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Original Article

Suppressive effect of *Siraitia grosvenorii* extract on dicyclanil-promoted hepatocellular proliferative lesions in male mice

Sayaka Matsumoto[1], Meilan Jin[1], Yasuaki Dewa[1], Jihei Nishimura[1], Mitsuyoshi Moto[1], Yuji Murata[2], Makoto Shibutani[1] and Kunitoshi Mitsumori[1]

[1]Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan
[2]Biochemical Laboratory, Saraya Company Ltd., Kashiwara, Osaka 582-0028, Japan

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ABSTRACT — Dicyclanil (DC) generates reactive oxygen species (ROS) due to *Cyp1a1* induction, and DNA damage caused by oxidative stress is probably involved in hepatocarcinogenesis in mice. To clarify the modifying effect of the *Siraitia grosvenorii* extract (SGE), which has antioxidative properties, we employed a 2-stage liver carcinogenesis model in partially hepatectomized male ICR mice. Mice maintained on diet containing DC at a concentration of 1,500 ppm for 9 weeks after a single intraperitoneal injection of diethylnitrosamine (DEN) at a dose of 30 mg/kg and they were given water containing 2,500 ppm of SGE for 11 weeks including 2 weeks as pre-administration on DC. SGE inhibited the induction of γ-glutamyltranspeptidase-positive hepatocytes, lipid peroxidation, and gene expression of *Cyp1a1*, all of which were caused by DC. To examine whether SGE indirectly inhibits *Cyp1a1* expression induced by inhibition of aryl hydrocarbon receptor (Ahr)-mediated signal transduction caused by DC, mice with high (C57BL/6J mice) and low affinities (DBA/2J mice) to Ahr were given DC-containing diet and/or SGE-containing tap water for 2 weeks. *Cyp1a1* gene expression was significantly lower in C57BL/6J mice administered DC + SGE than in C57BL/6J mice administered DC alone; there was no difference in the *Cyp1a1* expression between DBA/2J mice administered DC + SGE and DC alone. These results suggest that SGE suppresses the induction of *Cyp1a1*, leading to inhibition of ROS generation and consequently inhibited hepatocarcinogenesis, probably due to suppression of Ahr activity.

Key words: *Siraitia grosvenorii* extract, Dicyclanil, Chemoprevention, Aryl hydrocarbon receptor, *Cyp1a1*, Oxidative stress

INTRODUCTION

Siraitia grosvenorii (*Cucurbitaceae*), a traditional medicinal herb grown in China, has been used as a folk medicine for treatment of lung congestion, cold, and sore throat. The major component of this plant extract is triterpene glycosides which are predominantly composed of mogrosides. These glycosides are 400 times sweeter than sucrose (Kasai *et al.*, 1998). In addition, since the triterpene glycoside in *Siraitia grosvenorii* contains sapogenin with a triterpenol structure and the glucosidic bond is β-bond-like fiber, it is not decomposed and digested by amylase in humans and cannot be much absorbed and converted into energy, thereby contributing to lower calories (Song *et al.*, 2007). The non-caloric sweetening property of *Siraitia grosvenorii* extract (SGE) makes it useful as a substitute for sugar to prevent obesity and diabetes. Recent researches on the triterpene glycoside contained in SGE have focused more on its *in vitro* and *in vivo* anticarcinogenic and antioxidative effects. For example, SGE reduces the atherogenic potential of low-density lipoprotein (LDL) by inhibition of copper-mediated oxidation and human umbilical vein endothelial cell-mediated oxidation (Takeo *et al.*, 2002). In particular, 11-oxo-mogroside V in SGE has been shown to inhibit the tumor-promoting activity of 12-*O*-tetradecanotiphorbol-13-acetate in a two-stage skin carcinogenesis model in mice and the tumor-initiating activity of peroxinitrite (Takasaki *et*

Correspondence: Kunitoshi Mitsumori (E-mail: mitsumor@cc.tuat.ac.jp)

al., 2003). Additionally, 11-oxo-mogroside V in SGE has been also reported to have inhibitory effects on reactive oxygen species (ROS) and DNA oxidative damage (Chen *et al.*, 2007). In an *in vivo* experiment, the development of diabetic nephropathy was prevented in diabetic mice treated with SGE due to its antioxidative action (Song *et al.*, 2007). However, the precise mechanisms underlying physiological and pharmacological properties responsible for the antioxidative and anticarcinogenic effects of SGE remain unclear.

Dicyclanil (DC), 4,6-diamino-2-cyclopropylamino-pyrimidine-5-carbonitrile, is a pyrimidine-derived insect growth regulator that inhibits molting and development of insects and is used in the field of veterinary medicine to prevent myiasis in sheep. It has been evaluated in the 54th meeting of the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) that DC is a non-genotoxic hepato-carcinogen (WHO 2000). In our previous study using a two-stage hepatocarcinogenesis model in which male mice were fed a diet containing DC at a concentration of 1,500 ppm for 13 or 26 weeks after partial hepatectomy and dimethylnitrosamine (DMN) initiation treatment, the number and area of γ-glutamyltranspeptidase (GGT)-positive foci, a marker enzyme for preneoplastic hepatocytes in the liver (Carter *et al.*, 1985; Cameron *et al.*, 1978), was found to increase significantly in the DMN + DC group as compared to that in the DMN group (Moto *et al.*, 2006a). Significant increases in mRNA expressions of some metabolism- and oxidative stress-related genes such as *Cyp1a1*, *Por*, *Sod1*, *Txnrd1*, and *Ogg1* were also observed in the DMN + DC group. The *in vitro* measurement of ROS generation from mouse liver microsomes revealed a significant increase in ROS production in the presence of DC. These results suggest that DC induces oxidative stress, which is probably derived from its metabolic pathway, and plays an important role in DC-induced hepatocarcinogenesis in male mice.

In the present study, to investigate the antioxidative properties of SGE, we examined the modifying effect of SGE on liver tumor-promoting effect of DC in male ICR mice and investigated the possible molecular mechanism of the tumor-modifying effect of SGE.

MATERIALS AND METHODS

Animals and chemicals

In the present study, we performed 2 experiments. We purchased 5-week-old male ICR mice for Experiment 1 and 5-week-old male C57BL/6J and DBA2 mice for Experiment 2 from Japan SLC Inc. (Shizuoka, Japan).

All animals were maintained on a powdered basal diet and tap water during the 1-week accommodation period. During the course of the experiment, they were maintained under the conventional conditions (room temperature, 22 ± 2°C; light/dark cycle, 12 hr). Their body weights and food and water consumptions were measured once a week. The experiment was carried out in accordance with the Guide for the Animal Experimentation of the Tokyo University of Agriculture and Technology.

SGE was kindly provided by Saraya Co., Ltd. (Osaka, Japan). In extraction procedure, fresh *Siraitia grosvenorii* was washed and crushed; then, anextraction was done using hot water at a temperature range of 80-90°C. After filtration, the extract obtained was evaporated under reduced pressure. The moisture content of the extract was about 40 w/w%. To prepare the *Siraitia grosvenorii* glycoside, *Siraitia grosvenorii* extract in paste form was diluted and the sweet components were selectively adsorbed onto a reversal phase column. Using an ethanolic solution, the sweet components that had been adsorbed onto the columns were eluted. Ethanol was then removed. Afterwards, spray drying was used to change the *Siraitia grosvenorii* glycosides from a liquid state into powder form. The concentration of mogroside V was about 31 w%. (Hossen *et al.*, 2005) As a preliminary study to select the appropriate dose of SGE, male ICR mice (5 animals per group) were fed a diet containing 0, 25, 250 or 2,500 ppm SGE for 4 weeks. As a result, SGE showed a dose-dependent decrease in *Cyp1a1* expression, and the decreasing expression was marked in the 2,500 ppm group. From these results, the dose of 2,500 ppm SGE was decided to be appropriate in Experiment 1.

DC was kindly provided by Novartis Animal Health Inc. (Basel, Switzerland), and *N*-diethylnitrosamine (DEN) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Experiment 1

Experimental design

A two-stage hepatocarcinogenesis model in mice was employed. Total of 24 mice was divided into the following 3 groups, each comprising 8 animals: DEN alone (gr. I), DEN + DC (gr. II), and DEN + DC + SGE (gr. III). To enhance the hepatocellular proliferation, all animals in grs. I to III were subjected to two-thirds partial hepatectomy, and after 12 hr, they were administered a single intraperitoneal injection of DEN at a dose of 30 mg/kg. Pretreatment with triterpene has been reported to alter the redox system of tissues by scavenging the free radicals

and improving the antioxidant status of the liver (Sunitha *et al.*, 2001); hence, the animals in gr. III were given tap water containing 2,500 ppm SGE 1 week before initiation treatment, while those in grs. I and II were given tap water alone. One week after the initiation treatment, animals in grs. II and III were fed a diet containing DC at a concentration of 1,500 ppm until 9 weeks (Fig. 1). Three mice died due to the hepatectomy and initiation treatment. For liver sampling at 11 weeks, the mice were sacrificed by exsanguination of the posterior vena cava under ether anesthesia after measuring their body weights. The livers were excised, macroscopically examined, weighed, and cut into sections. For all mice, one liver section was fixed with 4% paraformaldehyde for 24 hr for histological and immunohistochemical examinations. Another liver section was embedded in an optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek Japan Co., Tokyo, Japan) before freezing for evaluation of GGT-positive cells, a marker of preneoplastic foci in mouse liver. The remaining liver samples were stored at −80°C for subsequent gene and protein expression analyses.

Histological and histochemical examinations

For histological examinations, liver tissues were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The histochemical staining of GGT was performed using the method proposed by Rutenberg *et al.* (1969) with some modifications. The frozen tissues were sectioned with a cryotome and fixed with methanol. After air drying, the tissue sections were coated with freshly prepared solution containing the substrate L-glutamic acid-γ-(4-methoxy-β-naphthylamide) (Sigma-Aldrich, St. Louis, MO, USA) and fast blue BBN (Wako Pure Chemical Industries, Japan) in 0.1 M Tris-buffered saline (pH 7.4). Following incubation, the slides were transferred into a 0.1 M cupric sulfate solution. The thin sections were then stained with hematoxylin and mounted in Apathy's mounting medium. On GGT evaluation, the number of positive cells per area was calculated from the total area of the tissue sections by using a computer-assisted image analyzer (NIH image).

Immunohistochemical examinations

Cell proliferation was assessed by immunohistochemical staining for proliferating cell nuclear antigen (PCNA). Thin sections of paraffin-embedded tissues (3 μm) were incubated with monoclonal anti-PCNA antibody (1 : 50; PC10, DAKO, Glostrup, Denmark), followed by Histofine Simple Stain Mouse MAX PO (M) procedures (Universal Immunoperoxidase Polymer for staining mouse tissue sections; Nichirei, Tokyo, Japan). Hydrogen peroxide with a coloring agent diaminobenzidine (DAB) was used as the substrate. The number of PCNA-positive cells per 2,000-3,000 cells on each slide was counted from 10 different areas.

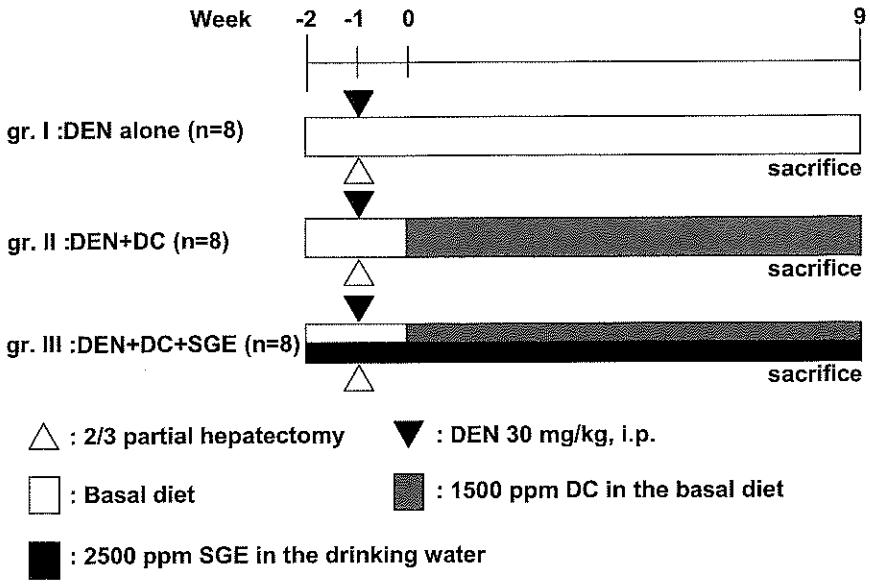

Fig. 1. Experimental design in Experiment 1.

cDNA microarray analysis

The gene expression in the liver samples obtained from mice in groups II and III was analyzed using 2 types of low-density pathway-specific microarrays (Mouse Stress and Toxicity Pathway Finder Gene Array and Mouse Drug Metabolism Gene Array: GEArray; SuperArray Bioscience, Frederick, MD, USA). A list of all genes in this microarray is available on the web site (http://www.superarray.com/gene_array_product/HTML/MM-012.html). Liver samples were selected from one mouse in each group. Total RNA was extracted using TRIzol (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized from 3 μg of total RNA by using the AmpoLabeling-LPR Kit (SuperArray Bioscience Corp.) with the conversion of total RNA biotinylated cDNA probes. The array membranes were hybridized overnight with biotin-labeled probes at 60°C. The filters were then washed twice with 2 × saline sodium citrate buffer (SSC)/1% sodium dodecyl sulfate (SDS), followed by washing twice with 0.1 × SSC/1% SDS at 60°C for 15 min each. Chemiluminescence was detected by subsequent incubation of the filters with alkaline phosphatase-conjugated streptavidin and CDP-Star substrate, followed by exposure to Hyperfilm™-ECL X-ray film (Amersham Biosciences UK Ltd., Buckinghamshire, UK). The image data obtained from GEArray were analyzed using the GEArray Expression Analysis Suite software (http://www.geasuite.superarray.com/index.jsp); correction for background noise was performed by subtracting the minimum value and normalizing to the value of the housekeeping genes (β-actin). For each spot, the ratio of intensities between grs. II and III was analyzed. The genes in gr. III were considered significant if the value of changes was less than 0.5-fold or greater than 2.0-fold as compared to that in gr. II. Based on the results of the cDNA microarray analyses in the present study and those of our previous study regarding the DC-induced hepatocarcinogenesis (Moto *et al.*, 2006b), the genes involved in detoxification and oxidative stress were selected for the analyses of quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR).

Real-time RT-PCR

Quantitative real-time RT-PCR was carried out to validate the genes listed in Table 1. Briefly, total RNA from 4 animals, including the mice used for cDNA microarray analysis in each group, was extracted using TRIzol (Invitrogen Corp.), according to the manufacturer's instructions. After measuring the total RNA concentration using an electrophotometer and determining the RNA quality by spectrometry, cDNA was synthesized from 2 μg of RNA in the presence of dithiothreitol (DTT), dNTPs, random primers, RNaseOUT (Invitrogen Corp.), and SuperScript™ III Reverse Transcriptase (Invitrogen Corp.) in a 20 μl total reaction mixture. Quantitative real-time RT-PCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The real-time RT-PCR reaction was performed according to the SYBR Green PCR Master Mix protocol. The PCR primers were designed using the Primer Express software (Applied Biosystems). The amount of target genes, normalized to an endogenous control (β-actin) and relative to a control, was determined by the 2_ΔΔCt method (Livak and Schmittgen, 2001).

Lipid peroxidation

Lipid peroxidation was measured by the formation of thiobarbituric acid-reactive substances (TBARS) (Ohkawa *et al.*, 1979). Liver tissues (1.5-2.0 g) stored at −80°C were homogenized in 1.15% KCl on ice. Liver homogenate (30%; 0.1 ml) was mixed with 8.1% SDS, 20% acetate buffer solution, 0.8% 2.6-di-*t*-butyl-4-methylphenol (BHT), and 0.8% thiobarbituric acid (TBA); the reaction mixture was incubated at 95°C for 30 min, and the reaction was terminated by placing samples under cold water. Centrifugation was conducted at 3,000 rpm for 10 min after adding 0.5 ml distilled water and 2.5 ml *n*-butanol and pyridine (15 : 1 v/v). Absorbance of the resulting solution in *n*-butanol phase was measured spectrophotometrically at a wavelength of 532 nm by using the Synergy HT Multi-Detection Microplate Reader (BioTek). Malondialdehyde (MDA), obtained by acid hydrolysis of 1,1,3,3-tetraethoxypropane (TEP), was used as the standard for the quantification of TBARS. Data was expressed as nmol of MDA per gram of the liver.

Experiment 2

To examine whether SGE indirectly inhibits CYP1A1 expression by inhibiting aryl hydrocarbon receptor (Ahr)-mediated signal transduction caused by DC, the following experiment was carried out using genetically defined mice with high affinity (C57BL/6J mice) and low affinity (DBA/2J mice) to Ahr. Compared to the C57BL/6J mice, the DBA/2J mice require a 10-20 times higher 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) dose to manifest toxicity (Chapman *et al.*, 1985). The combination of these animal models has been used in typical CYP1A inducers, including benz[a]anthracene, β-naphthoflavone, and TCDD, in order to examine the effects of these chemi-

Table 1. Sequences of primers used in the real-time RT-PCR analysis

Symbol	Gene Name	Forward primer	Reverse primer	Accession No.
Cyp1a1	Cytochrome P450, family 1, subfamily a, polypeptide 1	AGGATGTGTCTGGTTACTTTG	AGAAACATGGACATGCAAG	NM_009992
Cyp1a2	Cytochrome P450, family 1, subfamily a, polypeptide 2	GCTACTTGTGCATGGCCTA	AAGCCATTCAGTGAGGTGTC	NM_009993
Cyp2a5	Cytochrome P450, family 2, subfamily a, polypeptide 5	GCCAACGTTATGGTCCTGTATTC	GTCCGCACAGCACCACAA	NM_007812
Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1	TCAAAAAGACCAAAGGCCAGC	TCCGCAATGACATTGCAGG	NM_021282
Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	AAGCACAGATTCTCCCCTTGG	CAGCAATCCCCCAGATCAAA	NM_007824
Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1	GACTTGAAGATTCAACATACC	TCACAGCTTTGTCAACATCA	NM_013467
Gstμ2	Glutathionr S-transferase mu 2	TTCCCAATCTGCCCTACTTGA	TCTCCACACAGGTTGTGCTTTC	NM_008182
Gstα2	Glutathionr S-transferase alpha 2	ACATGAAGGAGAGAGCCCTGAT	GCAGTCTTGGCTTCTCTTTGGT	NM_008183
Nrf2	NF-E2 related factor 2	CGACAGAAACCTCCATCTACTGAA	CCTCATCACGTAACATGCTGAAG	NM_010902
HO-1	Heme oxigenase 1	TCGTGCTCGAATGAACACTCTG	AAGGCGGTCTTAGCCTCTTCTG	NM_010442
por	Cytochrome P450 oxidoreductase	GCCTGCCTGAGATCGACAAG	GGGTCGCCTTCTCCGTATGT	NM_008898
Gpx2	Glutathione peroxidase2	GCTGCCCTACCCTTATGATGAC	CGCACGGGACTCCATATGAT	NM_030677
Ahr	Aryl hydrocarbon receptor	CGCTGAAACATGAGCAAATTGG	ACAGCTTAGGTGCTGAGTCACGG	NM_01364
Arnt	Aryl hydrocarbon receptor nuclear translocator	GATGCGATGATGACCAGATGTG	CAGTGAGGAAAGATGGCTTGTAGG	NM_009709
βactin	beta actin	AGATTACTGCTCTGGCTCCTAGCA	GCCACCGATCCACACAGATG	NM_007393

cals on the expressions of cyp1A1 and Ahr (Prochaska and Talalay, 1998).

A total of 20 male mice, including C57BL/6J and DBA/2J mice, were divided into the following 6 groups, each comprising 3 or 4 animals: untreated (grs. IV and VII of C57BL/6J and DBA/2J mice, respectively), DC alone (grs. V and VIII of C57BL/6J and DBA/2J mice, respectively), and DC + 2,500 ppm SGE (grs. VI and IX of C57BL/6J and DBA/2J mice, respectively) groups. The mice in grs. IV and VII were fed powdered basal diets and tap water for 3 weeks. The mice in grs V, VI, VIII, and IX were fed powdered basal diets for the first week, and a diet containing 1,500 ppm DC for the following 2 weeks. The mice in grs. VI and IX were given tap water containing 2,500 ppm SGE for 3 weeks, including 1 week of pretreatment period, while the mice in grs. V and VIII were given tap water for 3 weeks (Fig. 3A). All surviving animals were sacrificed by exsanguination of the posterior vena cava under ether anesthesia after measuring the body weights, and the liver samples were frozen in liquid and stored at −80°C. The quantitative real-time RT-PCR was carried out to measure the gene expression of *Cyp1a1*.

Statistical evaluation

The data obtained by measuring body and liver weights, GGT-positive and PCNA-positive cells, TBARS, and real-time RT-PCR analysis were expressed as mean ± S.D.. We compared the difference between grs. I, IV, VII and II, III, V, VI, VIII, IX and that between grs. II, V, VIII and III, VI, IX by using Student's *t* test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Experiment 1

There was no remarkable difference in the final body weight among the three groups. The relative weights of the liver significantly increased in grs. II and III compared with those in gr. I (Table 2). The relative weight of the liver in gr. III was not significantly different from that in gr. II.

Histopathologically, hypertrophy of centrilobular hepatocytes with vacuolation was observed in mice from grs. II and III. Degenerative lesions and single cell necroses were also found in these groups. The severities of these changes in grs. II and III were almost the same.

On histochemical staining of GGT, GGT-positive reaction was observed as a single cell but not as a focus. In grs. II and III, the number of GGT-positive hepatocytes significantly increased compared with that in gr. I. On the contrary, this ratio in gr. III was significantly lower than that in gr. II (Table 2).

With regard to the immunohistochemistry of PCNA, a significant increase in the ratio of PCNA-positive hepatocytes was observed in grs. II and III as compared to gr. I. This mean ratio in gr. III showed a slight but not significant decrease as compared to that in gr. II (Table 2).

The microarray analysis of the liver of mice from gr. III showed that 23 out of 96 genes in the pathway of Mouse Stress and Toxicity and 25 out of 96 genes in the pathway of Drug Metabolism were over- or underexpressed as compared to gr. II (Data not shown). Among them, 19 out of 23 genes in the pathway of Mouse Stress and Toxicity and 22 out of 25 genes in the pathway of Drug Metabolism were underexpressed by less than 0.5-fold. To clarify the mechanism of DC-induced hepatocellular tumors in mice based on the data of the present microarray analysis (underexpressed 19 genes in the pathway of Mouse Stress and Toxicity and 22 in the pathway of Drug Metabolism) and those of our previous studies, we selected 14 target genes that were normalized to β-actin as the internal control for the real-time RT-PCR analysis (Table 1). The changes in the selected genes observed by real-time RT-

Table 2. Body and liver weights, PCNA positive rations, GGT positive cells, and TBARS in Experiment 1

Group	gr. I	gr. II	gr. III
Body weight (g)	43.4 ± 2.1	41.9 ± 3.6	40.2 ± 2.2
Liver weight (g)	2.2 ± 0.1	2.6 ± 0.2#	2.3 ± 0.3
Relative liver weight (%)	5.0 ± 0.4	6.5 ± 0.3#	5.8 ± 0.5#
PCNA labeling index (%)	5.6 ± 1.1	19.6 ± 1.1##	16.5 ± 2.6##
GGT positive cell (number/cm^2)	20.6 ± 11.4	235.2 ± 26.4##	157.1 ± 26.6##,*
TBARS (nmol MDA/g Liver)	1045.6 ± 104.3	1354.6 ± 89.0##	1102.9 ± 129.2#,*

#, ##; significantly different from the gr. I at $p < 0.05$, 0.01, respectively (Student's t-test).
*; significantly different from the gr. II at $p < 0.05$ (Student's t-test). Data show mean ± S.D. values.

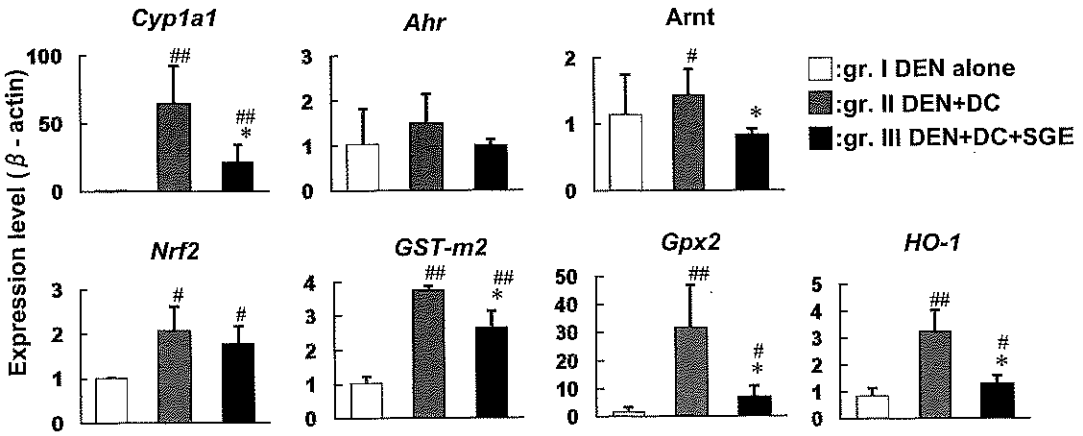

Fig. 2. mRNA expression analysis by real-time RT-PCR of the livers from mice in the grs. I (open column), II (grey column), III (black column) in Experiment 1. Individual gene expression levels were normalized using β-actin. Bars represent the mean ± S.D. of 4 mice. #, ## ; represents significantly different from gr. I at $p < 0.05$, 0.01, * ; represents significantly different from gr. II at $p < 0.05$ (Student's *t*-test).

PCR are shown in Fig. 2. The gene expressions of phase I enzymes such as *Cyp1a1*, significantly decreased in gr. III as compared to gr. II. In addition, the gene expressions of phase II enzymes such as *Gstm2*, *HO-1*, and *Gpx-2* significantly decreased in gr. III as compared to gr. II. There were no remarkable fluctuations in the gene expression of *Nrf2* and *Ahr* between grs. II and III. The results of real-time RT-PCR were consistent with those in the microarray analysis.

TBARS, an *in vivo* oxidative stress marker, significantly increased in grs. II and III compared to gr. I, but the value of gr. III was significantly lower than that in gr. II (Table 2).

Experiment 2

The gene expression of *Cyp1a1* significantly increased in gr. V (DC treated C57BL/6J mice) and gr. VIII (DC treated DBA/2J mice) as compared with that in the corresponding untreated groups (grs. IV and VII). The gene expression level of *Cyp1a1* in gr. VI (DC + SGE treated C57BL/6J mice) was significantly lower than that in gr. V (DC-treated C57BL/6J mice); however, there was no difference in the expressions between gr. VIII (DC treated DBA/2J mice) and gr. IX (DC+SGE treated DBA/2J mice) (Fig. 3B).

DISCUSSION

In Experiment 1, the administration of SGE to the DC-treated ICR mice subjected to DEN initiation and partial hepatectomy significantly decreased the *Cyp1a1* expression levels. The cytochrome P450 superfamily, which is responsible for the development of subsequent toxicity (Bock, 1994; Hankinson, 1985; Silvergeld *et al.*, 1989), is generally known to generate ROS as a byproduct of microsomal oxidation. *Cyp1a1*, a member of this family, has been reported to be the most active CYP enzyme for catalyzing procarcinogens (Guengerich and Shimada, 1991; Puntarulo and Cederbaum, 1998). In particular, the induction of *Cyp1a1* results in excessive generation of ROS due to the depletion of cellular antioxidants (Morehouse *et al.*, 1984; Stohs *et al.*, 1990). Therefore, the upregulation of *Cyp1a1* by the DC treatment could have increased the generation of ROS as a byproduct of microsomal oxidation. In the present study, we observed that SGE suppressed the induction of *Cyp1a1* and speculated that SGE indirectly decreased the amount of ROS generated by the metabolic pathway of DC. Significant decreases in the gene expression of phase II enzymes, antioxidant enzymes, and the level of TBARS that is a marker for lipid peroxidation (Carvalho *et al.*, 2007) were observed in gr. III of Experiment 1, indicating a decline in the amount of ROS generated. Furthermore, we observed that the number of GGT-positive cells, a marker of preneoplastic hepatocytes, decreased in gr. III as compared to gr. II. In our previous study of the mouse two-stage hepatocarcinogenesis model in DC, GGT-positive reaction was observed as a single cell but not as a focus in DC-treated groups in a short-term (13 weeks) study, while a significant increase in the number and area of GGT-positive foci was found in these groups in a long-term (26 weeks) study (Moto *et al.*, 2006a). This finding may suggest that

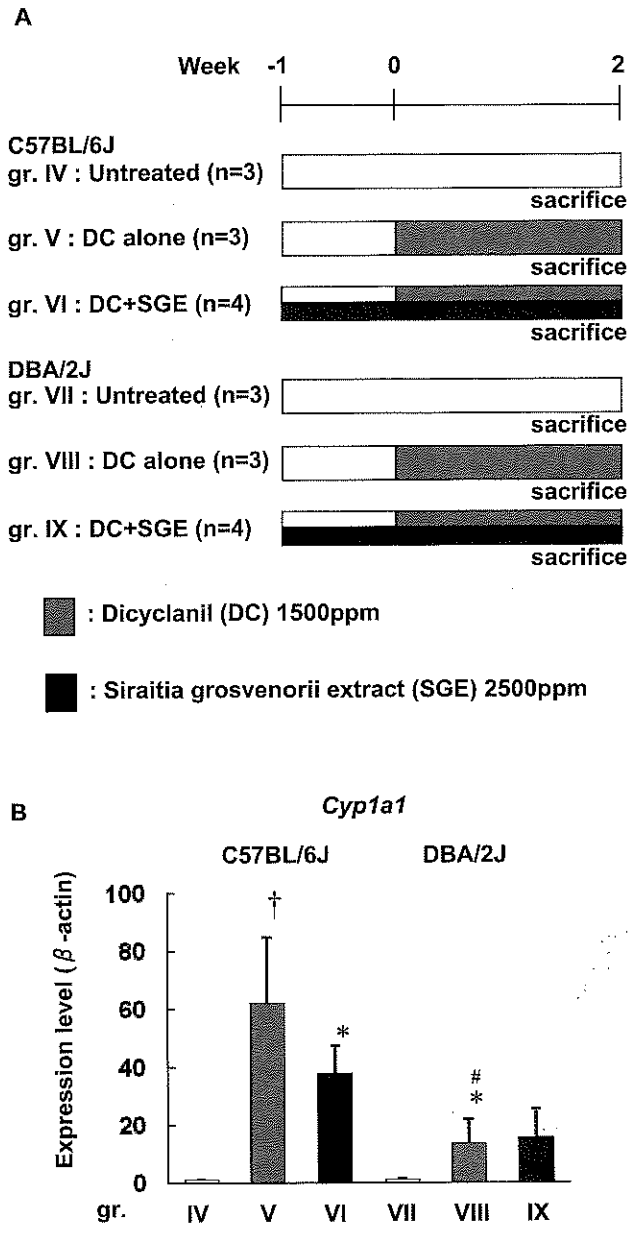

Fig. 3. **A.** Experimental design in Experiment 2.
B. mRNA expression of *Cyp1a1* of the livers from mice in grs. IV, VII (open column), V, VIII (grey column), VI, IX (black column). The gene expression levels was normalized using β-actin. Bars represent the mean ± S.D. of 3 mice. *; significantly different from gr. V at $p < 0.05$ respectively (Student's *t*-test). †; significantly different from gr. IV at $p < 0.01$ (Student's *t*-test). #; significantly different from gr. VII at $p < 0.01$ (Student's *t*-test).

GGT-positive cells, like GGT-positive foci, probably have a potential to function as a marker for preneoplastic hepatocytes in the liver. Based on the results of the present study and our previous studies, it can be considered that the development of preneoplastic cells promoted by DC after DEN initiation was suppressed by the SGE-induced inhibition of ROS generation.

Nrf2 plays a pivotal role in inducing the expression of the phase II detoxifying enzyme and the antioxidant protein that responds to oxidative stress (Itoh *et al.*, 2003). In the present study, the gene expressions of Gstμ2, a phase II enzyme; Gpx2, an antioxidant enzyme; and HO-1, a potent oxidative stress-responsive protein, significantly decreased in gr. III as compared to gr. II. The inducible expression of these genes depends on Nrf2 (Chanas *et al.*, 2002; Banning *et al.*, 2005; Cho *et al.*, 2006). DC-induced upregulations of the gene expression of phase I and II enzymes with a significant increase in the gene expression of Nrf2, while SGE significantly downregulated these gene expressions, along with a decrease in the gene expression of *Nrf2*.

The findings of Experiment 1 indicated that SGE suppressed the induction of *Cyp1a1*, and it might be an important part of the chemopreventive activity of SGE *in vivo*. The induction of *Cyp1a1* has been reported to depend on Ahr, which is a ligand-activated transcription factor in most cell and tissue types (Denison and Heath-Pagliuso, 1998). The interaction of the transformed Ahr complex with a specific dioxin responsive element of the nuclear DNA elicits the activation of certain genes, including *Cyp1a1*, in the cytochrome P450 superfamily (Neuhold *et al.*, 1989). The *Cyp1a1* induction has also been linked to the generation of ROS in not only the classical Ahr ligand, such as TCDD (Park *et al.*, 1996; Knerr *et al.*, 2006) and coplanar polychlorinated biphenyl congeners (Schlezinger *et al.*, 2006), but also the non-Ahr ligand. It is not clear whether DC induces *Cyp1a1* in association with Ahr. With regard to SGE, there is a report on the tumor-protective effect resulting from the function of antioxidant activities, but the mechanism underlying this effect has not been completely elucidated. Other natural antioxidants such as flavonoids (Fukuda *et al.*, 2007), catechins (Palermo *et al.*, 2003), and galangin (Hung *et al.*, 2006) have also been reported to suppress the Ahr transformation and downstream expression of *Cyp1a1*. In addition, resveratrol (Ciolino and Yeh, 1999), curcumin (Ciolino *et al.*, 1998), and certain vegetable constituents (Amakura *et al.*, 2003) act as antagonists of Ahr; this property is responsible for the inhibitory effects of these agents against the Ahr-induced carcinogenesis. Anti-oxidative properties include two mechanisms; directly scav-

enging ROS and indirectly suppressing ROS generation by inhibiting Ahr expression and above-referenced natural antioxidants, flavonoids, curcumin, have been reported to have both anti-oxidative properties (Kandaswami and Middleton, 1994; Fukuda *et al.*, 2007). The results of our study strongly suggest that SGE has a latter anti-oxidative property.

Thus, we performed Experiment 2 to examine the effect of SGE on Ahr and *Cyp1a1* expression induced by DC in genetically defined mice with high affinity (C57BL/6J mice) or low affinity (DBA/2J mice) to Ahr (Prochaska *et al.*, 1998). The aim of this experiment was to examine whether SGE indirectly inhibits *Cyp1a1* expression due to the DC-induced inhibition of Ahr-mediated signal transduction. In Experiment 2, the gene expression of *Cyp1a1* in both C57BL/6J and DBA/2J significantly increased in DC-treated groups (grs. V and VIII) as compared with that in the corresponding untreated groups (grs. IV and VII). However, the gene expression of *Cyp1a1* was significantly decreased in gr. VIII (DC treated DBA/2J mice) as compared to gr. V (DC treated C57BL/6J mice). This suggests the possibility that the induction of *Cyp1a1* in the metabolic pathway on DC may depend on Ahr. This is the first report to demonstrate that DC can induce the expression of *Cyp1a1* gene in an Ahr-dependent manner. In addition, the fact that the gene expression of *Cyp1a1* in group VI (DC + SGE treated C57BL/6J mice) significantly declined as compared to that in gr. V (DC treated C57BL/6J mice), while there were no significant differences in *Cyp1a1* between grs. VIII (DC treated DBA/2J mice) and IX (DC + SGE treated DBA/2J mice), indicates that SGE inhibits Ahr-induced *Cyp1a1* activity and suggests that SGE has antagonistic effects against Ahr. However, regarding the antagonistic effect of SGE on Ahr, the underlying molecular mechanism of action of SGE on Ahr has not been elucidated.

In conclusion, the results of our study strongly suggest the possibility that SGE plays an important role in suppressing the DC-induced generation of ROS in the metabolic process. This mechanism of the generation of ROS is probably derived from the activation of Ahr involving *Cyp1a1*. SGE might act on a certain component of this mechanism, leading to the suppression of the Ahr activity and downstream expression of *Cyp1a1*. The decreased incidence of GGT-positive cells in gr. III in Experiment 1 clearly suggests that SGE inhibits DC-induced hepatocarcinogenesis in a long-term experiment of mice initiated with DEN. SGE is used as a food item and a natural medicine in humans. Because of its low cost, proven chemopreventive potential, and pharmacological safety (Jin *et al.*, 2007), daily intake of SGE may possibly facilitate the maintenance of its constant blood concentration and prevent the generation of ROS. Due to a complex chemical structure of triterpene which is one of the main ingredients of SGE, digestive absorption of SGE and its subsequent metabolic pathway in the blood have not been determined. Though *in vitro* studies have demonstrated that the 11-oxo function of the B ring in the 11-oxo-mogroside V structure may be relevant with regard to its antioxidant activity (Chen *et al.*, 2007), extensive research is required to clarify the bioactive elements and the structure-effect relationship responsible for the antioxidant effects of SGE.

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