SHORT COMMUNICATION

Increased hepatic fibrogenesis in the cholesterol-fed mouse

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Summary

1. Mice when fed a cholesterol/choline-supplemented diet for 4 weeks developed histologically fatty livers. This lipid overloading was associated with an increase in hepatic concentration of connective tissues.

2. Both histological and biochemical abnormalities regressed on stopping the cholesterol diet for another 4 weeks. With continuing feeding for 24 weeks these abnormalities were sustained.

3. In the absence of available evidence that cholesterol is 'toxic' to the liver, it is concluded that lipid loading alone increases hepatic fibrogenesis.

Key words: cholesterol, collagen, fibrogenesis, steatosis.

Introduction

It is well accepted that in man fatty liver can be produced by a variety of metabolic disturbances including malnutrition, gross obesity, excessive alcohol intake, or by the action of drugs like corticosteroids. However, there is much argument about the consequences produced by continuous and excessive lipid loading on the liver. Although fatty livers produced by alcohol alone or in association with malnutrition have been observed to progress to hepatic fibrosis and cirrhosis, not all cases of fatty liver will result in a fibrosed liver. A large proportion of fatty infiltration of the liver is histologically reversible [1]. This led to the belief that steatosis and fibrosis are caused by different factors, although some conditions (e.g. alcohol excess) can lead to both steatosis and fibrosis. Attempts have been made also to classify lipid in hepatocytes morphologically into type A ('fatty infiltration') or type B ('fatty degeneration'), to predict or relate to the outcome of liver recovery [2].

In an experiment where mice were fed a diet containing 1% (w/w) cholesterol for 4 weeks, their livers were found to enlarge to four to five times the normal size. Macroscopically, they appeared pale and yellowish. Histologically, the liver had a normal architecture, the bile ducts and portal tracts were normal but lipid-laden hepatocytes were ubiquitous. It appears that cholesterol overloading in the mice produces simple steatosis. It also provides a unique situation whereby any change in hepatic collagen synthesis can be assessed with the development and regression of steatosis and in the absence of other features of hepatic injury or inflammation. The present study is designed to examine this association. Hepatic hydroxyproline and glycosaminoglycan, which are the major components of the matrix of fibrous tissues (collagen), have been measured and correlated with histological appearance of steatosis.

Materials and methods

Materials

Cholesterol was obtained from Sigma Chem. Co., St Louis, U.S.A. and recrystallized twice from 95% (v/v) ethanol at 60°C, pulverized, dried in vacuo and stored under nitrogen (melting point 149.2°C, migrating as a single spot with thin-layer chromatography and a single peak with gas–liquid chromatography (g.l.c.)). Laboratory chow (SG-I) was ground to a powder. Choline (Sigma, St Louis, U.S.A.) was used without further purification.
Preparation of diet

Cholesterol (1.0%, w/w) and choline (0.45%, w/w, approx. 0.16 mg/J or 65 mg/100 calories) were thoroughly mixed with powdered chow and fed to experimental mice. Auto-oxidative products of cholesterol have been shown to be toxic to many cells [3]. To prevent auto-oxidation of cholesterol with prolonged storage, fresh diets were prepared every 2-3 days. Experimental diets were sampled from feeding cans at days 2 and 3 of feeding; hexane extracts were studied by g.l.c. which confirmed a single major peak of cholesterol without other cholesterol metabolites (<1%). Control animals were fed an identical diet without the addition of cholesterol.

Experimental design

Seventy male albino SPF mice weighing 20-30 g were used. Ten were used as untreated controls before the feeding experiment (zero time). Thirty were fed the cholesterol/choline-supplemented diet and 30 pair-fed the control choline-supplemented diet. They were housed under identical conditions. At 4 weeks, 10 mice from the cholesterol/choline diet group and 10 from the control group were killed. Under intraperitoneal phenobarbital anaesthesia the livers were removed, biopsies taken for histology, weighed and kept at -70°C until used for analysis. The first group was then further divided into two groups. These 20 mice were allowed to continue with their experimental (n = 10) or control (n = 10, pair-feeding) diets for 24 weeks. The other 20 were fed the control diet alone (non-pair-feeding) for another 4 weeks before they were killed.

Analytical methods

Tissue cholesterol. Approximately 75 mg of freeze-dried whole liver was pulverized and the lipids extracted with chloroform/methanol (2:1, v/v) mixture. Cholesterol was determined by g.l.c. as described by Salen & Grundy [4].

Glycosaminoglycans. Dried defatted liver samples were subjected to papain digestion [5] and the protein was precipitated with 10% (w/v) trichloroacetic acid, centrifuged at 30 000 g for 20 min, and the supernatant extracted twice with diethyl ether. The glycosaminoglycans were precipitated by an ethanol (80%, v/v)/sodium acetate (2.0 g/l) mixture at 4°C for 24 h. The precipitate was collected after centrifugation at 30 000 g for 20 min, vacuum dried over phosphorus pentoxide, and acid hydrolysed by HCl (1.5 mol/l) at 120°C in sealed vials for 16 h. Hexosamines were determined by g.l.c. as previously described [6].

Hydroxyproline. Dried defatted liver samples were hydrolysed in HCl (6 mol/l) at 140°C for 4 h in sealed vials and analysed for hydroxyproline as described by Woessner [7].

Statistical methods

Results were compared by the two-tailed Student’s unpaired t-test.

Results

All mice appeared well externally and gained weight normally. At 4 and 24 weeks the livers from cholesterol-fed animals weighed significantly more (2P < 0.001) than those of the controls. There was no difference, however, in the liver weights at 4 and 24 weeks, nor was there any difference in the histological appearance of diffuse fatty infiltration without hepatocellular necrosis or fibrosis. There was massive cholesterol accumulation in the livers of mice fed cholesterol for 4 and 24 weeks (20-25-fold increase).

Both hepatic hydroxyproline and glycosaminoglycans (as reflected by the total hexosamines) contents were increased at 4 and 24 weeks (2P < 0.025). No significant difference was found between the cholesterol-fed group at 4 and 24 weeks (Table 1).

After the cholesterol supplement to the diet had been withdrawn for 4 weeks there was a return to control values of liver weight, hydroxyproline and glycosaminoglycans, as well as normal liver histology. The hepatic cholesterol was also much lowered, but remained slightly elevated above the control values (2P < 0.05).

Discussion

Previous studies have attempted to correlate dietary and hepatic fat with the development of liver fibrosis. For example, prolonged feeding of a high-fat, low-protein and lipotropic-deficient diet to the rat has been reported to result in hepatic cirrhosis [8]. In these experiments, factors other than fat are operative. Similarly, animal models in which alcohol has been used to induce fatty livers have also the concomitant toxic effect of alcohol itself [9]. These additional factors have confounded a logical interpretation of the possible association between steatosis and fibrosis. The present study, with purified cholesterol, combined with choline supplementation and a protein–calorie balanced diet, has demonstrated
TABLE 1. Sequelae of cholesterol feeding to mice

Values are expressed as means ± SEM (n = 10). 8 week feeding for cholesterol-fed group: mice were fed the cholesterol diet for 4 weeks followed by the control diet for 4 weeks. P values refer to comparison between cholesterol-fed group vs corresponding controls: *2P < 0.05; **2P < 0.025; ***2P < 0.001.

<table>
<thead>
<tr>
<th>Time of feeding (weeks)</th>
<th>0 (n = 10)</th>
<th>4 (n = 10)</th>
<th>8† (n = 10)</th>
<th>24 (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver weight (g)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Control diet</td>
<td>1.7 ± 0.3</td>
<td>1.8 ± 0.3</td>
<td>1.9 ± 0.4</td>
<td>2.0 ± 0.2</td>
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<tr>
<td>Cholesterol diet</td>
<td></td>
<td>6.7 ± 0.8***</td>
<td>2.3 ± 0.6</td>
<td>7.1 ± 0.7***</td>
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<tr>
<td><strong>Liver histology</strong></td>
<td></td>
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<tr>
<td>Control diet</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td></td>
<td>Fatty liver</td>
<td>Normal</td>
<td>Fatty liver</td>
</tr>
<tr>
<td><strong>Tissue cholesterol (μmol/g of whole liver)</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Control diet</td>
<td>42 ± 3</td>
<td>38 ± 3</td>
<td>37 ± 3</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td></td>
<td>760 ± 66***</td>
<td>69 ± 18*</td>
<td>844 ± 80***</td>
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<tr>
<td><strong>Total hexosamines in insoluble glycosaminoglycans (μmol/g of dried defatted liver)</strong></td>
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<tr>
<td>Control diet</td>
<td>2.36 ± 0.10</td>
<td>2.42 ± 0.09</td>
<td>2.37 ± 0.11</td>
<td>2.51 ± 0.13</td>
</tr>
<tr>
<td>Cholesterol diet</td>
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<td>4.76 ± 0.31**</td>
<td>2.55 ± 0.32</td>
<td>5.02 ± 0.39**</td>
</tr>
<tr>
<td><strong>Hydroxyproline (μmol/g of dried defatted liver)</strong></td>
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<tr>
<td>Control diet</td>
<td>3.92 ± 0.31</td>
<td>3.87 ± 0.24</td>
<td>4.08 ± 0.35</td>
<td>4.16 ± 0.34</td>
</tr>
<tr>
<td>Cholesterol diet</td>
<td></td>
<td>6.96 ± 0.92</td>
<td>4.56 ± 0.83</td>
<td>7.14 ± 0.76**</td>
</tr>
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</table>

that, in the absence of any hepatotoxin, fat in the hepatocytes alone can induce an increase in connective tissue synthesis. This increase, in the relatively short feeding period employed, was reversible.

Cholesterol overloading can lead to widespread metabolic changes in many cells. Fat loading of hepatocytes may be associated with a decrease in other intracellular components. Theoretically, therefore, the observed increase in collagen content may reflect only a decrease in other components in the dry defatted liver. However, there is substantial evidence to support the view that there is a true increase in collagen content with the development of a fatty liver. For example, Lee & Ho [10] described the detection by electron microscopy of an increased number of fibroblasts within bundles of collagen fibres surrounding hepatocytes in cholesterol-induced fatty livers in the rabbit. Similar observations have been made in the fatty livers of mice fed a cholesterol/cholic acid-supplemented diet [11]. In addition, rats fed a high-fat diet had increased liver connective tissue and fatty liver slices were shown to have an accelerated rate of incorporating [14H]proline into collagen [12]. The mechanism of enhanced fibrogenesis with the development of fatty liver and in the absence of a hepatotoxin is not well understood. It has been postulated that the mere mechanical distension and rupture of the hepatocyte may stimulate the surrounding fibroblast [13].

It has also been suggested that fat deposition per se does not cause fibrosis in the human liver [14]. Unless cholesterol overload can be proven as ‘toxic’ to the liver, the present study gives evidence that fat itself does increase fibrous tissue production. With prolonged feeding to 24 weeks, such abnormalities were sustained although progression to cirrhosis was not observed. It may take much longer periods of feeding for cirrhosis to evolve. Alternatively, it may require another fibrogenic factor acting synergistically to produce cirrhosis. Whatever the additional factor may be, the concept that nutritional factors influence hepatic fibrogenesis should not be completely dismissed.

Acknowledgment

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References


