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A mouse model of non-alcoholic steatohepatitis and hepatocellular carcinoma induced by western diet and carbon tetrachloride.

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Running head: NASH and HCC model induction by Western Diet and CCL₄.

Abstract

Non-alcoholic steatohepatitis (NASH) is a severe form of non-alcoholic fatty liver disease (NAFLD). Obesity is a known risk factor of NASH, which, in turn, increases the risk of developing cirrhosis (liver scarring) and hepatocellular carcinoma (HCC). In addition to being a potentially life-threatening condition, public health concerns surrounding NASH are amplified by the lack of FDA-approved treatments. Although various preclinical models reflecting both the histopathology and the pathophysiological progression of human NASH exist, most of these models are diet-based and require 6–13 months for NASH symptom manifestation. Here, we describe a simple and rapid-progression model of NASH and NASH-driven HCC in mice. Mice received a western diet equivalent (WD; i.e., a high-fat, high-fructose and high-cholesterol diet), high-sugar water (23.1 g/L fructose and 18.9 g/L glucose), and weekly intraperitoneal injections of carbon tetrachloride (CCl₄) at a dose of 0.2 μL/g of body weight. The resulting phenotype, consisting in liver fibrosis and HCC, appeared within 24 weeks of diet/treatment initiation and presented similar histological and transcriptomic features as human NASH and NASH-driven HCC, thereby supporting the adequacy of this preclinical model for the development and evaluation of drugs that can prevent or reverse these diseases.

Key words

western diet, carbon tetrachloride (CCl₄), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), hepatocellular carcinoma (HCC), NASH-driven HCC.

1. Introduction

Liver cancer is a major cause of cancer-related mortality worldwide, with hepatocellular carcinoma (HCC) accounting for about 90 % of all liver cancer types **(1)**. Various risk factors have been associated with the development and progression of HCC, including hepatitis B and C viruses, diabetes, alcohol, smoking, obesity and non-alcoholic fatty liver disease (NAFLD) **(2)**.

NAFLD is the most common chronic liver disease, with the spectrum of liver pathology ranging from simple steatosis (abnormal retention of lipid droplets within hepatocytes) to more severe non-alcoholic steatohepatitis (NASH), mainly characterized by steatosis, inflammation, hepatocyte injury (ballooning), and varying degrees of liver fibrosis **(3)**. NAFLD is strongly associated with metabolic syndrome, a cluster of conditions including abnormal glucose regulation, insulin resistance, dyslipidemia, hypertension and - critically - obesity **(4,5)**. A meta-analysis showed a high prevalence of obesity among patients with NAFLD (51.34%) or NASH (81.83%) **(6)**. With the obesity epidemic spreading worldwide, obesity-induced NAFLD/NASH is now emerging as one of the most prevalent risk factors for HCC **(4,5)**. NAFLD has been reported to be the primary cause contributing to the increased HCC incidence in the United States **(7)**, and the number of NAFLD-related HCC

cases is increasing rapidly in China, Japan, France, Germany, Italy, Spain and the UK **(8)**.

NAFLD- and NASH-driven HCC is a complex and dynamic evolution from (i) steatosis to (ii) steatohepatitis, (iii) advanced fibrosis/cirrhosis, and, finally, (iv) cancer (Figure 1A) **(5)**. Interventions that slow or reverse the development of NAFLD/NASH therefore have the potential to prevent liver cancer. To date, the sole treatment options for NAFLD/NASH consists in weight loss and lifestyle modifications **(9)**, a fact that highlights the considerable unmet medical need for novel therapeutic approaches and pharmacological agents against NAFLD/NASH. However, the lack of robust animal models necessary for preclinical testing appears as a major obstacle to the development of such drugs. Although several diet- or diet/treatment-based NASH-driven HCC mice models have been established (Figure 1B), their use for preclinical studies has been limited **(10-16)**. Indeed, most of these models require a relatively long period (6–13 months) before appearance of the disease phenotype and would therefore result major delays and costs in the context of drug development for human diseases **(10-16)**.

Here, we describe a NASH model in mice, which closely replicates the etiology, disease progression stages, histology and transcriptomic features of human NASH and NASH-driven HCC **(10)**. To this end, mice received a western diet equivalent (WD; high-fat, high-fructose, and high-cholesterol) and high-sugar water (with high glucose and fructose content) to mimic the primary cause (i.e.,

caloric excess) and induce the key risk factors (obesity and insulin resistance) of human NASH **(11)**. In addition, a low dose of carbon tetrachloride (CCl₄) was administered weekly to accelerate the WD-induced NASH histological changes, resulting in a disease phenotype consistent with human NASH within 12 weeks of diet/treatment initiation, and the development of NASH-driven HCC within 24 weeks **(10)**. HCC is diagnosed and monitored by using B-ultrasound in this model. Figure 2A-B show representative B-ultrasound images of healthy liver and NASH-driven HCC. Following B-ultrasound confirmation, the mice are sacrificed, and their livers are collected and photographed (Figure 2C-D).

2. Materials

2.1. Animals

Male C57BL/6J mice (8-week-old) are adapted to their environment for at least one week before the beginning of the study. An enriched environment minimizes anxiety, agonistic behaviors and stress. Animals are housed in a 12 h light/dark cycles, with a maximum of 5 mice per cage. All procedures are performed according to protocols approved by the local Animal Experimental Ethics Committee.

2.2. Diet and treatment

1. Western Diet (WD) containing 21.00 % milk fat, 40.54 % sucrose, and 1.25 % cholesterol (Diet energy is 4500kcal/kg, composed of 42 % fat, 42.7 % carbohydrate, and 15.2 % protein).

2. High-sugar water is prepared with D-(-)-fructose (23.1 g/L fructose) and D-(+)-glucose (18.9 g/L glucose).
3. Disposable sterile 450mL sipper sack bags.

2.3. CCl₄ injection

1. 9-week-old C57BL/6J male mice.
2. CCl₄ (0.2 μL/g of body weight) is diluted with corn oil.
3. Personal protective equipment according with the rules of the animal facility (long-sleeved laboratory coat, rubber gloves, cover shoes, disposable work cap, and face mask) and a chemical fume hood
4. 15mL centrifuge tubes
5. Shaver and laboratory animal weighing scale
6. 1mL syringes and 27G needles
7. Container for dangerous chemical waste

2.4. Equipments and other materials

1. The Vevo® 3100 (FUJIFILM VisualSonics) imaging system is an ultrasound imaging system used to perform diagnostic analyses on laboratory animals. Use the B-mode of the imaging system to detect tumors on the liver with the MX550D transducer.
2. The Vevo® imaging station includes an animal physiological monitoring system (with heating function) to secure the animal and monitor

temperature, electrocardiogram data, blood pressure, heart and respiratory rate of animals during an imaging session.

3. According to the equipment of the animal facility, required equipment and materials for the inhalation anesthesia procedure are an oxygen concentrator, flowmeter, vaporizer, scavenging system, induction chamber, and medical masks.
4. Isoflurane
5. Shaver and depilatory cream
6. Ophthalmic liquid gel
7. Ultrasound transmission gel
8. Animal scale
9. Common disposables, such as cotton buds, medical tapes and gauze

3. Methods

3.1. Western diet (WD) and high-sugar water feeding

1. Replace the normal chow diet (RCD) with WD for 9-week-old mice.
2. Simultaneously, replace the normal water by high-sugar water (See **Note 1**).
3. Measure weight of remaining WD and add fresh food on a weekly basis (See **Note 2**).
4. Prepare 450 mL high-sugar water with disposable sterile 450mL sipper sack bags for each cage on a weekly basis (See **Notes 3–5**).
5. Change the bedding on a weekly basis (See **Notes 6–8**).

6. Feed mice WD and high-sugar water for 24 weeks. The mice should be euthanized at the 24-week timepoint, after confirmation of tumor development by ultrasound.
7. Monitor the body weight of mice on a weekly basis.

3.2. CCl₄ preparation and administration to mice

1. Dilute the CCl₄ in corn oil (dilution 1:10–1:15 volume/volume) under the chemical fume hood, with adequate personal protective equipment to reduce risk of toxic exposure to CCl₄ for the experimenter (See **Notes 9–12**).
2. Weigh mice prior to the intraperitoneal injection to calculate volume of CCl₄ to be administered (CCl₄ dose is 0.2 μ L/g of body weight) (See **Note 13**).
3. Gently restrain mouse and perform the injection with the adequate volume of CCl₄. It is recommended to perform intraperitoneal injections in the lower part of the left-side of the abdomen, laterally to the midline, using a 27G needle, and with the mouse's head pointing down to minimize risk of injury to the bladder or intestine (See **Note 14**).
4. Following the injection, discard used material (tubes, needles, and syringes) in a container for hazardous chemical waste.
5. Put the mice back in the cage and inspect 1 hour after injection and daily thereafter (See **Notes 15–16**).

3.3. Ultrasonographic assessment of HCC

1. The Vevo® 3100 imaging system is used to monitor HCC development and determine the end-time of the experiment (See Notes 17–18): switch on the Vevo® Monitor and set the heated platform to 38°C; switch on the gas anesthesia station and set the air flow at 1.5–2 L/min and isoflurane vaporization at 2–3 % for general anesthesia induction with the chamber; transfer the mouse to the face mask for imaging, with isoflurane vaporization set at 1.5–2 % for anesthesia maintenance during the examination, which should last approximately 20 min per mice; turn on the Vevo® 3100 imaging system.
2. Remove fur from the abdominal region with a shaver, apply ophthalmic gel on the eyes, and place the mice on the imaging platform.
3. Use the depilatory cream to remove the fur more thoroughly (leave the cream for approximately 1–2 minutes), then clean the area with warm water and wet gauze.
4. Finally, apply an ultrasound transmission gel as needed, on the skin of the abdomen; position the transducer on the ultrasound gel in transverse orientation and start imaging in B-mode; apply necessary pressure to visualize the different regions of the liver.
5. At the end of the imaging procedure, let the mice recover on a heating lamp before placing them back in their original cages.

4. Notes

1. Animal food (RCD and WD) is recommended to ensure sterility. Food must be stored $\leq 4^{\circ}\text{C}$ and used within 6 months after opening.
2. Add about 100g WD/cage/week, replace the food weekly to avoid diet spoilage.
3. To avoid the contamination of high-sugar water, it is recommended to use sterilized 450 mL sipper sack bags and replace the water/high-sugar water on a weekly basis.
4. The animal drinking valves should be cleaned and decontaminated every time water is changed.
5. Since the mice in a given cage (max. 5 mice/cage) may drink more than 400 mL of water per week, it is suggested to add at least 450 mL sipper sack bags.
6. It is better to change cages on a weekly basis to avoid wet litter and dampness in the environment resulting from urination. A suboptimal environment may limit the mice's ability to move and impede their normal daily activity.
7. Aggression is innate to males of the C57BL/6 strain, especially after changing them to new cages. Environmental enrichment (e.g., addition of wooden sticks, cotton slivers, and/or a mouse hut to the cage) may contribute to reducing stress and aggression.
8. In addition, with the aim of limiting aggressive behavior, it is recommended

to purchase younger mice (e.g., 4-week-old) and provide them with ample time to adapt to housing conditions.

9. CCl₄ vapors are toxic to humans. The injection of CCl₄ should therefore be performed in a chemical fume hood.

10. In addition, wearing a long-sleeved laboratory coat, rubber gloves, and a medical mask is recommended for the CCl₄ injection procedure.

11. Remove fur to inject more precisely with a shaver.

12. In addition, the solution of CCl₄ diluted in corn oil should be at room temperature prior to injection.

13. The volume of CCl₄ to be injected should be calculated based on the actual body weight of each mouse. A correspondence table of injection volumes for a range of body weights is recommended to streamline the calculation process.

14. Mice should be restrained with the head pointing down, in order to move the abdominal viscera away from the injection site.

15. Animals should be inspected for abnormalities (like abdomen swelling, infection, bleeding), 1 hour after injection and every day thereafter.

16. Water bags should be correctly placed on the cages to avoid unwanted water leaking and resulting deterioration of the environment.

17. Although the diet/treatment-induced HCC is expected to occur after approximately 24 weeks, factors (e.g., differences between animal facilities)

may impact on diet/treatment efficacy and alter experiment duration.

18. B-ultrasound is recommended to confirm the development of HCC tumors at the 24-week timepoint, before mice sacrificing.

Conflicts of interest

GK has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Tollys, and Vascage. GK has been consulting for Reithera. GK is on the Board of Directors of the Bristol Myers Squibb Foundation France. GK is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics and Therafast Bio. GK is in the scientific advisory boards of Hevolution, Institut Servier and Longevity Vision Funds. GK is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis and metabolic disorders. Guido Kroemer's wife, Laurence Zitvogel, has held research contracts with Glaxo Smyth Kline, Incyte, Lytix, Kaleido, Innovate Pharma, Daiichi Sankyo, Pilege, Merus, Transgene, 9 m, Tusk and Roche, was on the on the Board of Directors of Transgene, is a cofounder of everImmune, and holds patents covering the treatment of cancer and the therapeutic manipulation of the microbiota. GK's brother, Romano Kroemer, was an employee of Sanofi and now consults for Boehringer-Ingelheim. The funders had no role in the design of the study; in the writing of the manuscript, or in the decision to publish the results.

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Figure 1. NAFLD and NASH-driven HCC mice models. **A.** NASH-induced HCC is the product of a complex and dynamic evolution, comprising a spectrum of liver disorders ranging from simple steatosis to NASH, which can subsequently proceed to fibrosis, cirrhosis and HCC. **B.** Several diet- or diet/treatment-induced mouse models have been used to study NASH (the model described in this chapter is in red).

Figure 2. Representative images of NASH-driven HCC and normal livers. B-ultrasound was used to analyze the livers of mice treated with RCD/Oil (A) and WD/CCl₄ (B) prior to euthanasia (white circles highlight the tumors). At the end of the experiment, the mice livers from RCD/Oil (C) and WD/CCl₄ (D) treated groups were collected and photographed (the black circles highlight tumors ≥ 2 mm in diameter).



