice a week for 1, 2, 3, 4, and 5 months. Control rats received vehicle alone [13]. After each treatment period, TAA was stopped and the animals were sacrificed after one week. Fully developed micro- and macro-nodular cirrhosis was confirmed by histology and circulating biochemical parameters. Blood was collected by direct heart puncture and serum separated. Care was taken to protect the tissue and serum from light exposure for retinoid estimations.

Histology

Liver tissue was fixed in 10% buffered formalin and processed. Four-micron sections were cut and stained with hematoxylin and eosin and observed under light microscope.

Preparation of liver homogenate

The liver was washed thoroughly of blood, minced and homogenized with 8 volumes of homogenization buffer containing 230 mM mannitol, 70 mM sucrose, and 3 mM Hepes, pH 7.4, using a Porter-Elvehjem homogenizer and used for the measurement of retinoids [14].

Retinol, retinaldehyde, and retinoic acid extraction and quantitation by high performance liquid chromatography

The liver homogenate corresponding to approximately 1 mg protein was mixed with an equal volume of 100% ethanol and 0.025× volume of 0.1 N HCl. Neutral and acidic retinoids were extracted twice with 3× volume of hexane. Extracted fractions were dried under nitrogen and reconstituted in 100% ethanol for high performance liquid chromatography (HPLC) separation. Retinoids were separated on a Shim-pack CLC-SIL silica column running at 1 ml/min using the mobile phase (hexane/dioxane/acetic acid, 92:8:0.1). Retinol, retinaldehyde, and retinoic acid were monitored at 350 nm [15]. Quantitation was performed by relating the area of the peak to areas obtained by the analysis of known quantities of retinoid standards and expressed as picomoles per milligram protein. For serum retinoids, 0.5 ml serum was mixed with equal volume of ethanol and 0.025 ml of 0.6 N HCl and extracted as mentioned above and expressed as picomoles per milliliter of serum. The recovery of these retinoids by this method was around 85%.

Saponification of retinyl esters

For saponification of retinyl esters to retinol, the liver homogenate corresponding to 0.1 mg protein was mixed with an equal volume of 5% ethanolic potassium hydroxide and 1 M sodium chloride. The mixture was incubated at 65°C for 2 h in dark [5]. The reaction was stopped by adding equal volume of 100% ethanol and extracted as
Retinoid metabolizing enzyme activity measurements

Liver cytosol was prepared by differential centrifugation as described [16]. Cytosol was used for assay of enzymes involved in retinoid inter-conversion. Retinaldehyde dehydrogenase activity was estimated by measuring the retinoic acid formed from retinaldehyde. The reaction mixture consists of cytosolic protein corresponding to approximately 1 mg, 200 μM NAD, and 0.5 μM retinaldehyde (in ethanol) (all final concentration) in a total volume of 0.2 ml. This was incubated at 37 °C for 20 min and the reaction stopped by the addition of ethanol, followed by extraction and quantitation by HPLC. Cytosolic incubations was also been carried out in the absence of NAD to look for retinaldehyde oxidase activity. For retinaldehyde reductase activity, 2 mM reduced nicotinamide adenine dinucleotide (NADH) was added instead of NAD and estimated by measuring the retinol formed from retinaldehyde. Retinol dehydrogenase activity was measured by incubating the cytosolic protein corresponding to approximately 1 mg along with 200 μM NAD and 1 μM retinol in a total volume of 0.2 ml at 37 °C for 20 min and here again the reaction was stopped by the addition of ethanol [15,17] followed by extraction and quantitation by HPLC. Specific activity is expressed as picomoles of product formed per minute per milligram protein.

Estimation of hydroxy proline

Hepatic hydroxy proline content was measured as described [18]. The amount of hydroxy proline was calculated using standard curve obtained from commercial hydroxy proline and expressed as microgram of hydroxy proline per gram wet weight of liver tissue.

Serum parameters

Serum separated from the blood obtained by direct heart puncture was used for the assay of alanine amino transferase, aspartate amino transferase [19], alkaline phosphatase [20], and total bilirubin [21].

Protein estimation

Protein was estimated by Lowry’s method using BSA as standard [22].

Table 1
Serum markers for liver damage in control, phenobarbitone control, CCl₄, and TAA treated rats after 3 months

<table>
<thead>
<tr>
<th>Serum markers</th>
<th>Control</th>
<th>PB-control</th>
<th>CCl₄</th>
<th>TAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine amino transferase (IU/L)</td>
<td>138 ± 2</td>
<td>224 ± 24</td>
<td>671 ± 56*</td>
<td>560 ± 38*</td>
</tr>
<tr>
<td>Aspartate amino transferase (IU/L)</td>
<td>130 ± 10</td>
<td>144 ± 29</td>
<td>715 ± 68*</td>
<td>721 ± 44*</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>80 ± 10</td>
<td>116 ± 25</td>
<td>398 ± 52*</td>
<td>245 ± 29*</td>
</tr>
<tr>
<td>Total bilirubin (μmol/L)</td>
<td>15 ± 4</td>
<td>14.5 ± 2.5</td>
<td>63 ± 4*</td>
<td>58.5 ± 5*</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>8.16 ± 0.23</td>
<td>7.8 ± 0.9</td>
<td>3.12 ± 0.13*</td>
<td>3.48 ± 0.29*</td>
</tr>
</tbody>
</table>

* P < 0.05 when compared to control.
Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed with the non-parametric Mann–Whitney test. A P value of less than 0.05 was taken to indicate statistical significance. Statistical calculations were performed using SPSS software for windows (version 9.0).

Results

Liver cirrhosis was established by histology and serum markers of liver damage in animals treated with CCl₄ or TAA. Intragastric and intraperitoneal administration of CCl₄ or TAA showed micro- and macro-nodular cirrhosis with extensive fibrosis, after 3 months of treatment.

Fig. 2. Hepatic hydroxy proline content from CCl₄ and TAA treated rats at 1, 2, 3, 4, and 5 months, compared to phenobarbitone treated controls. The assays were done as described in the text. Each value represents mean ± SD of separate experiments (n = 5). *P < 0.05, when compared to control.

Fig. 3. Retinaldehyde (A), retinoic acid (B), and total retinol (C) levels in liver from control, phenobarbitone controls, CCl₄, and TAA treated animals at different time periods. All the estimations were carried out as described in Materials and methods. Each value represents mean ± SD of separate experiments (n = 5). *P < 0.05 as compared to control.
and phenobarbitone control (Table 1). Development of after 3 months of treatment as compared to the control (Fig. 1). Serum markers for liver cirrhosis such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and total bilirubin were found to be increased significantly in rats treated with CCl₄ or TAA after 3 months of treatment as compared to the control and phenobarbitone control (Table 1). Development of liver fibrosis was followed by measuring hepatic hydroxy proline content, which is a marker for fibrosis [23]. As seen in Fig. 2, hydroxy proline content increases significantly after 3 months of CCl₄ treatment and stayed elevated until 5 months. In TAA treated rats, hydroxy proline content increased gradually from 2 to 5 months of treatment period.

The levels of retinaldehyde and total retinol (retinol plus retinyl esters) in the liver showed a decrease during the time course of treatment as compared to the controls. However, retinoic acid levels were increased 11-fold by 1 month after treatment with CCl₄ or TAA, and stayed elevated at 2 months. By the third and fourth month, the increase had reduced to 4-fold compared to control, and by the fifth month it was a 2.5-fold increase (Fig. 3).

Alterations in the retinoid levels in the liver might be due to changes in the activity of retinoid metabolizing enzymes. Hence, these were measured at different time points during development of cirrhosis after treatment with CCl₄ or TAA. Fig. 4 illustrates the enzymes involved in metabolism of retinoids and also the transfer between the circulation and the liver. As seen in Fig. 4, retinaldehyde is converted to retinoic acid (the active metabolite of retinol) by two enzymes, namely retinaldehyde dehydrogenase (NAD dependent) and retinaldehyde oxidase (NAD independent). In our study, the activity of both these enzymes decrease gradually from the first month until five months after CCl₄ or TAA treatment as compared to controls (Figs. 5A and B).

Conversion of retinaldehyde to retinol is carried out by the enzyme retinaldehyde reductase, which uses NAD as a cofactor. The activity of this enzyme was then measured in the liver cytosol of animals treated with both CCl₄ and TAA. As seen in Fig. 5C, enzyme activity decreased gradually from the second month until five months after treatment when compared to controls. Retinol dehydrogenase is the enzyme which catalyses conversion of retinol to retinaldehyde, and this enzyme activity was decreased by 1 month after treatment and stayed low for the second month. By the third month the levels returned to near control levels, and did not alter on the fourth month. By the fifth month of treatment, a slight increase in enzyme activity was seen when compared to controls.

Another contributor to hepatic retinoid levels can be the circulation, and circulating retinoid concentration in serum was measured at different time points in rats treated with CCl₄ or TAA over a five-month period. In contrast to the hepatic retinoid concentration, a 2-fold increase in levels of retinol and retinaldehyde was evident after 1 month of treatment as compared to the controls. The retinol level came back to control level by 3 months and decreased further by 5 months of treatment. However, retinaldehyde levels decreased significantly at 2 months of treatment and continued to stay below control levels till the end of the study (Figs. 6A and C). Retinoic acid levels in the serum were decreased as compared to the control and stayed lower throughout the treatment period in both the CCl₄ and TAA treated rats (Fig. 6B).

**Discussion**

The liver is a major storage organ of retinol, which is stored mainly as retinyl palmitate in hepatic stellate cells or fat storing cells [24]. In addition to the liver, studies have shown the presence of low levels of retinol and RBP mRNA in adipocytes, kidney, lungs, spleen, brain, stomach, small intestine, pancreas, and testis [25]. One mechanism suggested for uptake of carotenoids by extra-hepatic tissue involves degradation of carotenoids-containing
chylomicrons by lipoprotein lipase in blood resulting in formation of chylomicron remnants, and uptake into tissue [26]. Though the transport and storage of retinoids in stellate and parenchymal cells has been well studied [5,27], the factors controlling tissue uptake, recycling back to the liver, and excretion of the retinoids from the liver are not fully understood. In the context of cirrhosis, studies have demonstrated significant decrease in hepatic and blood vitamin A concentration in patients with liver cirrhosis [28–30]. However, there is scarce information on the alterations in retinoid metabolism during development of cirrhosis [28–30]. However, in our study the decrease in hepatic levels of retinol and retinaldehyde were evident within a month of treatment with the hepato-toxic agents. Retinoic acid, on the other hand showed an opposite effect, with levels increasing within a month of treatment. It has been suggested that an increase in metabolism of retinol to retinoic acid in hepatic stellate cells may be responsible for the activation of these cells, which play a role in fibrosis [31]. However, retinoic acid can also activate transforming growth factor-β (TGF-β)

![Fig. 5. Retinaldehyde oxidase (A), retinaldehyde dehydrogenase (B), retinaldehyde reductase (C), and retinol dehydrogenase (D) activity in liver from control, phenobarbitone control, CCl₄, and TAA treated animals at different time periods. All the estimations were carried out as described in Materials and methods. Each value represents mean ± SD of separate experiments (n = 5). *P < 0.05 as compared to control.](image-url)
by inducing the activation and production of latent TGF-β in hepatic stellate cells [2]. In addition, retinoic acid can also regulate α2(1) collagen expression in these cells through the binding of RAR-β and RXR-α to retinoic acid response elements (RAREs) [33] and inhibit matrix metalloproteinases by its action on tissue inhibitors of matrix metalloproteinases [34].

The most probable cause for changes in retinoid levels observed in the liver is due to alterations in activity of enzymes involved in retinoid metabolism. In our study, there were significant decreases in activity of retinaldehyde oxidase, retinaldehyde dehydrogenase, and retinaldehyde reductase during the development of cirrhosis. However, these alterations could not explain the changes in retinoid levels seen in the liver. For example, activity of both retinaldehyde oxidase and retinaldehyde dehydrogenase would result in the production of retinoic acid, and their decreased activity would suggest a decrease in retinoic acid in the liver. In contrast, we find a significant increase in liver retinoic acid levels during cirrhosis. The decrease in activity of retinaldehyde reductase may be a consequence of the decreased availability of retinaldehyde, which was observed during and after development of experimental liver cirrhosis. Interestingly, we also found that the phenobarbitone treatment per se affected enzyme activity, with decreases in activity of retinaldehyde dehydrogenase, retinaldehyde oxidase, and retinaldehyde reductase. Though this may make interpretation of data difficult in the case of the CCl4 model, the enzyme alterations are also seen in the cirrhotic animals with TAA. This indicates that the decrease in enzyme activity is not exclusively due to the phenobarbitone treatment, and these alterations do occur in development of cirrhosis.

Though the liver is a major organ in maintenance of retinoid homeostasis, it is now becoming evident that the dynamics of retinoid transfer between circulation and the liver also may play a role. The simultaneous measurement of retinoid levels in serum and liver are important, since a recent study in embryonal carcinoma cell lines suggests that precise shuttling of retinoic acid bound to CRABPs, rather than intracellular retinoic acid concentrations per se can explain alterations in cell behavior [35–37]. Low levels of serum retinol have been shown in patients with primary biliary cirrhosis, hepatocellular carcinoma, and alcoholic liver disease [11,38]. In a 7 year follow-up study of cirrhotic patients, the prediagnostic levels of serum retinol from patients who develop hepatocellular carcinoma is lower.
than those who did not [29] suggesting that retinoid deficiency may promote hepatocarcinogenesis.

A parallel estimation of the levels of vitamin A metabolites in serum and liver during development of cirrhosis, would thus help in the better understanding of vitamin A alterations in liver disease, and since the changes in hepatic retinoid levels did not seem to occur as a result of enzyme activity changes, the levels of retinoids in serum were then measured. In our study, serum levels of retinol and retinaldehyde increased in parallel with their decrease in the liver. These changes were evident at the first month after treatment. The retinoic acid level also decreased in serum, parallel to the increase seen in hepatic concentration. This indicates that there might be a possible mobilization of retinol and retinaldehyde from the liver into the circulation, and an inverse mobilization of retinoic acid early in the development of cirrhosis.

In conclusion, this study shows the alterations in the level of retinoids in the liver during the early stages of liver cirrhosis, might be due to changes in retinoid dynamics between the liver and the circulation. These changes do not seem to be linked to alterations in retinoid metabolizing enzymes of liver such as retinaldehyde dehydrogenase, retinaldehyde oxidase, and retinol dehydrogenase. However, further studies are required to understand the effect of liver cirrhosis on molecular mechanisms mediating retinoid exchange between the circulation and the liver.

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References