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Effect of enzymatically modified isoquercitrin on preneoplastic liver cell lesions induced by thioacetamide promotion in a two-stage hepatocarcinogenesis model using rats

Yuta Fujii[a], Masayuki Kimura[a], Yuji Ishii[b], Ryuichi Yamamoto[a], Reiko Morita[a,c], Shim-mo Hayashi[d], Kazuhiko Suzuki[a], Makoto Shibutani[a,*]

[a] Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan
[b] Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[c] Pathogenetic Veterinary Science, United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan
[d] San-Ei Gen F.F.I, Inc., 1-4-9 Hirano-machi, Chuo-ku, Osaka 540-8688, Japan

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A B S T R A C T

To investigate the protective effect of enzymatically modified isoquercitrin (EMIQ) on the hepatocarcinogenic process, we used a two-stage hepatocarcinogenesis model in *N*-diethylnitrosamine-initiated and thioacetamide (TAA)-promoted rats. We examined the modifying effect of co-administration with EMIQ on the liver tissue environment including hepatic macrophages and lymphocytes and on the induction mechanism of preneoplastic cell apoptosis during early stages of hepatocellular tumor promotion. TAA increased the number and area of glutathione *S*-transferase placental form $(GST-P)^+$ liver cell foci and the numbers of proliferating and apoptotic cells in randomly selected areas in liver sections. Co-administration with EMIQ suppressed these effects. TAA also increased the numbers of $ED2^+$, cyclooxygenase-2^+, and heme oxygenase-1^+ liver cells, as well as the number of $CD3^+$ lymphocytes. These effects were also suppressed by EMIQ. EMIQ increased liver levels of thiobarbituric acid-reactive substance and 8-hydroxydeoxyguanosine, and $TUNEL^+$ apoptotic cells, death receptor 5 $(DR5)^+$ cells and 4-hydroxy-2-$nonenal^+$ cells within GST-P^+ foci. Outside the GST-P^+ foci, EMIQ decreased the numbers of apoptotic cells and $DR5^+$ cells. These results suggest that TAA-induced tumor promotion involves activation of hepatic macrophages producing proinflammatory factors. EMIQ may suppress the TAA-induced tumor-promoting activity by an anti-inflammatory mechanism mediated by suppressing the activation of these macrophages. Furthermore, EMIQ may suppress tumor-promoting activity differentially between the inside and outside of GST-P^+ foci. Within GST-P^+ foci, EMIQ facilitates the apoptosis of preneoplastic cells through the upregulation of DR5. Outside the GST-P^+ foci, EMIQ suppresses apoptosis and the subsequent regeneration of non-transformed liver cells.

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1. Introduction

Thioacetamide (TAA) is a well-known hepatocarcinogen that induces oxidative stress in liver cells by generation of reactive oxygen species (ROS), and subsequent liver cirrhosis and liver cell tumors in rodents (Uskoković-Marković et al., 2007). Chronic administration of TAA typically causes repeated apoptosis or necrosis, which is followed by regeneration of liver cells, causing regenerative nodules and eventually tumors (Gervasi et al., 1989; Mangipudy et al., 1995).

Quercetin (302.23 g/mol; Fig. 1), an antioxidant, has a preventive effect on TAA-induced liver cell necrosis, and may have a preventive effect on TAA-induced hepatotoxicity by modulating the apoptosis pathway (de David et al., 2011). It is also reported quercetin has anti-inflammatory potential (Granado-Serrano et al., 2012). However, orally administered quercetin is poorly absorbed, with the bioavailability administered in capsule form to human being less

Abbreviations: Aldh1a1, aldehyde dehydrogenase family 1 member A1; Bax, Bcl2-associated X protein; BNF, β-naphthoflavone; Col1a1, collagen, type I, alpha 1; Cox-2, cyclooxygenase 2; Cu, copper; Cxcl10, chemokine (C-X-C motif) ligand 10; Cx3cl1, chemokine (C-X3-C motif) ligand 1; DAB, diaminobenzidine; DEN, *N*-diethylnitrosamine; DR5, death receptor 5; EMIQ, enzymatically modified isoquercitrin; Fadd, Fas (TNFRSF6)-associated via death domain; Fe, iron; Gstm1, glutathione *S*-transferase mu 1; GST-P, glutathione *S*-transferase placental form; HO-1, heme oxygenase-1; Hprt, hypoxanthine guanine phosphoribosyl transferase; Mmp, matrix metalloproteinase; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; Ptgs2, prostaglandin-endoperoxide synthase 2; ROS, reactive oxygen species; Serpine1, serine (or cysteine) peptidase inhibitor, clade E, member 1; TAA, thioacetamide; TAMs, tumor-associated macrophages; TBARS, thiobarbituric acid-reactive substances; Tgfb2, transforming growth factor, beta 2; Tnfrsf10b, tumor necrosis factor receptor superfamily, member 10b; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; 4-HNE, 4-hydroxy-2-nonenal.

* Corresponding author. Tel.: +81 42 367 5771; fax: +81 42 367 5771.
E-mail address: mshibuta@cc.tuat.ac.jp (M. Shibutani).

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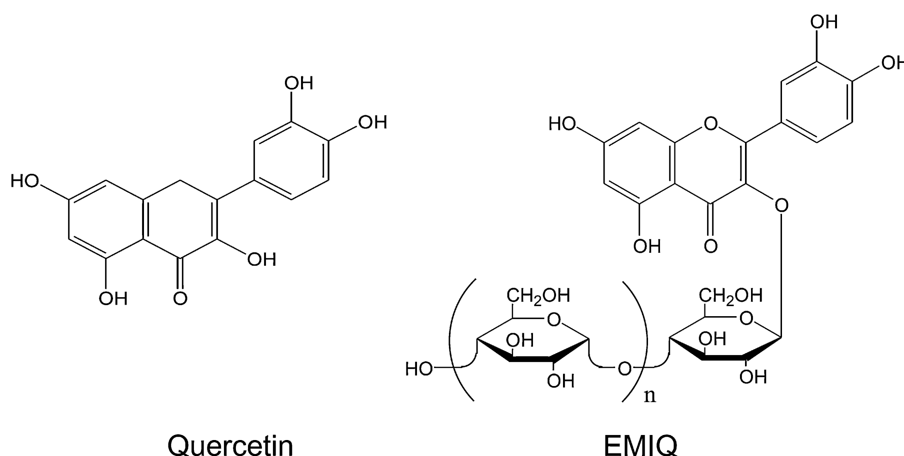

Fig. 1. Chemical structure of quercetin and enzymatically modified isoquercitrin (EMIQ).

than 1% (Gugler et al., 1975). Enzymatically modified isoquercitrin (EMIQ) is a quercetin-glycoside mixture consisting of isoquercitrin and its α-glucosylated derivatives, with 1–10 additional linear glucose moieties (Akiyama et al., 2000; Fig. 1). EMIQ is produced from rutin by enzymatic glycosylation (Formica and Regelson, 1995). Average molecular weight of EMIQ is 800. EMIQ is soluble in water and absorbed better than quercetin. EMIQ has previously shown to prove no carcinogenic effects in any organs in rats (Salim et al., 2004; US FDA, 2007). EMIQ is expected to be more effective than quercetin. EMIQ is effective as an antioxidant in vivo and we have previously shown that it has chemopreventive potential against the development of glutathione *S*-transferase placental form-positive (GST-P$^+$) foci in rats (Morita et al., 2011; Nishimura et al., 2010; Shimada et al., 2010). These foci induced with oxfendazole, phenobarbital, or β-naphthoflavone (BNF), all of which cause oxidative cellular responses as a mechanism of tumor promotion, were suppressed by co-administered EMIQ. In humans, it is reported that intake of EMIQ proved to be effective for the relief of ocular symptoms caused by pollinosis (Hirano et al., 2009). In addition, EMIQ-based beverages for specified health uses has recently been subjected to development in Japan for the uses such as improvement of obesity (Ishikura et al., 2012; Egawa et al., 2012). Therefore, it should be necessary to accumulate evidences on the potency of EMIQ as reported with quercetin.

Recently, we investigated the mechanisms underlying hepatocellular tumor promotion by copper overloading (Cu) and its possible enhancement by iron (Fe) in rats (Mizukami et al., 2010). In that study, we found that co-overloading of both Cu and Fe enhanced Cu-induced liver tumor promotion by increasing Cu-overload-related single liver cell toxicity and regeneration, and increasing the cytokine imbalance involving increased production of cyclooxygenase (Cox)-2 and heme oxygenase-1 (HO-1) by Kupffer cells (Mizukami et al., 2010). We have also reported suppression of tumor-promoting activity of BNF by EMIQ through the anti-inflammatory effects (Kuwata et al., 2011; Shimada et al., 2010). It was suggested that BNF-induced oxidative stress causes single liver cell toxicity, allowing subsequent concomitant apoptosis and regeneration involving inflammatory responses, contributing to tumor-promotion activity (Kuwata et al., 2011). Kupffer cells may protect against inflammatory stimuli induced as a result of oxidative cellular stress caused by BNF, causing fluctuations in proinflammatory cytokine levels (Kuwata et al., 2011).

The present study was performed to clarify whether EMIQ exerts the preventive effect on TAA-induced hepatotoxicity found in quercetin and further on the hepatocarcinogenic process using a two-stage liver carcinogenesis model in *N*-diethylnitrosamine (DEN)-initiated and TAA-promoted rats. For this purpose, we focused on liver tissue environment including hepatic macrophages and lymphocytes and the modulation of apoptosis within and outside the preneoplastic liver cell foci during the early stages of hepatocellular tumor promotion.

2. Materials and methods

2.1. Chemicals

Thioacetamide (TAA; CAS no. 62-55-5, purity >98%) was purchased from Wako Pure Chemical Industries (Osaka, Japan). *N*-Diethylnitrosamine (DEN; CAS No. 55-18-5, purity >99%) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Enzymatically modified isoquercitrin (EMIQ, purity 95%) was provided by San-Ei Gen F.F.I., Inc. (Osaka, Japan).

2.2. Animals and experimental design

Animals were given free access to powdered diets, and were kept under standard conditions (room temperature, 22 ± 3 °C; relative humidity, 56 ± 11%, 12-h light/dark cycle). Thirty-six 5 week-old male F344/NSlc rats, purchased from Japan SLC, Inc. (Hamamatsu, Japan), were acclimated to laboratory conditions for 1 week, and subjected to a medium-term rat liver bioassay by the following procedure (Ito et al., 2003): all rats received an intraperitoneal injection of 200 mg DEN/kg body weight. After 2 weeks, 12 of these rats were fed a basal diet (DEN-alone), 12 were treated with TAA (0.02% in drinking water; DEN + TAA) and 12 were treated with TAA plus 0.5% EMIQ in the diet (DEN + TAA + EMIQ), for 6 weeks. To promote hepatocellular proliferation, all animals were subjected to a two-thirds partial hepatectomy 3 weeks after DEN initiation. Eight weeks after DEN initiation, all animals were killed under deep anesthesia by exsanguinations from the abdominal aorta, and livers were removed. Two slices from the quadrate liver lobe were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4; Wako Pure Chemicals Industries, Ltd.) for 2 days, after which they were processed for histopathological examinations. Approximately 20 mg of liver tissue was directly quick frozen in liquid nitrogen and stored at −80 °C until analysis. Because 0.025% TAA was shown to induce strong tumor promotion activity in rat livers (Gervasi et al., 1989), we chose to use a dose of 0.02% in this study. Because the no-observed-adverse-effect level for EMIQ is at least 500 mg/kg body weight/day based on the 2-year carcinogenicity study in rats (Salim et al., 2004; US FDA, 2007), and 0.2% EMIQ in drinking water (180–200 mg/kg body weight/day) has shown to prove anti-tumor promoting activity in rats (Nishimura et al., 2010; Shimada et al., 2010; Morita et al., 2011), we chose to use a corresponding dietary dose of 0.5% in this study.

All procedures of this study were conducted in compliance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006) and according to the protocol approved by the Animal Care and Use Committee of the Tokyo University of Agriculture and Technology.

2.3. Immunohistochemistry and apoptosis assay

Fixed liver slices were dehydrated in graded ethanol, embedded in paraffin and sectioned for immunohistochemistry using the horseradish peroxidase avidin–biotin complex method, utilizing a VECTASTAIN® Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). Deparaffinized sections were blocked against endogenous peroxidase with 0.3% H_2O_2 in methanol for 30 min. Incubation with the primary antibody was performed at 4 °C for 16 h, followed by incubation with the biotinylated secondary antibody for 30 min and with avidin peroxidase conjugate for 30 min at room temperature. Sections were developed in 0.05%

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