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Involvement of multiple cell cycle aberrations in early preneoplastic liver cell lesions by tumor promotion with thioacetamide in a two-stage rat hepatocarcinogenesis model

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ABSTRACT

Thioacetamide (TAA) induces oxidative stress and hepatocarcinogenicity in rats. We previously reported that TAA promotion caused various disruptions in cell cycle protein expression in rats, including downregulation of $p16^{Ink4a}$, which is associated with intraexonic hypermethylation in hepatocellular proliferative lesions. This study further investigated the contribution of cell cycle aberrations associated with early hepatocarcinogenic processes induced by TAA using antioxidants, enzymatically modified isoquercitrin (EMIQ) and α-lipoic acid (ALA), in a two-stage rat hepatocarcinogenesis model. TAA-promotion after initiation with *N*-diethylnitrosamine increased the number and area of hepatocellular foci immunoreactive for glutathione *S*-transferase placental form (GST-P) and the numbers of proliferating and apoptotic cells. Co-treatment with EMIQ and ALA suppressed these increases. TAA-induced formation of $p16^{Ink4a-}$ foci in concordance with GST-P^+ foci was not suppressed by co-treatment with EMIQ or ALA. TAA-promotion increased cellular distributions of cell proliferation marker Ki-67, G_2/M and spindle checkpoint proteins (phosphorylated checkpoint kinase 1 and Mad2), the DNA damage-related protein phosphorylated histone H2AX, and G_2-M phase-related proteins (topoisomerase IIα, phosphorylated histone H3 and Cdc2) within GST-P^+ foci, and co-treatment with EMIQ or ALA suppressed these increases. These results suggest that downregulation of $p16^{Ink4a}$ may allow selective proliferation of preneoplastic cells by TAA promotion. However, antioxidants did not counteract this gene control. Moreover, effective suppression of TAA-induced cellular population changes within preneoplastic lesions by antioxidants may reflect facilitation of cell cycling and accumulation of DNA damage causing the activation of cell cycle checkpoints, leading to G_2 and M phase arrest at the early stages of hepatocarcinogenesis promoted by TAA.

1. Introduction

Cell proliferation is controlled by many proteins that accelerate or slow down the speed of the cell cycle. When DNA damage or improper chromosomal segregation occurs, the cell cycle checkpoints, i.e. the G_1/S, S-phase, G_2/M and spindle checkpoints, stop the cell cycle for repair (Molinari, 2000). Mitotic aberrations such as chromosomal missegregation and cytokinesis failure, which occurs as a result of checkpoint dysfunction, can produce tetraploid/aneuploid cell populations that may eventually cause genomic instability (Ichijima et al., 2010).

In a two-stage rat hepatocarcinogenesis model, the number and area of altered liver cell foci that are immunoreactive for glutathione *S*-transferase placental form (GST-P) were shown to increase following tumor promotion with hepatocarcinogens, in accordance with their hepatocarcinogenic potential. Therefore, these foci have been confirmed as preneoplastic lesions of liver cells (Shirai, 1997), and understanding of the tumor promotion processes may provide insights in the carcinogenic response (Taniai et al., 2009).

Thioacetamide (TAA) is a well-known hepatotoxin that is believed to produce oxidative stress in liver cells and that is thought to induce liver cirrhosis (Nozu et al., 1992). TAA requires oxidative bioactivation, leading first to its *S*-oxide and then to its chemically reactive *S*,*S*-dioxide, which ultimately modifies amine lipids and proteins causing cytotoxicity (Hajovsky et al., 2012). Chronic administration of TAA leads to repeated apoptosis and necrosis and regeneration of liver cells, which leads to regenerative nodules and subsequently liver cell adenomas and carcinomas in rodents (Gervasi et al., 1989; Mangipudy et al., 1995). TAA also induces

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karyomegaly and cytomegaly reflecting aneuploidy or polyploidy in liver cells, which is suggestive of cell cycle aberrations (Uehara et al., 2008; Tsuchiya et al., 2012). This genomic aberration may lead to chromosomal instability and subsequent tumorigenesis and acquisition of a malignant phenotype (Adler et al., 2009). In addition, we recently reported that TAA tumor promotion caused up- or downregulation of G_1/S (cyclin-dependent kinase inhibitors) and G_2/M checkpoint proteins in GST-P$^+$ foci, contributing to clonal selection by acquisition of multiple aberrant phenotypes and causing checkpoint disruption in a two-stage rat hepatocarcinogenesis model (Tsuchiya et al., 2012).

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). It is an effective antioxidant in vivo and we have previously found that it is chemopreventive against the development of GST-P$^+$ foci induced in rats by oxfendazole, phenobarbital or β-naphthoflavone (Nishimura et al., 2010; Morita et al., 2011; Shimada et al., 2010; Kuwata et al., 2011).

α-Lipoic acid (ALA), a metabolic antioxidant that increases intracellular glutathione levels and regenerates other antioxidants such as vitamins C and E (Packer et al., 1995; Jia et al., 2009), has been reported to prevent or ameliorate of several ailments such as diabetes, polyneuropathy, cataract, neurodegeneration and nephropathies (Amudha et al., 2007a,b; Takaoka et al., 2002) because of its antioxidant properties (Winiarska et al., 2008). ALA also reduced the generation of chromate-induced DNA damage in mice, making ALA a potential new experimental therapy for reducing the occupational cancer risk in humans (Kumar et al., 2009).

The aim of this study was to further clarify the contribution of cell cycle aberrations to the early stages of hepatocarcinogenesis induced by promotion with TAA in an initiation promotion model in rats. For this purpose, we used EMIQ and ALA as antioxidants to suppress the tumor-promoting activity of TAA.

2. Materials and methods

2.1. Chemicals

Thioacetamide (TAA; CAS no. 62-55-5, purity: >98%) was purchased from Wako Pure Chemicals Industries (Osaka, Japan), α-lipoic acid (ALA; CAS no. 1077-28-7, purity: >98%) was purchased from Sigma–Aldrich Japan K. K. (Tokyo, Japan), and *N*-diethylnitrosamine (DEN; CAS no. 55-18-5, purity: >99%) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). EMIQ (purity: 95%) was obtained from San-Ei Gen F.F.I., Inc. (Osaka, Japan).

2.2. Animals and experimental design

A total of 48 five-week-old male F344/NSlc rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and acclimatized to a powdered basal diet (Oriental Yeast Co., Tokyo, Japan), and tap water for 1 week. They were housed in cages on clean racks at up to 4 rats per cage, maintained in an air-conditioned room (40–70% humidity, 20–26 °C) with a 12-h light/dark cycle. To analyze the early stages of liver tumor promotion, all animals were subjected to a two-stage hepatocarcinogenesis promotion model using a medium-term liver bioassay (Shirai, 1997). After a 1-week acclimatization period, all rats were initiated by a single intraperitoneal injection of DEN (200 mg/kg body weight). Two weeks later, rats were divided into four groups; 12 rats were fed the basal diet and tap water (DEN-alone group), 12 rats were fed the basal diet and water containing 0.02% TAA (DEN + TAA group), and two groups of 12 rats were fed a diet containing 0.5% EMIQ or 0.2% ALA and water containing 0.02% TAA (DEN + TAA + EMIQ and DEN + TAA + ALA groups). All animals were subjected to two-thirds partial hepatectomy at week 3 after the initiation treatment. After 6 weeks of treatment with TAA, EMIQ and ALA, all rats were killed under deep anesthesia by exsanguination from the abdominal aorta, and the livers were removed. Two slices of liver were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4; Nacalai Tesque, Inc., Kyoto, Japan) for immunohistochemistry, and the remaining pieces of the livers were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

The animal protocols were reviewed and approved by the Animal Care and Use Committee of Tokyo University of Agriculture and Technology.

2.3. Immunohistochemistry

Immunohistochemistry of liver sections was performed using the avidin–biotin–peroxidase complex method (Vectastain Elite ABC system; Vector Laboratories, Burlingame, CA, USA) with the antibodies listed in Table 1 and 3,3′-diaminobenzidine/H_2O_2 as a chromogen, followed by light counterstaining with hematoxylin. Antigen retrieval conditions for each antibody are shown in Table 1.

The number and area of GST-P$^+$ foci larger than 200 μm in diameter in the liver sections at the early stages of tumor promotion were measured as previously reported (Taniai et al., 2009). The number of cells immunoreactive for Ki-67 was counted in 10 randomly selected fields under 200× magnification, and the number of cells immunoreactive for active caspase 3 was counted in 5 randomly selected fields under 100× magnification, and the ratio of immunoreactive cells to total liver cells was calculated in each field selected irrespective of GST-P$^+$ foci. The numbers of cells immunoreactive for phosphorylated histone H2AX (γH2AX), phosphorylated checkpoint kinase 1 (p-Chk1), mitotic arrest deficient-2 (Mad2), Ki-67, topoisomerase IIα (TopoIIα), phosphorylated histone H3 and cell division cycle 2 (Cdc2) were counted in 10 randomly selected fields at 400× magnification, and the ratio of immunoreactive cells to total liver cells was calculated for both the inside and outside regions of the GST-P$^+$ foci.

The immunoreactivity patterns for $p21^{Cip1}$, $p16^{Ink4a}$, and cell division cycle 25 homolog C (Cdc25c) were classified as positive or negative in the GST-P$^+$ foci when compared with the surrounding liver cells, and the percentage ratio of positive foci among all GST-P$^+$ foci was calculated.

2.4. Real-time RT-PCR analysis

Total RNA was isolated from the liver samples of 6 rats per treatment group using RNeasy Mini kits (Qiagen, Hilden, Germany). Total RNA was reverse transcribed using ThermoScript reverse transcriptase (SuperScript III First-Strand Synthesis System; Invitrogen, Carlsbad, CA, USA). Semi-quantitative real-time RT-PCR with Power SYBR® Green PCR Master Mix (Applied Biosystems Japan Ltd., Tokyo, Japan) was performed using Applied Biosystems StepOnePlus™ Real-Time PCR System (Applied Biosystems Japan Ltd.). The PCR primer sequences are listed in Table 2. The PCR primers for each gene were designed using the Primer Express 3.0 software following Applied Biosystems' instructions for optimal primer design. The relative differences in gene expression were calculated using the cycle time (C_t) values that were first normalized with those of *Hprt*, the endogenous control in the same sample, and then relative to a control C_t value by the $2^{-\Delta\Delta C_T}$ method.

2.5. Statistical analysis

All data are expressed as means ± SD. Bartlett's test for multigroup analyses was used to test the homogeneity of variance between the groups (DEN alone, DEN + TAA, DEN + TAA + EMIQ