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Dietary flavonoids suppress azoxymethane-induced colonic preneoplastic lesions in male C57BL/KsJ-*db*/*db* mice

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ABSTRACT

Obesity is known to be a risk factor for colon carcinogenesis. Although there are several reports on the chemopreventive abilities of dietary flavonoids in chemically induced colon carcinogenesis, those have not been addressed in an obesity-associated carcinogenesis model. In the present study, the effects of 3 flavonoids (chrysin, quercetin and nobiletin) on modulation of the occurrence of putative preneoplastic lesions, aberrant crypt foci (ACF), and β-catenin-accumulated crypts (BCACs) in the development of colon cancer were determined in male *db*/*db* mice with obesity and diabetic phenotypes. Male *db*/*db* mice were given 3 weekly intraperitoneal injections of azoxymethane (AOM) to induce the ACF and BCAC. Each flavonoid (100 ppm), given in the diet throughout the experimental period, significantly reduced the numbers of ACF by 68–91% and BCAC by 64–71%, as well as proliferation activity in the lesions. Clinical chemistry results revealed that the serum levels of leptin and insulin in mice treated with AOM were greater than those in the untreated group. Interestingly, the most pronounced suppression of development of preneoplastic lesions and their proliferation were observed in the quercetin-fed group, in which the serum leptin level was lowered. Furthermore, quercetin-feeding decreased leptin mRNA expression and secretion in differentiated 3T3-L1 mouse adipocytes. These results suggest that the present dietary flavonoids are able to suppress the early phase of colon carcinogenesis in obese mice, partly through inhibition of proliferation activity caused by serum growth factors. Furthermore, they indicate that certain flavonoids may be useful for prevention of colon carcinogenesis in obese humans.

1. Introduction

Epidemiological studies have shown that obesity is an important cofactor for several types of cancer, including colorectal cancer [1]. Recently, a prospective population based study of about 90,000 subjects conducted by the American Cancer Society confirmed that obesity is directly associated with an increased risk of death from colon cancer [2]. In addition, animal studies have also suggested that obesity enhances tumor development [3], while calorie restriction was reported to inhibit a broad range of spontaneous, transplanted, and chemically induced neoplasms [4]. In fact, the hypothesis that obesity-associated disarrayed metabolic and hormonal plasma parameters, including insulin, triglycerides, glucose and free fatty acids, may promote ACF development and colon cancer has been proposed in the past decade [5]. However, the underlying mechanisms of obesity-related colon carcinogenesis are not fully understood until now.

Leptin, a 16-kDa protein encoded by the *ob* gene, was first documented in 1994 as a regulator of body weight gain and energy balance, with its activities displayed in the hypothalamus [6]. It is well known that serum leptin levels are highly elevated in obese individuals [7] and the protein is mainly secreted by white adipocytes [8]. C57BL/KsJ-*db*/*db* (*db*/*db*) mice are often used as a genetically altered animal model with the genotypes of obesity and diabetes mellitus [9]. In this mouse strain, a mutation in the cytoplasmic domain of the long form of the leptin receptor (Ob-Rb) results in loss of expression of this isoform [10]. In the absence of Ob-Rb, the mice eat excessively and are already obese at 4 weeks of age. Furthermore, they are also demonstrate hyperleptinemia, hyperinsulinemia, hyperglycemia, and hyperlipidemia, as well as increased levels of cholesterol in plasma [11]. The synthesis of leptin in adipocytes, which may be involved in neoplastic processes, is influenced by insulin, tumor necrosis factor-α, glucocorticoids,

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; BCAC, β-catenin-accumulated crypt; IGF-1, insulin-like growth factor-1; PCNA, proliferating cell nuclear antigen; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor.

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reproductive hormones, and prostaglandins [12]. In addition, leptin can act as a growth factor in colonic epithelial cells [13], while it also modulates the proliferation of colonic cryptal cells [14]. Since leptin might be one of the biological factors involved in the development of colorectal cancer associated with obesity/diabetes, *db*/*db* mice are quite useful as a model for elucidating the relationship between colon carcinogenesis and obesity/diabetes.

Flavonoids comprise a structurally diverse class of polyphenolic compounds ubiquitously found in plants and produced as a result of plant secondary metabolism [15]. They have several biological effects, such as anti-oxidative and anti-inflammatory activities [16]. We previously reported that chrysin [17], quercetin [18], and nobiletin [19] showed chemopreventive effects toward azoxymethane (AOM)-induced colon carcinogenesis in rats. In addition, administration of green tea polyphenols, including epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate, resulted in a significant reduction in body weight gain and body fat accumulation in rodents [20,21]. Furthermore, an *in vitro* study found that certain flavonoids inhibit the growth of 3T3-L1 pre-adipocytes [22]. However, there are known no studies of the effects of flavonoids on obesity-associated carcinogenesis.

In the present study, we first determined the modulatory effects of six different flavonoids; flavone, chrysin, apigenin, luteolin, quercetin, and nobiletin, on leptin secretion from 3T3-L1 cells. Next, we evaluated the effects of dietary chrysin, quercetin, and nobiletin on the occurrence of AOM-induced aberrant crypt foci (ACF) and β-catenin-accumulated crypts (BCACs), putative precursor lesions for colonic adenocarcinoma [23,24], in *db*/*db* male mice. We also investigated those three flavonoids to determine their effects on clinical chemistry related to the occurrence of colorectal cancer [25]. Since we previously observed high proliferation activities in preneoplastic colonic lesions and non-lesional crypts in *db*/*db* mice [26], the effects of these flavonoids in regard to proliferation activity in ACF and BCAC were analyzed using an immunohistochemical methods.

2. Materials and methods

2.1. Cell culture

3T3-L1 mouse pre-adipocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine serum (BS), as well as 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Intracellular lipid accumulation and leptin secretion

3T3-L1 cells (1×10^4/200 μl/well) were seeded into 96-well plates under the growth conditions described above. After reaching confluence, they were incubated for an additional 24 h (designated as day 0). Then, adipocyte differentiation was induced by treatment with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (1 μM), and insulin (10 μg/ml), components of an Adipogenesis Assay Kit (Chemicon International, Temecula, CA), in DMEM containing 10% FBS for 48 h. The medium was then replaced by DMEM containing 10% fetal bovine serum (FBS) and insulin (5 μg/ml), and changed to fresh medium every 2 days, according to a method previously described by Maeda et al. [27], with some modifications. On day 2, each flavonoid (10, 50, and 100 μM) was dissolved in dimethyl sulfoxide (DMSO), then added to DMEM containing FBS and insulin. The final concentration of DMSO was 0.1% (v/v), which was found to have no effect on cell growth (data not shown). After 12 days, the medium was collected and subjected to ELISA to determine the levels of leptin. The cells were stained with the Oil Red-O component of an Adipogenesis Assay Kit, according to the manufacturer's instructions. Stained oil droplets in 3T3-L1 cells were extracted with dye extraction solution and absorbance of the extracts was measured at 490 nm.

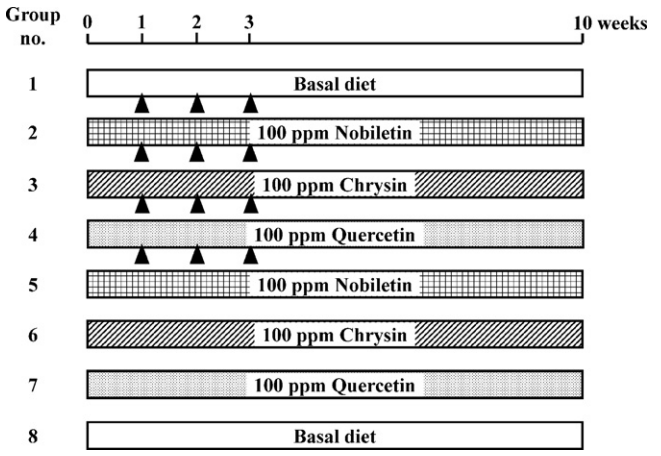

Fig. 1. Experimental protocol for the *in vivo* study. All mice were divided into the following eight experimental and control groups. They were given three weekly intraperitoneal injections of AOM (15 mg/kg body weight, ▲) or saline. Mice in groups 2 through 7 were fed the experimental diets containing each of the flavonoids (100 ppm) for the entire 10-week experimental period. Groups 1 and 8 were given the basal diet without the flavonoids during the study.

2.3. Mice, diet, and carcinogens

Male *db*/*db* mice were obtained from Jackson Laboratories (Bar Harbor, ME) at the age of 4 weeks and maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. On arrival, all mice were randomized and transferred to plastic cages (2 or 3 mice/cage), and given free access to drinking water and a pelleted basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle), and temperature (23 ± 2 °C). All mice were quarantined for 1 week before starting the experiment. Nobiletin (>98% purity) was obtained from Nard Chemicals (Hyogo, Japan), while other flavonoids were purchased from WAKO Pure Chemicals (Osaka, Japan). Experimental diets were prepared by mixing each flavonoid (100 ppm) separately with powdered CRF-1 every week during the study. Azoxymethane (AOM), a colonic carcinogen, was purchased from Sigma Chemical Co. (St. Louis, MO).

2.4. Experimental procedures

As shown in Fig. 1, all mice were divided into the following eight experimental and control groups: AOM alone (group 1, $n = 9$); AOM + chrysin (group 2, $n = 10$); AOM + quercetin (group 3, $n = 10$); AOM + nobiletin (group 4, $n = 10$); chrysin alone (group 5, $n = 5$); quercetin alone (group 6, $n = 5$); nobiletin alone (group 7, $n = 5$); and untreated (group 8, $n = 5$). All mice were given 3 weekly intraperitoneal injections of AOM (15 mg/kg body weight) or saline, while those in groups 2 through 7 were fed the experimental diets containing the flavonoids (100 ppm) for the entire 10-week experimental period. Groups 1 and 8 were given the basal diet without flavonoids during the study. At week 10, all mice were euthanized after overnight fasting by an intraperitoneal injection of sodium pentobarbital (1 mg/kg body weight). Blood samples were taken from the portal vein before the mice were killed. A complete necropsy was done, and all organs, including the colon, were removed, with the liver, kidneys, pancreas, and epididymal adipose tissue weighted.