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Essential role of constitutive androstane receptor in *Ginkgo biloba* extract induced liver hypertrophy and hepatocarcinogenesis

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ABSTRACT

Ginkgo biloba extract (GBE) is commonly used as a herbal supplement. The National Toxicology Program (NTP) study of GBE reported clear evidence of hepatocarcinogenicity in mice. To clarify the mode of action (MOA) for hepatocarcinogenesis by GBE, we investigated the involvement of the constitutive androstane receptor (CAR) in hepatocarcinogenesis induced by GBE using CAR-knockout (CARKO) and wild type (WT) mice. We used the same lot of GBE that was used for the NTP study. In 1-week GBE dietary treatment, hepatocellular DNA replication was increased in WT mice but not in CARKO mice. In 4- or 13-week treatment, greater hepatic Cyp2b10 induction and hepatocellular hypertrophy were observed in WT mice, whereas these effects of GBE were much smaller in CARKO mice. In a two-stage hepatocarcinogenesis model initiated by diethylnitrosamine, 27-week treatment with GBE resulted in an increase of eosinophilic altered foci and adenomas in WT mice. By contrast, foci and adenomas were clearly less evident in CARKO mice. These results indicate that GBE-induced hepatocarcinogenesis is mainly CAR-mediated. Since CAR-mediated MOA for hepatocarcinogenesis in rodents is considered to be qualitatively implausible for humans, our findings would be helpful to evaluate the carcinogenic characterization of GBE to humans.

1. Introduction

Ginkgo biloba extract (GBE) is commonly used as a herbal supplement to improve brain function and memory (Chan et al., 2007), and it is one of the most frequently used natural products in the United States and many European countries (Barnes et al., 2008; Garcia-Alvarez et al., 2014). GBE is composed of a number of ingredients (flavonol glycosides and terpene lactones) and constituent concentrations vary widely in commercially available GBE products (Kressmann et al., 2002). The National Toxicology Program (NTP) selected one GBE product from a variety of GBE products and reported concerns of genotoxicity and clear evidence of hepatocarcinogenicity from the study of GBE in mice (NTP, 2013). In reaction to the NTP report, the International Agency for Research on Cancer (IARC) classified GBE as a Group 2B substance (possibly carcinogenic to humans) (Grosse et al., 2013). Recently, we reported that GBE was not genotoxic in *in vivo* genotoxicity studies (Maeda et al., 2014). In this study, we used the same lot of GBE that was used for the NTP study. No remarkable increases in *gpt* or spi$^-$ mutation frequencies were observed in DNA extracted from the livers of B6C3F$_1$ *gpt* delta mice that had been exposed to GBE up to 2000 mg/kg bw/day for 90 days. Furthermore, no induction of DNA damage and chromosomal aberrations were detected in liver comet assays and bone marrow micronucleus assays using C3H mice treated with GBE for 3 days. On the basis of the lack of *in vivo* genotoxicity by GBE, we considered that GBE induces liver tumors through non-genotoxic mechanisms. However, detailed mode of actions (MOAs) for hepatocarcinogenesis with GBE is still unknown.

The existing data from the NTP study demonstrated that GBE induced centrilobular hepatocyte hypertrophy and drug metabolizing enzymes (Guo et al., 2010). In addition, Umegaki et al. (2002, 2007) reported GBE greatly induces CYP2B-catalyzed 7-

Abbreviations: GBE, *Ginkgo biloba* extract; CAR, Constitutive Androstane Receptor; WT, wild-type; CARKO, CAR knockout.

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pentoxyresorufin O-dealkylase activity (known to be specific for CYP2B enzyme) in rats and mice. These data suggest that the MOA of both GBE-induced liver hypertrophy and carcinogenesis involves the constitutive androstane receptor (CAR). CAR is a nuclear receptor that plays critical roles in regulating enzymes involved in xenobiotic metabolism such as members of the cytochrome P-450 (CYP) family (Ueda et al., 2002). Phenobarbital (PB) is a non-genotoxic hepatocarcinogen in rodents (IARC, 2001). PB also induced centrilobular liver hypertrophy accompanied by the induction of various CYPs, mainly CYP2B (Whysner et al., 1996). This PB-dependent drug metabolizing enzyme induction and hepatocellular hypertrophy is accepted as a CAR-mediated event (Wei et al., 2000). Mouse CAR is essential for PB-induced liver tumor development (Yamamoto et al., 2004). Key events in the PB-induced liver tumor MOA comprise CAR activation which results in altered gene expression, increased cell proliferation, clonal expansion leading to altered foci and subsequently in the formation of liver adenomas/carcinomas (Elcombe et al., 2014). In humans, epidemiologic studies of patients undergoing long-term PB therapy showed no increase in the incidence of hepatic cancer (CarloVecchia and Negri, 2014; IARC, 2001; Lamminpää et al., 2002). Hence, if xenobiotics with hepatocarcinogenic potential in rodents have robust data for a PB-like MOA, it can be concluded that their carcinogenic responses are not relevant to humans (Cohen, 2010; Elcombe et al., 2014; Holsapple et al., 2006). We previously reported a short-term (3-day) study that showed GBE induced hepatocellular hypertrophy is CAR mediated (Maeda et al., 2014). 3-day treatment with GBE induced mild to moderate centrilobular liver hypertrophy in wild type C3H (WT) mice, whereas it induced slight hypertrophy in CAR knockout (CARKO) mice. However, this short-term study was not sufficient to assess CAR involvement in liver carcinogenesis. Therefore, we investigated CAR involvement in liver hypertrophy/carcinogenesis in 1-, 4-, and 13-week treatment studies and a two-stage hepatocarcinogenesis model study using CARKO mice. In the present study, we used GBE of the same lot that was used for the NTP study. As a difference between the NTP study and our study, we used C3H mice more susceptible to liver tumor than B6C3F$_1$ mice (Goldsworthy and Fransson-Steen, 2002).

2. Materials and methods

2.1. Chemicals

The GBE used in the present study was the same lot (Lot 020703) that was used for the NTP 2-year study (NTP, 2013) and was a gift provided by the National Institute of Environmental Health Sciences (North Carolina, USA). This GBE was produced by Shanghai Xing Ling Science and Technology Pharmaceutical Company, Ltd (Shanghai, China) and contains 31.2% flavonol glycosides, 15.4% terpene lactones (6.94% bilo-balide, 3.74% ginkgolide A, 1.62% ginkgolide B, 3.06% ginkgolide C), and 10.45 ppm ginkgolic acids.

2.2. Animals

Male C3H/HeNCrl background CARKO mice were generously provided by Dr. Negishi, National Institute of Environmental Health Sciences, NC (Kodama et al., 2004; Yamamoto et al., 2004). Male C3H/HeNCrlCrlj mice were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and were used as the wild type (WT) mice in the present study. All protocols were approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences, and all studies followed the guidelines of the National Institute of Health Sciences for use of laboratory animals.

2.3. Experimental procedures

2.3.1. Experiment 1: subchronic feeding study

Experiment 1 (Exp. 1) was conducted to clarify the role of CAR in mouse liver hypertrophy and in the induction of liver metabolism enzymes by GBE. 6-week-old male WT (5 animals/group) and CARKO mice (5 animals/group) were administered 0 (control), 100 (low dose), 1000 (middle dose) or 10,000 ppm (high dose) GBE in the basal diet (powdered CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) for 4 or 13 weeks. Hepatocyte proliferation was evaluated in an additional set of control and high dose groups (5 animals/group) fed the diet for 1 week.

2.3.2. Experiment 2: two-stage hepatocarcinogenesis study

Experiment 2 (Exp. 2) was performed to investigate the role of CAR in mouse hepatocarcinogenesis pathways in accordance with the standard protocol for a two-stage hepatocarcinogenesis model (Diwan et al., 1986). This protocol was similar to the one followed in the study of CAR-mediated hepatocarcinogenesis by PB using CARKO mice (Yamamoto et al., 2004). All of the mice were administered a single intraperitoneal injection of 90 mg/kg body weight diethylnitrosamine (DEN) at 5 weeks of age. One week after DEN initiation, 25 WT mice/group and 22 to 26 CARKO mice/group were fed GBE in the basal diet at the same doses as in Exp. 1 for 27 weeks.

All of the mice in the 2 experiments were checked daily for their clinical condition. Body weight and food intake were measured once a week throughout the experiments. From these records daily chemical intake (mg/kg/day) was calculated. At the termination of each experiment, all surviving mice were euthanized under deep anesthesia with isoflurane (Mylan Inc., Tokyo, Japan). After necropsy, the livers were weighed and dissected for the analysis described below in both experiments. Thyroid gland was dissected for histopathology.

2.4. Clinical chemistry

In the 4- and 13-week studies for Exp. 1, blood samples were separated into serum, and the serum samples were stored at −30 °C until being used. Serum alanine transferase (ALT), total bilirubin (T-Bil), and γ-glutamyl transpeptidase (γ-GTP) levels were analyzed at SRL, Inc. (Tokyo, Japan).

2.5. Histopathology

In Exp. 1, the left and median lobes of the liver were fixed in 10% buffered formaldehyde solution, embedded in paraffin wax, and stained with hematoxylin-eosin (H&E). The severity of each histological finding was evaluated based on a 0–4 grading scale (0: none; 1: slight; 2: mild; 3: moderate; 4: severe).

In Exp. 2, all lobes of the livers were quickly removed and fixed in 10% buffered formaldehyde solution, and the whole liver was dissected into 18 sections. After dissection, all sections were routinely processed and stained with H&E for histopathological analysis. Proliferating lesions in the liver (hepatocellular adenomas and foci of hepatocellular alteration) were classified into 3 types, that is, “eosinophilic”, “basophilic”, and “other type”. Mixed or clear types were classified as “Other type”. The incidence and multiplicity (average number per animal) for each type of proliferative lesion were calculated. The classification of liver proliferating lesions was in accordance with the criteria of the International Harmonization of Nomenclature and Diagnostic Criteria of Lesions in Rats and Mice (Thoolen et al., 2010).

In both experiments, thyroid glands were fixed in 10% buffered formaldehyde solution, embedded in paraffin wax, and stained