Mild Hepatic Fibrosis in Cholesterol and Sodium Cholate Diet-Fed Rats

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ABSTRACT. To date, the majority of research on hypercholesterolemia has focused on the effects of a high cholesterol diet on atherosclerosis and coronary heart disease. The toxic effects of cholesterol on the liver and the relationship between the intake of a high cholesterol diet and hepatic fibrosis, however, have not been investigated clearly or histopathologically. Male Wistar rats were fed a diet supplemented with 1.0% cholesterol and 0.3% sodium cholate for 12 weeks. Rats were sacrificed and analyzed via blood biochemistry, traditional microscopy and immunohistochemistry. Following the feeding of this diet, the rates of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase and total cholesterol in the rats were elevated consistently from week 3 and throughout the remainder of the experiment. From microscopic observation, hepatic necrosis, macrophage infiltration and steatosis increased markedly throughout the experiment. Hepatic fibrosis and myofibroblast proliferation were detected at weeks 9 and 12. Mast cell appearance was proportional to the degree of hepatic damage. These findings suggest that hepatic fibrosis is inducible by a high cholesterol diet and is likely the result of the interaction between several different cell types (i.e., macrophages, myofibroblasts, and mast cells) in an inflammatory milieu. Hypercholesterolemia should be considered as a risk factor for hepatic fibrosis as well as atherosclerosis and coronary artery disease.

KEY WORDS: cholesterol, hepatic fibrosis, myofibroblast, rat, sodium cholate.

MATERIALS AND METHODS

Animals and treatment: Male Wistar rats, weighing 150–160 g, were used in this study. All experimental procedures were performed in accordance with the NIH guidelines for the care and use of laboratory animals. The rats were housed in an air-conditioned room (22–24°C) with a 12 hr light-dark cycle. They had free access to water and a manipulated powder diet containing 1.0% cholesterol (w/w) and 0.3% sodium cholate (w/w) for 12 weeks. This diet was a modification of the AIN-76 semipurified diet which was stored at 4°C to prevent autoxidation. Composition of the diet is shown in Table 1. Sodium cholate was used to induce sufficient hypercholesterolemia in the study rats. Four rats were sacrificed at weeks 3, 6, 9 and 12, respectively. Blood was obtained from the abdominal aorta at the time of sacrifice. The standard diet-fed normal rats (n=4), without any treatment, were sacrificed at week 0 as a control.

Serum measurements: The blood was centrifuged at 1,200 × g for 20 min at 4°C to separate serum which was immediately frozen for analysis for the following levels: Aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and total cholesterol (TC). These levels were determined using an automated blood chemistry analyzer (Hitachi 7600, Tokyo, Japan).

Histopathology and immunohistochemistry: The liver samples from each rat were removed rapidly at necropsy and fixed in 10% neutral buffered-formalin, processed routinely and embedded in paraffin. Sections were cut in 4 µm in thickness. The sections were stained with hematoxy-
lin and eosin (H-E) and with Azan stain for collagen fibers and toluidine blue for mast cells. For immunohistochemical analysis, sections of liver were deparaffinized in xylene, rehydrated in a graded alcohol series, and incubated in a solution of 3% hydrogen peroxide for 10 min. Tissue sections were washed with phosphate-buffered saline (PBS), and then incubated with primary antibodies; the primary antibodies used were anti-rat monocyte/macrophage (clone ED1, Chemicon International, Temecula, CA, U.S.A.) and monoclonal anti-\( \alpha \) smooth muscle actin (\( \alpha \)-SMA) (clone 1A4, Sigma Co., St. Louis, MO, U.S.A.). The antigen-antibody complex was visualized by the labeled streptavidin-biotin (SAB) method using a Histostatin-plus bulk kit (Zymed Laboratories Inc., San Francisco, CA, U.S.A.) with 3,3’-diaminobenzidine (Zymed Laboratories Inc., San Francisco, CA, U.S.A.). Tissue sections were then rinsed in distilled water and counterstained with Mayer’s hematoxylin. Non-immunized goat serum, which was used instead of the primary antibody, served as the negative control.

**Statistical analysis and cell counting:** Data is expressed as mean ± SEM. Mast cells with distinct nuclei were counted in the three portal areas of each of the three pieces of liver from each rat at a magnification of \( \times \) 400 [16]. Mast cells increase generally in the portal area as hepatic injuries develop. For statistical analysis, the Student’s \( t \) test was employed. Values of \( P<0.05 \) were considered significant.

**RESULTS**

**Effects of intake of high cholesterol diet on body weight and serum biochemistry:** Rats receiving a diet supplemented with 1.0% cholesterol and 0.3% sodium cholate gained weight over the 12-week period (data not shown). Serum was analyzed in an attempt to clarify the relationship between the feeding of a high cholesterol diet and the levels of hepatic enzymes, lipids and proteins in blood (Table 2). AST, ALT, LDH and TC levels in the treated rats increased consistently starting at week 3 and throughout the remainder of the experiment. AST, ALT and LDH levels increased about double during that time and the TC level increased by five-fold times.

**Histopathology:** As a high cholesterol diet was fed to the rats, hepatic damage was characterized by steatosis and mild fibrosis (Table 3). At week 3, many lipid droplets were visible in the cytoplasm of 66% of the hepatocytes, in decreasing levels of concentration from the periportal areas toward the central veins (Fig. 1A and 1B). Necrotic foci were visible primarily in the periportal and midlobular areas of the hepatic lobule but, occasionally, also near the central veins. Cellular debris and lipid droplet from necrotic hepatocytes were phagocytosed by macrophages, which often exhibited a foamy appearance and occasionally coalesced into giant cells (Fig. 1C). Nuclei of giant cells were of regular and ovoid shape, and were situated in the periphery of the cells. Giant cells, however, started to disappear at week 6 and did
tive macrophages were observed almost coincided with the 2D). At weeks 9 through 12, the regions where ED1-positive cells increased and peaked at week 12; myofibroblasts were also detected in the same sites as in the first week. Within the lobules of the liver at the first week, which had been used widely in other studies [16, 17]. In the control rats, there were very few ED1-positive cells that were suspected to be Kupffer cells. In cholest erol-supplemented animals, the number of ED1-positive cells increased by week 3, predominantly within the central veins, as demonstrated by the Azan stain) were present as normal stroma in the portal areas and in the wall of the central veins at week 0. Later, they were deposited along the perisinusoidal spaces in steatotic lesions at week 9 (Fig. 1F) and also in the perportal and midlobular areas at week 12 (Fig. 1G). Mast cells with distinct nuclei were counted (Table 3). The majority of mast cells were identified in the portal areas (Fig. 1H). Increased numbers of mast cells coincided with hepatic damages (steatosis and fibrosis).

**Immunohistochemistry:** ED1-positive cells were considered as blood macrophages, or monocytes and Kupffer cells [16, 17]. In the control rats, there were very few ED1-positive cells that were suspected to be Kupffer cells. In cholesterol-supplemented animals, the number of ED1-positive cells increased by week 3, predominantly within the necrotic foci and around areas where hepatocytes contained abundant lipid droplets. These cells were small, round, and interpreted as infiltration macrophages. The increase in ED1-positive macrophages in these areas progressed during the course of study (Fig. 2A and 2B). In this study, we used an antibody against α-SMA to identify myofibroblasts which had been used widely in other studies [16, 17]. Within the lobules of the liver at the first week, α-SMA-positive myofibroblasts were observed only in the central veins, portal veins and hepatic arteries. At weeks 3 and 6, they were also detected in the same sites as in the first week. Beginning at week 9, the number of α-SMA-positive myofibroblasts increased and peaked at week 12; myofibroblasts were observed primarily in the perisinusoidal areas where steatosis and macrophage infiltration occurred (Fig. 2C and 2D). At weeks 9 through 12, the regions where ED1-positive macrophages were observed almost coincided with the distribution of α-SMA-positive myofibroblasts. However, myofibroblasts were not detected in conjunction with macrophages at weeks 3 and 6.

**DISCUSSION**

Hepatic damage induced by cholesterol diet in rats, mice and rabbits have been noted often, but not further investigated [6, 25, 34]. Our study was designed to investigate the relationship between the intake of a diet supplemented with 1.0% cholesterol and 0.3% sodium cholate and hepatic fibrosis in rats. Previous studies have shown that chow-based diets supplemented with varying amounts of cholesterol and sodium cholate can induce hypercholesterolemia in mice and rats, presumably by interfering with the hepatobiliary excretion of cholate [22, 25]. Cholate may result in hepatic steatosis and ultimately, cirrhosis. However, Lichtman et al. [22] reported that sodium cholate (0.5% by weight) did not cause significant elevation in the amount of serum ALT or AST nor a decrease in albumin as compared with mice which were fed a similar diet.

Studies conducted on rats and rabbits that were fed a high cholesterol or fat diet have demonstrated profound lipid accumulation in the perportal area or centrilobular [6, 9, 29]. While studying the development of atheromatous lesions, one paper had reported that a diet supplemented with high levels of cholesterol induced moderate to pronounced centrilobular liver fibrosis in rabbits and, in this study, the three stages of fibrosis were divided [6]. Collagen was deposited around the central veins in the first stage; in the second stage, an increase of collagen fibers, myofibroblasts and macrophages were observed; in the third stage, large numbers of myofibroblasts were observed with a heavy deposition of connective tissue protein in the pericentral sinusoids. Therefore, Buyssens et al. [6] concluded that cholesterol overload induced pericentral liver fibrosis in rabbits, but they didn’t explain why hypercholesterolemia induced the transformation of hepatic stellate cells. Other reports describe that hepatic sinusoidal and centrilobular fibrosis can be induced by diets supplemented with cholesterol and stilbestrol in rabbits or alcohol in rats [26, 34]. There is, however, a lack of information regarding the role of cholesterol in fibrosis in these studies. In addition, they did not mention inflammatory responses induced by a high

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Hepatic lesion (H-E stain)</th>
<th>No. of mast cells/×400⁹</th>
<th>Hepatic fibrosis (Azan stain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>6.0 ± 0.5</td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>Microsteatosis (moderate)</td>
<td>8.2 ± 0.8⁹</td>
<td>Normal</td>
</tr>
<tr>
<td>6</td>
<td>Macrosteatosis (moderate)</td>
<td>8.8 ± 1.6⁹</td>
<td>Normal</td>
</tr>
<tr>
<td>9</td>
<td>Macrosteatosis (severe)</td>
<td>9.1 ± 0.7⁹</td>
<td>Normal ~ mild</td>
</tr>
<tr>
<td>12</td>
<td>Fibrosis (mild)</td>
<td>12.2 ± 0.6⁹</td>
<td>Mild</td>
</tr>
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a) Data are expressed as mean ± SEM.
b) P<0.05, significant difference from the control value (week 0).
c) P<0.05, significant difference from the value of the number at week 9.
cholesterol diet. Nanji et al. [26] suggested that cholesterol supplementation prevented necrosis and inflammation but enhanced fibrosis in alcohol induced liver disease in rats. In contrast, our results showed that significant necrosis and resultant inflammation occurred in addition to hepatic fibrosis, suggesting that a high-cholesterol diet may in itself be hepatotoxic. We first observed hepatic steatosis and foamy macrophage infiltration in and around the areas where necrosis and steatosis occurred at weeks 3 and 6. At times, the macrophages formed giant cells. As time passed, hepatic necrosis and macrophage infiltration significantly increased at weeks 9 and 12.

Atherosclerosis is characterized by a chronic inflammatory and fibro-proliferative response, which involves the recruitment and activation of mononuclear leukocytes [32]. In part, this process is controlled by the upregulation of genes whose products range from chemoattractant cytokines to substances which accelerate the local and systemic inflammatory response [33]. Atherogenic diets generate the following responses, namely the activation of nuclear factor-kappa B and the induction of genes for macrophage-colony stimulating factor, murine homologue of monocyte chemotactic stress, growth related oncogene (GRO-α), hemoxygenase and serum amyloid A in the liver [19–21]. It is likely that these substances play a role in monocyte recruitment during atherogenesis. It has been previously shown that the feeding of a diet high in fat and cholesterol to atherosclerosis-susceptible C57BL/6J mice resulted in the accumulation of oxidized lipids in the liver and arteries. Then, oxidative stress developed an inflammatory response in both hepatic and aortic tissues [19, 20]. Indeed, large quantities of oxidized low density lipoprotein may induce a differential expression of genes relevant to atherogenesis when engulfed by macrophages [8, 15]. In our study, with the obvious exception of necrotic areas, ED-1-positive macrophages infiltrated only those areas of the liver where steatosis occurred at week 3.

Recently, complex interactions between macrophages, myofibroblasts and mast cells have been postulated to be significant in the development of hepatic fibrosis [16]. However, no one has yet investigated the relationship between hepatic fibrosis and these three types of cells (macrophage, myofibroblast and mast cell) in rats that were fed a diet supplemented with 1.0% cholesterol and 0.3% sodium cholate. Myofibroblasts may be transformed from quiescent hepatic stellate cells (HSCs) by various factors, including
inflammatory cytokines, oxidants and growth factors [12, 27, 28] that are produced by activated macrophages [27], Kupffer cells [11, 31], hepatocytes or stellate cells themselves [2] in response to parenchymal damage. Mast cell appearance has also been reported in the development of liver fibrosis in rodents and human [1, 3, 13]

In our study, in the animals that were treated with a high cholesterol diet, hepatic steatosis and macrophage infiltration developed at week 3. Steatosis was more pronounced from weeks 6 to 12, leading to a further increase in macrophage infiltration in these areas. a-SMA-positive myofibroblasts were not detected in the areas of steatosis and macrophage infiltration at weeks 3 or 6. However, they were observed in these areas at weeks 9 and 12. Moreover, collagen fibers were detected in the most areas of macrophage infiltration and the periportal and midlobular areas at week 12. From week 0 to 12, as parenchymal damage increased, the number of mast cells increased significantly within the portal areas especially during times in which fibrosis was prevalent. In addition, mast cell numbers increased significantly in comparison with those at week 9 (P<0.05).

Previous studies demonstrated that the proliferation of myofibroblasts is preceded by a local expansion of the macrophage population in the damaged regions of hepatic tissue [16, 17], which is the same result as the present study. Infiltrating macrophages produced various mediators such as transforming growth factor-beta (TGF-β), platelet-derived growth factor, tumor necrosis factor-alpha and basic fibroblast growth factor [14, 17]. Among these cytokines, TGF-
β, functions on numerous cell types, is thought to play an important role in the fibrotic processes. It was presumed that HSCs might be transformed into myofibroblasts particularly by TGF-β1, and that they produce an abnormal extracellular matrix which results in hepatic fibrosis [14, 17]. One hypothesis for the acceleration of fibrosis by cholesterol is the up-regulation of TGF-β1 gene expression [10]. Indeed, one study showed the up-regulation of TGF-β1 in the livers of cholesterol-fed rats [24]. However, the stimulus for the up-regulation of TGF-β1 is unknown. Nanji et al. [24] reported previously that macrophages are the most prominent source of TGF-β. In our study, we observed a marked increase of HSCs after macrophage infiltration as the rats were fed a cholesterol diet at weeks 9 and 12. Therefore, we concluded that activated TGF-β produced by macrophages is thought to play an important role in hepatic fibrosis by mediating the transformation of HSCs to myofibroblasts and the induction of an abnormal extracellular matrix.

The high incidence of obesity in Western countries is associated with increased levels of dietary fat or cholesterol; indeed, steatosis is often present in the liver of obese persons [18]. Therefore, obesity itself can be a causative factor for the development of several diseases including coronary heart disease, gallstone formation and fatty liver [23, 36]. In our study, we didn’t evaluate atherosclerosis or gallstone formation. The consequences of hepatic damage, however, are of interest in view of the association between a high cholesterol diet and liver disease. This study 1) correlates a high cholesterol diet with quantifiable hepatic damage at the cellular level, 2) supports existing concepts of oxidative stress and hepatic fibrosis, and 3) establishes the dietary hypercholesterolemic rat model as a visible option for further investigation along these lines.
In conclusion, our findings suggest that hepatic damage and fibrosis can be induced by a high cholesterol diet and is likely the result of the interaction between several different cell types (i.e., macrophages, myofibroblasts, and mast cells) in an inflammatory milieu. Hypercholesterolemia should be considered as a risk factor for hepatic damage as well as atherosclerosis and coronary artery disease.

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REFERENCES


