Video Article

The Murine Choline-Deficient, Ethionine-Supplemented (CDE) Diet Model of Chronic Liver Injury

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Abstract

Chronic liver diseases, such as viral hepatitis, alcoholic liver disease, or non-alcoholic fatty liver disease, are characterized by continual inflammation, progressive destruction and regeneration of the hepatic parenchyma, liver progenitor cell proliferation, and fibrosis. The end-stage of every chronic liver disease is cirrhosis, a major risk factor for the development of hepatocellular carcinoma. To study processes regulating disease initiation, establishment, and progression, several animal models are used in laboratories. Here we describe a six-week time course of the choline-deficient and ethionine-supplemented (CDE) mouse model, which involves feeding six-week old male C57BL/6J mice with choline-deficient chow and 0.15% DL-ethionine-supplemented drinking water. Monitoring of animal health and a typical body weight loss curve are explained. The protocol demonstrates the gross examination of a CDE-treated liver and blood collection by cardiac puncture for subsequent serum analyses. Next, the liver perfusion technique and collection of different hepatic lobes for standard evaluations are shown, including liver histology assessments by hematoxylin and eosin or Sirius Red stainings, immunofluorescent detection of hepatic cell populations as well as transcriptome profiling of the liver microenvironment. This mouse model is suitable for studying inflammatory, fibrogenic, and liver progenitor cell dynamics induced through chronic liver disease and can be used to test potential therapeutic agents that may modulate these processes.

Video Link

The video component of this article can be found at https://www.jove.com/video/56138/

Introduction

The liver is the largest glandular metabolic organ of the body and has many complex functions. Key roles for the liver include digestion, metabolism, detoxification, storage of essential nutrients, production of blood plasma protein components, and immunity mediated through resident macrophages or Kupffer cells. The liver has a great ability to regenerate even if up to 70-90% of its total mass is lost. In the event of acute liver injury, such as seen following a partial hepatectomy or acetaminophen poisoning, the remaining healthy hepatocytes proliferate to repair the damage in a highly coordinated process[1]. However, when the hepatocytes are chronically injured due to long-term viral infection, alcoholic or non-alcoholic fatty liver disease, the inflammatory microenvironment triggers the activation of fibrosis-driving hepatic stellate cells and the proliferation of liver progenitor cells (LPCs) with the potential to differentiate into either cholangiocytes or hepatocytes[2,3,4,5]. The precise origin, differentiation fate of LPCs, their contribution to liver regeneration, and hepatocarcinogenesis have been topics of intense debate and most likely depend on the injury severity and context[2]. The order of early regeneration-associated events is also controversially discussed, with some investigators stating that hepatic stellate cell activation and matrix remodeling is essential for generation of a LPC-favoring niche[6], while others report that LPC expansion and the so-called Ductular Reaction are required to trigger fibrogenesis[7]. There are numerous animal models

to study specific aspects of injury and regeneration, in an attempt to understand all the underlying factors that regulate disease progression and to ultimately develop new treatment strategies for patients[8].

The choline-deficient and ethionine-supplemented (CDE) dietary model was originally developed for use in rats and later modified for chronic liver injury induction in mice[9,10]. Dietary deficiency of choline results in impaired assembly and secretion of very low-density lipoproteins. Combined with the hepatocarcinogen DL-ethionine, this regimen leads to excessive hepatic fat loading, continuous inflammation, periportal fibrosis, LPC response and long-term to hepatocellular carcinoma development[11,12]. However, importantly, different mouse strains exhibit distinctive patterns of inflammatory, fibrogenic and LPC response dynamics[13]. This protocol describes chronic liver injury induction in C57BL/6J mice, the most commonly used inbred mouse strain.

In chronic liver disease research, typical analyses include histological assessments by hematoxylin and eosin as well as Sirius Red staining to visualize collagen depositions, immunohistochemical, or immunofluorescent detection of hepatic cell populations, and transcriptomic analyses of the liver microenvironment that orchestrates the induced cellular changes through complex growth factor and cytokine networks[14,15,16,17,18].

Protocol

1. Animal Experimentation

All animal studies described in this investigation were approved by the Curtin University Animal Ethics Committee (Approval number: AEC_2014_28) prior to commencement of the experiments and performed in accordance with the Australian code for the care and use of animals.

1. **Animals**
    1. Use six-week old male C57BL/6J mice for the experiments.

2. **Experimental design**
    1. Following arrival at the Animal Facility, allow mice to acclimatize for four days prior to commencement of any experiments.
    2. House mice on wheaten chaff bedding, which has been depleted of visible grains and stalks and keep mice on 12-hour day/night cycles in individually ventilated cages. Change bedding on days three and seven, then weekly thereafter.
    3. Provide mice with *ad libitum* access to the choline-deficient diet and drinking water supplemented with 0.15% of DL-ethionine. Keep the DL-ethionine-supplemented water at 4°C and replace drinking water every second day to ensure freshness and encourage drinking. Top up the choline-deficient chow every second day with a complete change of chow once a week.
    4. Observe mice at rest and during handling. Monitor standard signs of animal health, including overall appearance, posture, social interaction, grooming, coat condition, and body weight.
    5. Weigh mice daily during the first two weeks of the experiment to ensure any animals exhibiting more than 20% body weight loss are euthanized to limit undue suffering. This is typically the case in less than 5% of animals. After two weeks, the frequency of weighing can be reduced to three times weekly (*e.g.*, Monday, Wednesday, Friday).

3. **Liver perfusion and isolation**
    1. Anesthetize mice at indicated time points (in this study one, two, and six weeks on the CDE diet) with ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection. Test the pedal withdrawal reflex to ensure adequate anesthesia.
    2. Wet the fur with 70% ethanol and make a vertical midline incision in the abdominal wall up to the diaphragm using Mayo scissors. The rib cage can be removed for easier access to the heart.
    3. Collect blood by slow cardiac puncture using a 25 G x 1/2" regular wall needle to avoid collapsing of the heart. Allow the blood to clot in a microcentrifuge tube at room temperature to obtain serum samples after centrifugation at 2,000 x g for 10 min. Later, measure serum alanine transaminase levels by standard procedures[12].
    4. Move the stomach and small intestine to the side and expose the portal vein. Cut the heart using Mayo scissors to allow fluids to exit and perfuse the liver with sterile phosphate buffered saline (pH=7.4) by cannulating the portal vein using a 27 G x 1/2" regular wall needle. An evenly blanched liver indicates successful perfusion of all liver lobes.
    5. Carefully detach the liver and place it in a petri dish using forceps and Mayo scissors. Remove excess, non-liver tissue and the gallbladder.

4. **Liver preservation**
    1. After the total liver weight has been recorded, cut one small lobe into several small pieces of a few square millimeters, transfer them to sterile tubes, and snap-freeze in liquid nitrogen for later RNA and protein extraction, as per standard protocols. Snap-freeze these tissue pieces as soon as possible after organ collection to avoid any tissue degradation.
    2. Collect one lobe in 10% neutral buffered formalin for later processing and paraffin embedding, as per standard protocols.
    3. Place one lobe into a mold filled with optimum cutting temperature cryomatrix embedding resin and snap-freeze in liquid nitrogen.

Representative Results

Throughout the six-week time course of CDE-induced chronic liver injury, parameters were assessed on days 7 (induction phase), 14 and 21 (establishment phase) and 42 (maintenance phase). Compared to control mice, CDE-treated mice lost up to 20% of their initial body weight in an initial adaptation phase and tend to regain weight in the establishment and maintenance phases **(Figure 1)**. The body weight was inversely correlated with serum alanine transaminase levels, an indicator of hepatocellular injury **(Figure 2)**. Four micrometer-thin, formalin-fixed, and paraffin-embedded liver tissue sections were stained with hematoxylin and eosin to assess the hepatic architecture. Healthy livers displayed a normal architecture with orderly cords of hepatocytes radiating from the central vein, forming the liver parenchyma. In contrast, CDE-treated livers showed vastly disrupted liver architecture and the parenchymal infiltration of many basophilic cells in the induction and establishment phase and normalization in the maintenance phase **(Figure 3)**. To assess fibrogenesis, 4-micrometer thin, formalin-fixed, and paraffin-embedded liver sections were stained with the poly-azo dye Sirius Red, which visualizes hepatic collagen deposition. In the induction phase of CDE-induced chronic liver injury, collagen accumulated in the parenchyma in periportal regions. However, levels normalized in the establishment and maintenance phase **(Figure 4)**. Matrix-embedded and snap-frozen liver tissue was cut into 7-micrometer thin sections on a cryostat. Immunofluorescent staining for cell markers was then performed to analyze the temporal and spatial arrangement of specific hepatic cell populations during a time course. In healthy livers, the biliary and liver progenitor cell marker panCK only stained bile duct structures, while the inflammatory cell marker CD45 detected liver resident macrophages or Kupffer cells. In chronically injured livers, however, increased panCK staining reflected biliary and liver progenitor cell proliferation, which peaked and reached a steady state after around 14 days of CDE treatment. The number of CD45-positive inflammatory cells, representing both liver-resident and infiltrating cells, also peaked in the induction phase of CDE-induced injury and slowly normalized to control levels in the establishment and maintenance phases **(Figure 5)**. This expansion of inflammatory cell populations in chronically injured livers was accompanied by rapid changes in the hepatic microenvironment. A quick induction or significant increase in transcription levels of cytokines and growth factors was observed. These include tumor necrosis factor (TNF), tumor necrosis factor-like weak inducer of apoptosis (TWEAK), lymphotoxin-beta (LTβ), hepatocyte growth factor (HGF), transforming growth factor-beta (TGFβ), and interleukin-6 (IL-6), for example. Their transcript levels all peaked in the induction phase and normalized during the establishment and maintenance phase - similar to previously shown disease parameters **(Figure 6)**.

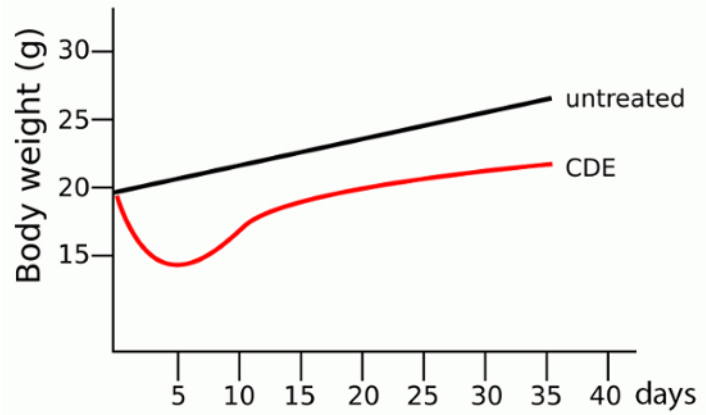

Figure 1. Body weight recordings of healthy and CDE-treated mice. Chronically injured mice lost up to 20% of their initial body weight in the induction phase of CDE feeding and recovered thereafter. A representative diagram is shown. Please click here to view a larger version of this figure.

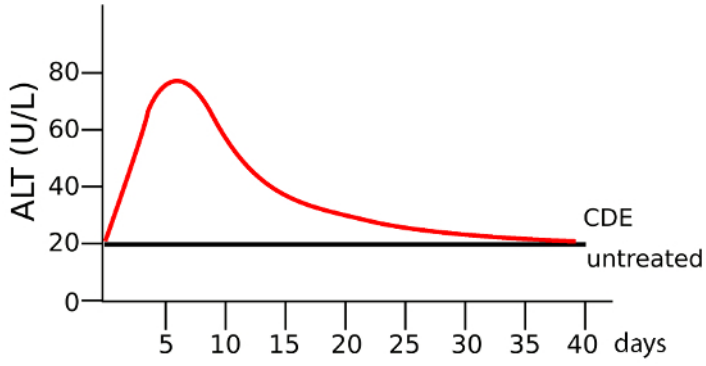

Figure 2. Serum alanine transaminase levels in healthy and CDE-treated mice. Serum alanine transaminase (ALT) levels remained unchanged in healthy mice during a six-week time course but peaked in the induction phase, with normalization in the establishment and maintenance phase in CDE-treated animals. A representative diagram is shown. Please click here to view a larger version of this figure.

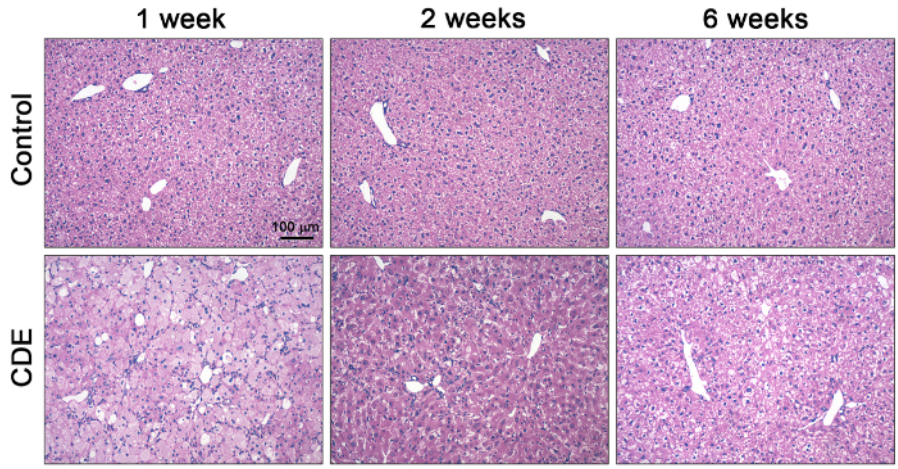

Figure 3. Haematoxylin and eosin stainings of liver sections. Formalin-fixed and paraffin-embedded liver tissue sections that were stained with H&E demonstrated orderly arrangements of hepatocyte cords in control mice. In contrast, the liver architecture was vastly disrupted in CDE-treated mice in the induction phase, with normalization towards the end of the six-week time course. The scale bar represents 100 µm. Please click here to view a larger version of this figure.

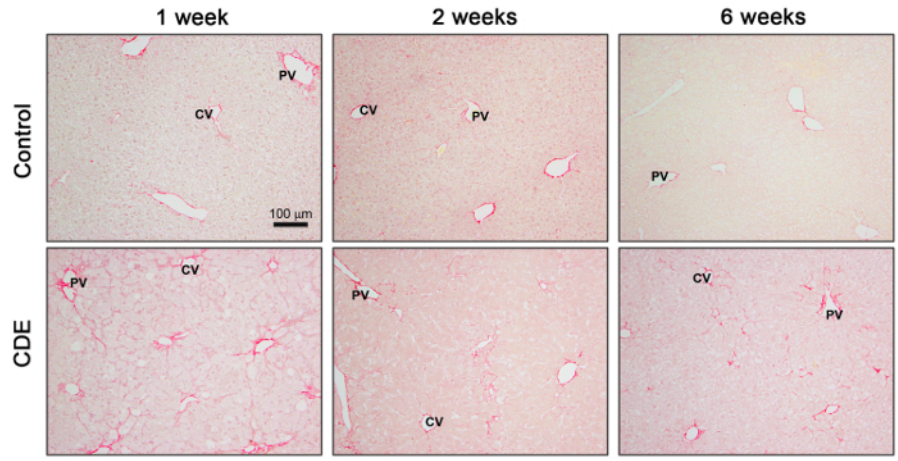

Figure 4. Sirius Red staining-facilitated collagen visualization in healthy and CDE-treated mice. Compared to control mice that only displayed minor perivenous collagen depositions, chronically injured animals showed collagen accumulation in periportal parenchyma regions in the induction phase, with slow normalization towards the maintenance phase of the CDE time course of six weeks. The scale bar represents 100 µm. Please click here to view a larger version of this figure.

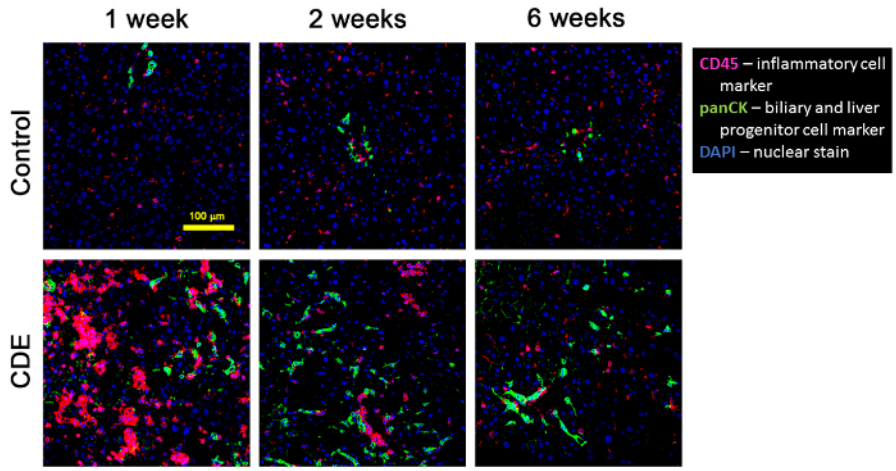

Figure 5. Immunofluorescent detection of inflammatory and biliary/liver progenitor cells. In healthy livers, only biliary structures stained with the biliary and liver progenitor cell marker panCK (red) and the CD45 antibody visualized liver-resident macrophages or Kupffer cells (green). In the induction phase of CDE feeding, the number of $CD45^+$ cells increased drastically which included liver-resident as well as infiltrating inflammatory cells. This inflammatory response normalized towards the end of the six-week time course. Biliary and liver progenitor cells that stain with the marker panCK increased in numbers up to week two and reached a steady state thereafter. DAPI was used for nuclear quantitation. The scale bar represents 100 µm. Please click here to view a larger version of this figure.

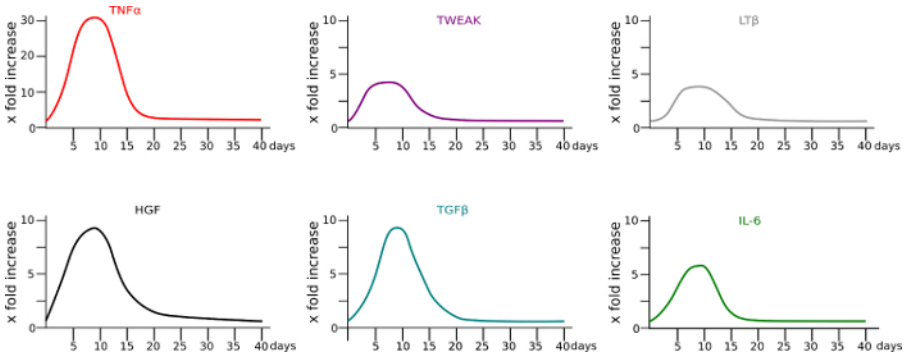

Figure 6. Transcriptomic analyses of cytokines and growth factors. RNA was isolated from snap-frozen pieces of CDE-treated liver tissues to perform transcriptomic analyses of the liver microenvironment. Representative diagrams of tumor necrosis factor (TNF), TNF-like weak inducer of apoptosis (TWEAK), lymphotoxin-beta (LTβ), hepatocyte growth factor (HGF), transforming growth factor-beta (TGFβ), and interleukin-6 (IL-6) are shown. Please click here to view a larger version of this figure.

Discussion

Chronic liver disease is often a silent disease with most patients being asymptomatic and it is one of the major contributors to morbidity and mortality worldwide. Chronic alcoholism and hepatitis C virus infection are the leading causes. Chronic liver injury is characterized by hepatic inflammation, fibrosis and in severe cases cirrhosis, carcinoma and ultimately liver failure. There is currently no cure available and although major advances have been made to understand the mechanisms of liver disease, new therapeutic avenues are still urgently required.

The CDE diet is a simple, time-effective, and easily administrable model for induction and study of fatty changes or hepatic steatosis, inflammation, fibrosis, LPC proliferation and, in long-term experiments, development of hepatocellular carcinoma[6,11,12,13,16,17]. Initially used in rats[19,20], it was later adapted to facilitate LPC studies in wildtype and genetically modified mice[9].

In our laboratory, we have found it critical to use wheaten chaff instead of other bedding alternatives. Even though it is depleted of visible grains and stalks, a few additional nutrients remain in the wheaten chaff bedding and counterbalance some of the harsh effects of CDE-induced chronic liver injury induction. In addition, this bedding provides environmental enrichment and encourages normal food search behavior in mice. The vast majority of adverse events were observed in the first week of CDE treatment and rarely seen thereafter. The likelihood of adverse events can be reduced by using mice that have a starting weight of at least 16 g. However, if the starting weight is more than 18-20 g, the number of induced liver progenitor cells may be lower. The CDE model can be used to study hepatic steatosis, inflammation, liver progenitor cell responses, fibrosis, and hepatocellular carcinoma development without the presence of liver cirrhosis, such as seen in some non-alcoholic fatty liver disease (NAFLD) and hepatitis B virus-infected patients[21,22]. In comparison, the thioacetamide (TAA) model of chronic liver injury represents a more aggressive regime. It induces mild fatty changes, inflammation, liver progenitor cells, and fibrosis which already progresses to early cirrhosis around the 6-week time point[12]. Hence, CDE feeding is suggested if NAFLD-associated processes are the study focus, whereas a TAA time course may be advantageous when different stages of fibrosis severity are of interest.

Disclosures

There is nothing to be disclosed by the authors.

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