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A diet-induced Sprague–Dawley rat model of nonalcoholic steatohepatitis-related cirrhosis☆,☆☆

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Abstract

Certain modified diets containing saturated fatty acids, cholesterol or fructose lead to the development of nonalcoholic steatohepatitis (NASH)-related fibrosis in rodents; however, progression to cirrhosis is rare. Experimental liver cirrhosis models have relied on genetic manipulation or administration of hepatotoxins. This study aimed to establish a reliable dietary model of NASH-related cirrhosis in a relatively short period. Male Sprague–Dawley rats (9 weeks of age) were randomly assigned to normal, high-fat (HF), or two types (1.25% or 2.5% cholesterol) of high-fat and high-cholesterol (HFC) diets for 18 weeks. All HFC diets contained 2% cholic acid by weight. Histopathological analysis revealed that the HFC diets induced obvious hepatic steatosis, inflammation with hepatocyte ballooning and advanced fibrosis (stage 3–4) in all 12 rats at 27 weeks of age. In contrast, all five rats given the HF diet developed mild steatosis and inflammation without fibrosis. The amount of cholesterol in the liver and hepatocellular mitochondrial and microsomal fractions was significantly higher in rats fed the HFC diets than the normal or HF diets. The HFC diets significantly suppressed mRNA levels of microsomal triglyceride transfer protein, adenosine triphosphate binding cassette transporter G5, bile acid CoA: amino acid N-acyltransferase and bile salt export pump, as well as the enzymatic activity of carnitine palmitoyltransferase in the liver. In conclusion, the HFC diets induced liver cirrhosis in conjunction with hepatic features of NASH in Sprague–Dawley rats within 18 weeks, and altered gene expression and enzyme activity to accumulate lipid and bile acid in the liver.

Keywords: Liver cirrhosis; Nonalcoholic steatohepatitis; High-fat and high-cholesterol; Cholic acid; Animal model

1. Introduction

Hepatic cirrhosis is defined anatomically as a diffuse process with fibrosis and nodule formation. Histologically, this is characterized by the replacement of the normal acinar liver structure with nodules separated by fibrous tissue. Regardless of cause, cirrhosis eventually results in three major events: hepatocellular failure, portal hypertension and hepatocellular carcinoma (HCC). The severity of these events determines the prognosis and treatment of cirrhosis [1–2].

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic steatosis in the absence of alcohol abuse and is now the most common etiology of chronic liver disease globally [3]. The more aggressive form of NAFLD, nonalcoholic steatohepatitis (NASH, defined as the presence of steatosis, lobular inflammation, hepatocyte ballooning and fibrosis), may advance to end-stage liver disease, including liver cirrhosis [4]. Patients with NASH-associated cirrhosis are at a high risk of developing HCC. A recent study in the United States reported that NASH was found to be the most rapidly growing indication for liver transplantation among patients with HCC [5]. It is also expected that the increased proportion of HCC secondary to nonviral liver disease in recent years is attributable to NASH in Japan [6]. The mechanisms involved in the progression of NASH to cirrhosis and HCC, however, remain largely unknown; thus, therapies to prevent or delay the disease progression remain limited.

Because of the high prevalence of NAFLD/NASH in the obese population, NAFLD/NASH can be considered one of the manifestations of metabolic syndrome. Some factors also contribute directly to the development of NASH, such as an increased consumption of high-fat foods and beverages high in fructose [3]. Recent reports suggest that dietary cholesterol intake and consequent increased hepatic

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cholesterol are critical factors in the development of NASH and fibrosis in animal models [7–8]. In addition, Ioannou et al. showed that dietary cholesterol intake was independently associated with the development of liver cirrhosis in a human epidemiological study [9]. Developing a better understanding of the contribution of dietary cholesterol to NASH pathogenesis is essential for developing methods for the prevention and treatment of NASH progression.

We previously reported a novel model of NASH-associated fibrosis using Sprague–Dawley (SD) rats fed a high-fat and high-cholesterol (HFC) diet. Our model developed steatohepatitis, including stage 1–4 fibrosis, within 9 weeks without reliance on genetic mutations and toxins [10]. In this study, we aimed to observe the histological and metabolic changes in our model rat fed the HFC diet for 18 weeks and to evaluate our model's suitability as a cirrhotic model.

2. Methods

2.1. Animals and experimental design

Eight-week-old male SD rats were obtained from Japan SLC (Hamamatsu, Japan) and housed individually in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Following a 1-week period of adaptation, the rats were randomly assigned to four groups receiving different diets for 18 weeks as follows: (a) a control group (n=5) fed a normal diet (MF; Oriental Yeast, Tokyo, Japan), (b) an HF group (n=5) fed a high-fat (68% MF, 30% palm oil, 2% cholic acid) diet with no cholesterol, (c) an HFC1.25 group (n=6) fed an HF diet with 1.25% cholesterol (68% MF, 28.75% palm oil, 1.25% cholesterol, 2% cholic acid) and (4) an HFC2.5 group (n=6) fed an HF diet with 2.5% cholesterol (68% MF, 27.5% palm oil, 2.5% cholesterol, 2% cholic acid). The rats in all groups had free access to food and water, and daily energy intake and body weight were assessed throughout the study.

At 27 weeks of age, the rats were fasted for 6 h and sacrificed under anesthesia with pentobarbital sodium. Organs were harvested, and blood samples were collected from the inferior vena cava. Liver tissues were either fixed in 10% neutral buffered formalin or snap frozen in liquid nitrogen and stored −80°C. This animal experiments were subjected to approval by the Animal Use Committee of University of Nagasaki, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals, University of Nagasaki.

2.2. Serum biochemical analysis

Serum concentrations of triglyceride (TAG), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, glucose, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using Triglyceride E test Wako, Cholesterol E test Wako, HDL Cholesterol E test Wako, Glucose C II test Wako and Transaminase C II test Wako (Wako Pure Chemical Industries, Osaka, Japan), respectively. Serum insulin levels were assayed using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Yokohama, Japan). Serum adiponectin levels were measured using a mouse/rat ELISA kit (Otsuka Pharmaceuticals, Tokyo, Japan). Serum leptin levels were determined with a Leptin ELISA kit (Morinaga Institute of Biological Science). The insulin resistance index was calculated using the homeostasis model of assessment [serum glucose (mg/dL)×serum insulin (ng/mL)/405], and the relative levels were evaluated among groups.

2.3. Histopathological examination

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin. All histopathological examinations were performed by a pathologist (K.T.) who was blinded to the experimental and serological data. Histological findings from the liver were scored using the NASH Clinical Research Network Scoring System based on 4 semiquantitative factors: steatosis (0–3), lobular inflammation (0–3), hepatocyte ballooning (0–2) and fibrosis (0–4). The grade and stage were further classified as follows: grade 0.5, the degree of histological finding was between 0 and 1; grade 3.5, between stages 3 and 4. The NAFLD activity score (NAS) was defined as the unweighted sum of the scores for steatosis, lobular inflammation and hepatocyte ballooning [11].

2.4. Lipid analysis in the liver and hepatocyte fraction

Hepatic lipids were extracted from frozen liver using the method of Folch et al. [12]. The extract was dissolved in isopropanol and analyzed for TAG and TC with a kit, as described above. Hepatic free fatty acid (FFA) was measured using a NEFA C test Wako (Wako Pure Chemical Industries). Diacylglycerol (DAG) was detected by thin layer chromatography (TLC). Briefly, equal amounts of lipid samples, which were extracted as mentioned above, were dried under a nitrogen stream, redissolved in chloroform, applied in equal amounts onto silica gel plates and then chromatographically separated with a solvent system consisting of hexane/diethylether/acetic acid [80:20:1 (v/v/v)]. After drying, the plates were visualized in an atmosphere of iodine vapor prior to scanning. The unprocessed images in jpeg format were quantified using ImageJ ver.1.43 software.

The mitochondrial and microsomal fraction of hepatocytes was prepared as described previously [13], and TC content was measured using a kit, as described above. Free cholesterol (FC) was measured using a free cholesterol E-test Wako (Wako Pure Chemical Industries).

2.5. Assay of hepatic enzyme activity

Liver portions were processed for preparation of mitochondria, and carnitine palmitoyltransferase (CPT) activity was measured spectrophotometrically as described previously [13].

2.6. Gene expression analysis

Total RNA from the liver was prepared using a Tripure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. Total RNA was reverse-transcribed using PrimeScript RT Master Mix (Takara Bio, Otsu, Japan). PCR was performed using cDNA with Thunderbird SYBR qPCR mix (Toyobo, Osaka, Japan) and specific primers (Greiner Japan, Tokyo, Japan) (Table 1) using an Applied Biosystems 7300 Real-time PCR system (Life Technologies Japan, Tokyo, Japan). The amplification program consisted of 1 cycle for 60 s at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. All quantifications were normalized with the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data were showed as relative expression to that of control-diet-fed rats, which was set to 1.

2.7. Statistical analysis

All values were expressed as means±standard error (S.E.). Differences between groups were tested for statistical significance using one-way analysis of variance and Bonferroni's *post hoc* test, or Fisher's exact probability test. Correlations between two variables were determined using Pearson correlation coefficient. Statistical significance was defined as $P<.05$. All calculations were performed using IBM SPSS statistics software, version 21.0 (IBM Co., Somers, NY) on a computer for Windows.