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Short communications

Dietary broccoli protects against fatty liver development but not against progression of liver cancer in mice pretreated with diethylnitrosamine

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ABSTRACT

Western-style high fat, high sugar diets are associated with non-alcoholic fatty liver disease (NAFLD) and increased liver cancer risk. Sulforaphane from broccoli may protect against these. Previously we initiated broccoli feeding to mice prior to exposure to the hepatocarcinogen diethylnitrosamine (DEN), and saw protection against NAFLD and liver cancer. Here we administered DEN to unweaned mice, initiating broccoli feeding two weeks later, to determine if broccoli protects against cancer progression. Specifically, male 15-day-old C57BL/6J mice were given DEN and placed on a Western or Western + 10%Broccoli diet from the age of 4 weeks through 7 months. Dietary broccoli decreased hepatic triacylglycerols, NAFLD, liver damage and *tumour necrosis factor* by month 5 without changing body weight or relative liver weight, but did not slow carcinogenesis, seen in 100% of mice. We conclude that broccoli, a good source of sulforaphane, slows progression of hepatic lipidosis, but not tumourigenesis in this robust model.

1. Introduction

In the United States, more than one-third of the adult population is obese (Ogden, Carroll, Kit, & Flegal, 2014). Excess intake of energy (typically from saturated fats and sugars) favours the development of obesity because of the loss of energy balance (Spiegelman & Flier, 2001). Liver is the major metabolic centre for both carbohydrate and lipid, thereby maintaining glucose homeostasis (Postic, Dentin, & Girard, 2004). An overload of dietary carbohydrate and fat disturbs the metabolic function of the liver and may lead to non-alcoholic fatty liver disease (NAFLD), a cluster

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Abbreviations: AHF, altered hepatic foci; ALT, alanine aminotransferase; *AMPK*, 5' AMP-activated protein kinase; Cyp, cytochrome P450; DEN, diethylnitrosamine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HA, hepatic adenomas; HCC, hepatocellular carcinomas; IFNg, interferon-γ; NAFLD, non-alcoholic fatty liver disease; *NASH*, non-alcoholic steatohepatitis; Nrf2, Nuclear factor erythroid-2-related factor; NQO1, NAD(P)H-quinone oxidoreductase 1; TNF, *tumour necrosis factor*; WBD, Western + Broccoli diet; WD, Western diet

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of related liver diseases that begins with simple steatosis, developing into non-alcoholic steatohepatitis (NASH), cirrhosis, and possibly hepatocellular carcinoma (HCC) (Angulo, 2002; Cohen, Horton, & Hobbs, 2011). Compared to non-obese people, the risk for hepatic steatosis is almost 5-fold higher in obese people (Bellentani et al., 2000). For obese males, their mortality risks from liver cancer are 4.5-fold higher than men with a normal BMI (Calle, Rodriguez, Walker-Thurmond, & Thun, 2003).

Broccoli, like other brassica vegetables, is rich in many bioactive compounds, including flavonoids, ascorbate, and especially the glucosinolate glucoraphanin (Jeffery et al., 2003). Glucosinolates are stable, sulphur-rich glycosides, until they are hydrolyzed by plant- or microbiome-derived myrosinase to form bioactive aglycones (Angelino et al., 2015). Sulforaphane, the aglycone of the broccoli glucosinolate glucoraphanin, activates nuclear factor erythroid-2-related factor (Nrf2), which in turn upregulates detoxification, ameliorates inflammation, and induces apoptosis (Jeffery & Araya, 2009).

To evaluate the relation between diet and liver diseases, including NAFLD and liver cancer, several animal models are available. We recently fed broccoli to mice receiving either a control or Western diet (WD), giving the hepatocarcinogen diethylnitrosamine (DEN) one week after initiating dietary treatments (Chen, Wallig, & Jeffery, 2016). At six months, hepatic lipidosis was lessened and there was a small but significant protective effect against liver cancer in those mice fed broccoli. These findings are in agreement with those of Kay and colleagues who found that Nrf2 upregulated by sulforaphane, inhibits LXRα-dependent hepatic lipogenesis (Kay et al., 2011). Multiple mechanisms have been proposed for protection against cancer by sulforaphane, with Nrf2 playing a key role. Our recent study is the first to show protection against liver cancer (Chen et al., 2016). However, most studies, including ours, do not identify whether the impact of dietary broccoli was on the initiation or progression of these liver diseases. The present study, a longitudinal, diet-facilitated (WD) and chemical-induced (DEN) liver cancer model, in which DEN was administered before dietary intervention, was used to evaluate progression of hepatic lipidosis and cancer through 7 months, separate from initiation. Our aim was to evaluate the role of broccoli in protection against progression of NAFLD and liver cancer.

2. Material and methods

2.1. Animal and study design

Six to eight week-old dams and male C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) for breeding. All mice used for breeding were maintained on rodent chow diet, except during gestation and lactation where the dams were fed AIN-93G. Fifteen-day-old male pups were used in this longitudinal study, given 25 mg DEN/kg body weight intraperitoneally in normal saline. The DEN-treated mice were housed individually, and randomly assigned to WD or Western + Broccoli diet (WBD) groups at 4 weeks of age, and given *ad libitum* access to water and feed. Feed was replaced daily. Animals were maintained under a 12-hour light/dark cycle at 22 °C and 60% humidity. Animal care was in compliance with the approved protocol by the Institutional Animal Care and Use Committee and the Division of Animal Resources at the University of Illinois, Urbana-Champaign, according to the National Institutes of Health guidelines. Six animals from each dietary group were sacrificed at 3, 5, and 7 months after starting the dietary treatments, for evaluating the progression of hepatic lipidosis and tumorigenesis. Body weight and feed intake were monitored each week.

2.2. Reagents and diets

Diethylnitrosamine was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Western diet was formulated by modifying AIN-93G to increase the sucrose and saturated fat content (Table S1). Freeze dried broccoli powder (*Brassica oleracea* L. var. Green Magic) was kindly provided by Dr. John A. Juvik, which contained 4 mmol sulforaphane/kg broccoli powder, determined by hydrolysis of endogenous glucoraphanin, as previously reported (Dosz & Jeffery, 2013). Other diet ingredients were purchased from the Harlan Laboratory (Indianapolis, IN, USA). Broccoli powder (10%, w/w) was incorporated into the WBD, which was balanced nutritionally by partly replacing the corn starch and cellulose with fibre and carbohydrate from broccoli (Table S1).

2.3. Hepatic triglyceride content

Liver lipid was extracted by the method of Folch (Folch, Lees, & Stanley, 1957) with some modifications. Liver tissue was homogenized with chloroform/methanol (2:1, v/v) with 0.01% butylated hydroxytoluene (BHT). Homogenates were washed with ultrapure water and centrifuged for the separation of organic and water phases. The chloroform phase was heated to 50 °C under a stream of nitrogen gas for evaporation. The remaining lipid extract was stored at –20 °C for determining hepatic triacylglycerol concentrations by the glyceride phosphate oxidase method using a reagent set (Pointe Scientific, Canton, MI, USA).

2.4. Hepatic lesion detection and NAFLD score

At sacrifice, livers were weighed and examined for macroscopic lesions. Visible nodules (diameter ≥ 1 mm) in each liver lobe were counted and the maximum diameter was measured using calipers. Mouse liver (median lobe) was fixed in 10% neutral buffered formalin for later paraffin section. Sections (3 μm) were stained with haematoxylin and eosin (H&E) for histological examination. All histology work was done at the Veterinary Diagnostic Laboratory (University of Illinois, Urbana, IL, USA). Liver sections with H&E staining were examined for microscopic lesions, including altered hepatic foci (AHF), hepatic adenomas (HA), HCC, and NAFLD scores. All pathology was carried out by a board-certified veterinary pathologist; the scoring criteria are shown in Table S2.

2.5. Serum alanine aminotransferase (ALT)

Serum ALT levels were determined using the Liquid ALT Reagent Kit (Pointe Scientific, Canton, MI, USA) according to the manufacturer's instructions.