Doctoral Dissertation

Food functionality of *Dioscorea japonica* targeting to prostaglandin E_2 synthetic pathway

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Contents

Abbreviations

Bcl-2	B-cell chronic lymphocytic leukemia/lymphoma 2
Cluc	Cypridina luciferase
COX	cyclooxygenase
cPGES	cytosolic prostaglandin E synthase
CRTH2	chemoattractant receptor homologous molecule expressed on Th2
DJE	*Dioscorea japonica* extract
DMBA	7, 12-dimethylbenz[*a*]anthracene
DMBADE	7, 12-dimethylbenz[*a*]anthracene-3, 4-diol-1,2-epoxide
DP	D-prostanoid receptor
EPA	eicosapentaenoic acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HE	hematoxylin and eosin
HETE	hydroxyeicosatetraenoic acid
HODEs	hydroxyoctadecadienoic acids
H-PGDS	hematopoietic prostaglandin D synthase
IL	interleukin
LC	Langerhans cell
LPS	lipopolysaccharide
mPGES-1	microsomal prostaglandin E synthase-1
mPGES-2	membrane-associated prostaglandin E synthase-2
MRM	multiple reaction monitoring
NF-κB	nuclear factor-kappa B
NSAIDs	non-steroidal anti-inflammatory drugs
PG	Prostaglandin
TPA	12-*O*-tetradecanoylphorbol 13-acetate
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Chapter Ⅰ

Introduction

In recent years, Japan has rapidly aging society, and accordingly the number of people suffering from various chronic diseases has been increased. Chronic diseases, including cancer, cardiovascular diseases, and neurological disease are linked with inflammation[1–3]. It is known that prostaglandin (PG) E_2 is one of the lipid mediators and induces an array of chronic inflammation. PGE_2 is widely distributed in many tissues and organs, and is also involved in several pathophysiological responses such as inhibition of gastric acid secretion, pain transmission, neurodegeneration, and carcinogenesis[4,5].

Arachidonic acid is cleaved from cell membrane by cytosolic phospholipase A_2, and are converted to PGH_2 as a common substrate of prostanoids which are PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxanA_2 (TXA_2) (Fig. 1). Cyclooxygenase (COX) is a rate-limiting enzyme of prostanoid synthesis, and catalizes two reactions, bis-dioxygenation of arachidonic acid to PGG_2 and the hydroperoxidation of PGG_2 to PGH_2[6]. COX has two isozymes, that is, constitutively expressed COX-1 and inducible COX-2. The former is important to produce homeostatic prostanoids, whereas, the latter is induced under pathophysiological condition and is involved in several diseases[7]. The terminal PGE_2 synthesizing enzyme, PGE synthase (PGES), lies downstream of COX, and catalyzes the isomerization of PGH_2 to PGE_2. PGES has at least three isozymes, microsomal PGES-1 (mPGES-1)[8], membrane-associated PGES-2 (mPGES-2)[9,10], and cytosolic PGES (cPGES)[11]. Among these, mPGES-1 requires glutathione for catalytic activity and is usually coupled with COX-2. In several types of cancer cells, COX-2 and mPGES-1 are coordinately expressed, and consequently, the excessive PGE_2 production worsens the symptoms[5,12,13].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used a class of medicines. NSAIDs are COX inhibitors and are used for pain relief and pyretolysis of

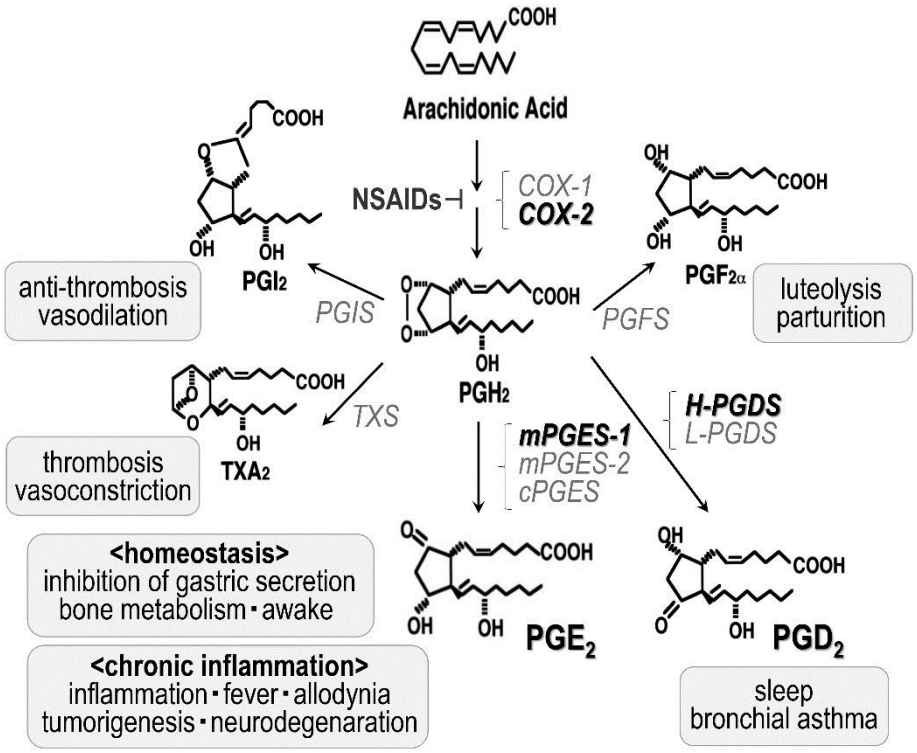


Fig. 1. Prostaglandin (PG) synthetic pathway.
PGH_2 is a common substrate for various lipid mediators, and are biosynthesized by specific enzyme catalysis into prostaglandins, thromboxane (TX). It binds to specific receptors and exerts various physiological effects. COX; cyclooxygenase, PGES; PGE synthase, PGDS; PGD synthase, PGFS; PGF synthase, PGIS; PGI synthase, TXS; TX synthase.

patients[13]. However, NSAIDs have been reported to cause side effects such as gastrointestinal adverse effect and cardiovascular risk[14]. NSAIDs-associated gastrointestinal effect is thought to arise from the inhibition of the constitutive COX-1. Therefore, a selective COX-2 inhibitor was developed with improved safety. However, the selective COX-2 inhibition, elevate cardiovascular risk by suppressed COX-2-dependent biosynthesis of PGI_2 in cardiovascular system[15,16]. Therefore, mPGES-1 is targeted to drug development as a next-generated NSAIDs. Recently, a few mPGES-1 inhibitors have been found[17], but none have been used clinically. Thus, we have an aim

to find food functionality to decrease PGE_2 production without side effects. Such safety and mild effects from food will be useful for prevention of chronic inflammation caused by PGE_2.

From these viewpoint, we focused on the PGE_2 synthetic pathway, and investigated the food functional effects of *Dioscorea japonica* on the related enzymes of PGE_2 synthesis. Chapter II describes the effects of *Dioscorea japonica* on the expression of COX-2 and mPGES-1 in human non-small cell lung carcinoma A549 cells. Chapter III demonstrates the food functionality of *Dioscorea japonica in vivo* using squamous cell carcinoma of mouse skin. Based on this study, it is proved that *Dioscorea japonica* may be effective for treating inflammation and carcinogenesis.

Chapter Ⅱ

Dioscorea japonica extract suppresses the expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 and induces apoptosis in lung cancer cells

In several types of cancer cells, both COX-2 and mPGES-1 are highly expressed, and the consequent excessive production of PGE_2 worsens the symptoms[5,12,13]. Overexpression of COX-2 is commonly observed in lung tumors as well as in many other tumors[18,19]. Similarly, inducible mPGES-1 expressed preferentially coupling with COX-2, and the overproduced PGE_2 develops cancer[12,20]. On the other hand, 15-hydroxyprostaglandin dehydrogenase (PGDH) is well known for its association with the biological inactivation of PGE_2, and induces cancer cell apoptosis in human non-small cell lung carcinoma cell line, A549[21]. NS-398, a selective COX-2 inhibitor[22,23], has been found to induce cancer cell apoptosis[24]. However, long-term use of NSAIDs cause life-threatening side effects, including gastrointestinal injury, renal pathology and cardiovascular adverse events[23].

Dioscorea japonica, a wild yam, is a relative of the *Dioscoreaceae* family native to Japan. *Dioscoreaceae* yam tubers are usually edible and are rich in many nutrients including carbohydrates, essential amino acids, vitamin C, minerals, and physiologically active components such as glycoprotein, polysaccharides, and steroidal saponins[25–28]. It is well known that *Dioscorea japonica* is good for nutritional fortification, and it has also been shown to mediate gastric mucosal protection and digestive enhancement. However, the other physiological effects of *Dioscorea japonica* have not been completely elucidated.

The aim of this study was to explore the novel functional effects of *Dioscorea japonica* on the regulation of the PGE_2 synthesis pathway. We also examined the possibility that *Dioscorea japonica* exerts anti-tumoral effects in lung cancer cells via down-regulation of PGE_2 synthesizing enzymes.

MATERIALS AND METHODS

Preparation of Dioscorea japonica powder and extract

Dioscorea japonica was obtained from Autoraimu Yoshio Ltd. (Niimi, Japan). *Dioscorea japonica* was pared away the outer skin and was dried under 40°C. The ground *Dioscorea japonica* was prepared to uniform powders through 250 μm mesh. The powders were eluted with 20–80% ethanol (v/v, EtOH), and the extracts were immediately dried-up with nitrogen gas and were completely filling with the gas protect oxidation. They were stored at -80°C until used for the experiments. When the *Dioscorea japonica* extract (DJE) was added to the medium for cell culture, the dried DJE was added to the medium for cell culture, the dried DJE was re-dissolved in DMSO as a vehicle. The final concentration of DMSO was 0.1% in the medium for control cells and DJE-treated cells.

Cell culture

Human non-small cell lung carcinoma cell line A549 and human colon carcinoma cell line (ATCC CCL-185) and human colon carcinoma cell line Caco-2 (ECACC 86010202) were obtained from RIKEN BioResource Center (Tsukuba, Japan). The cells were cultured in Dulbecco's modified Eagle’s medium (Sigma, MO, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were treated with 0–100 μg/ml DJE in dimethyl sulfoxide, 0–10 μM diosgenin (Sigma, St. Louis, MO) in dimethyl sulfoxide or were added the only vehicle as a control.

RNA purification and quantitative RT-coupled real-time PCR

A549 or Caco-2 cells after the treatment for 24h were collected and the total RNA was isolated using the acid guanidinium thiocyanate procedure and analyzed for gene expression by real-time quantitative RT-PCR (iQ5 real-time PCR system, Bio-Rad, CA, USA). After converting total RNA to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA), quantitative PCR was performed using Eva Green-Comparative Ct method (Bio-Rad). The following primer pairs were used for amplification of COX-2, mPGES-1, B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl-2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-TGCATTCTTTGCCCAGCACT-3' (COX-2 forward), 5'-AAAGGCG CAGTTTACGCTGT-3' (COX-2 reverse), 5'-TGATCACACCCACAGTTGAGC-3' (mPGES-1 forward), 5'-TGATGATGGCCACCACGTA-3' (mPGES-1 reverse), 5'-TG GATGACTGAGTACCTGAA-3' (Bcl-2 forward), 5'-CCAGGAGAAATCAAACAG AG-3' (Bcl-2 reverse), 5'-CGACATCCCTCCAAAATCAA-3'(GAPDH forward), and 5'-TTCACACCCATGACGAACAT-3' (GAPDH reverse). The cycling conditions were 30 s at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. Expression levels of COX-2, mPGES-1 and Bcl-2 mRNAs were normalized to those of GAPDH mRNA. The relative expression levels were shown against control and were mean ± S.D. of 5 separate experiments.

Western blotting

Collected A549 cells (5 x 10^5 cells) after the treatment with or without DJE for 48h were sonicated in 100 μl of 10mM Tris-HCl (pH 7.6) and then were centrifuged at 10,000 x *g*, and the supernatant (30 μg protein/well) was subjected to 10% sodium

dodecyl sulfate-polyaclylamidegel electrophoresis, and were transferred electrophoretically to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK). After treatment with blocking reagent (Roche, Penzberg, Germany), the membrane was incubated with rabbit anti-COX-2 antibody (1:1000, Immuno-Biological Laboratories Co., Ltd., Gunma, Japan), rabbit anti-mPGES-1 antibody (1:1000, Cayman Chemical Co. Ann Arbor, MI) or rabbit anti β-actin antibody (1:1000, Cell Signaling Technology, Boston, MA). The immunoreactive proteins were visualized using BM chemiluminescence Western blotting kit (Roche). The density of each protein bands was analyzed with Fujifilm Multi Gauge version 3.0 software (Fujifilm, Tokyo, Japan). Protein concentration was determined using a BCA protein assay kit (Thermo Scientific Inc., Rockford, IL).

COX activity assay

COX activity was analyzed as described previous report[29]. The lysates were incubated with 100mM Tris-HCl buffer (pH 7.4) containing 25mM linoleic acid, and the products were analyzed by reversed-phase HPLC using a COSMOSIL 5C18-MS-II column (5 mm particle, 4.6x250mm, Nacalai Tesque, Kyoto, Japan) with a solvent system of methanol/water/acetic acid (80:20:0.01, v/v). The produced 9- and 13-hydroxyoctadecadienoic acids (HODEs) which cochromatographed on reverse-phase HPLC as a single peak were quantified by the comparison of the area of the peak with that of an internal standard, 15-hydroxyeicosatetraenoic acid (HETE).

Enzyme immunoassay

After the treatment with or without DJE by 50% ethanol (100 μg/ml) for 48h, arachidonic acid (10 μM) was added to the culture medium of A549 cells. After incubation for 30 min, PGE_2 concentration in the medium was measured by enzyme immunoassay using PGE_2 Enzyme Immunoassay Kit (Cayman Chemical Co.) as instructed by the manufacturer.

Immunocytochemistry

The intracellular localization of transcription factor nuclear factor-κB (NF-κB) was analyzed by fluorescence immunocytochemistry. After the treatment for 48h, the cell was fixed with 4% paraformaldehyde for 10 min and 0.5% ethanol for 2 min. After blocking with Block Ace (Dainippon Seiyaku, Osaka, Japan), the cells were incubated with anti-NF-κB p65 (C22B4) rabbit antibody (Cell Signaling Technology), and then were incubated with Cyanine (Cy) 3-labeled donkey anti-rabbit IgG (1:400, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The cells were counter stained with 4', 6-diamidino-2-phenylindole (DAPI). The sections were analyzed using confocal laser scanning light microscopy (CLSM, FV1000, Olympus Corporation, Tokyo, Japan).

Promoter assay

COX-2 promoter assay was carried out as described previously[30]. COX-2/Cluc plasmid with COX-2 promoter region and Cypridina luciferase (Cluc) as a reporter gene was transfected into A549 cells. After the transfection, A549 cells were treated with 0.1 μM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and with or without DJE for 48h, and the luciferase activity in the culture medium was measured using BioLux™ Cypridina

Luciferase Assay Kit (New England BioLabs Inc., MA). A549 cells without the treatment of TPA and DJE was used as a control.

TUNEL assay

After treatment of with or without DJE for 48h, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was carried out identification of the extent of DNA fragmentation in A549 cells, using Peroxidase In Situ Apoptosis Detection Kit (EMD Millipore, Billerica, MA) as instructed by the manufacturer. TUNEL positive reactivity was observed with a light microscope (ECLIPSE 80*i*; Nikon Co., Tokyo, Japan).

Statistics

Data were statistically evaluated by unpaired Student's t-test (Fig. 3B and 5) and ANOVA with Bonferroni's (Fig. 3A, 4B and 7A) or Dunnett's (Fig2, 6B and 8) post hoc test at significance level of $p<0.05$ or 0.01.

RESULTS

Dose-dependent effect of DJE on the expression of COX-2 and mPGES-1 in A549cells

COX-2 and mPGES-1 are constitutively and highly expressed in human lung carcinoma A549 cells. A549 cells were treated with DJE by 50% ethanol at 0.1-100 μg/ml concentration, and mRNA expression of COX-2 and mPGES-1 in the cells was analyzed by real-time PCR. DJE suppressed the expression of COX-2 and mPGES-1 in a dose-dependent manner (Fig. 2). Treatment with 100 μg/ml DJE significantly decreased mRNA levels of COX-2 and mPGES-1 to 27% and 46% compared with the control, respectively. Therefore, this concentration of DJE (100 μg/ml) was used for the following

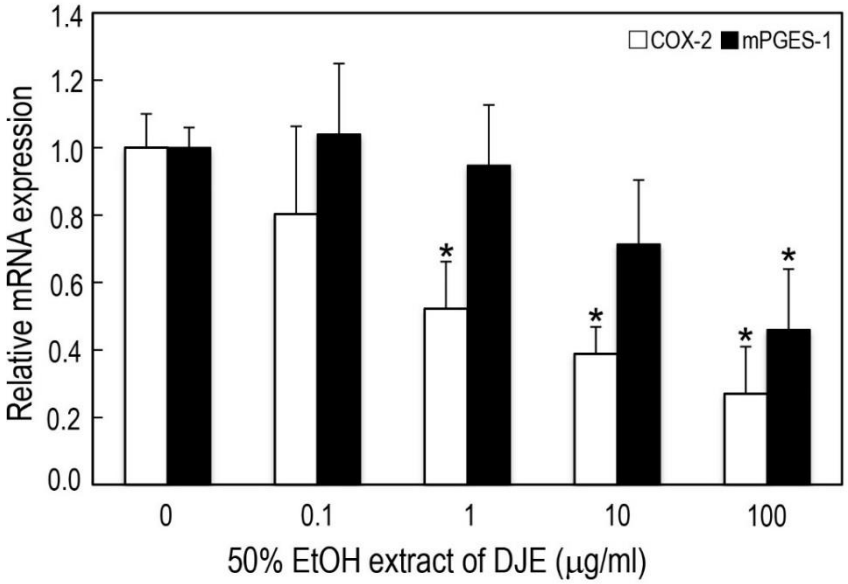


Fig. 2. Dose-dependent effect of DJE on the expression of COX-2 and mPGES-1 in A549 cells. DJE by 50% ethanol (0–100 μg/ml) was added to A549 cells. mRNA expression of COX-2 (open column) and mPGES-1 (closed column) was measured by real-time PCR, as described in the Materials and Methods section. The values are represented as a relative value against 0 μg/ml extract-treated cells and represent mean ± S.D. of 5 separate experiments. *p<0.05 compared with treatment with 0 μg/ml extract.

experiments. Addition of DJE made no significant difference on cell proliferation in the experimental period.

Suppression of the expression of COX-2 and mPGES-1 by DJE

Among the water-soluble extract of *Dioscorea japonica* and DJE by 20, 50, and 80% ethanol, DJE by 50% ethanol was most effective in suppressing the expression of COX-2 and mPGES-1 in A549 cells, whereas the water-soluble extract had little effect (Fig. 3A). Further, in human colon carcinoma model Caco-2 cells, treatment with DJE led to the suppression of COX-2 and mPGES-1 mRNAs to approximately 67% compared with the control (Fig. 3B).

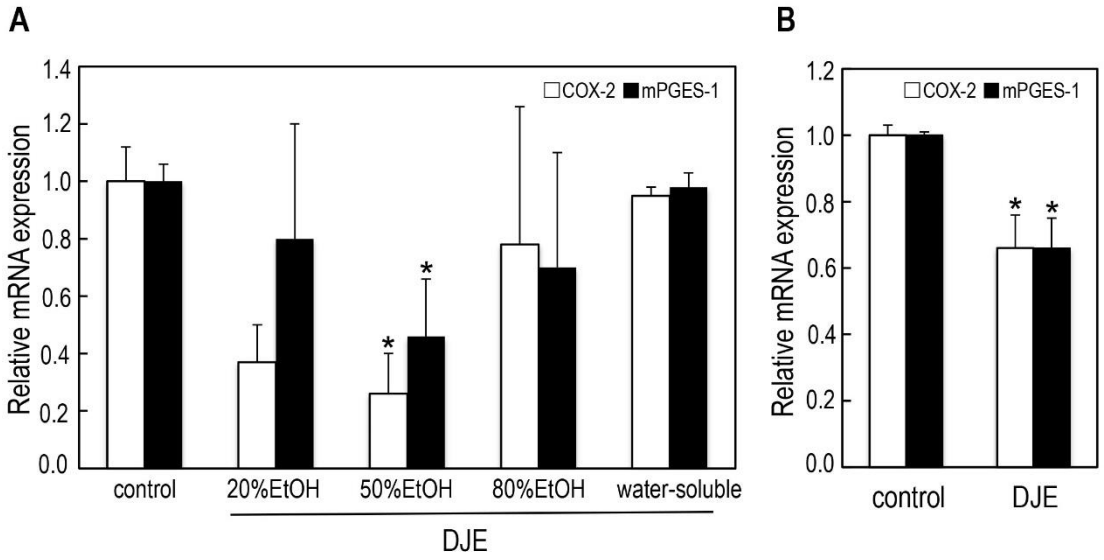


Fig. 3. Effect of water-soluble and Ethanol extract of *Dioscorea japonica* on the expression of COX-2 and mPGES-1 in A549 cells (A) and Caco-2 cells (B).
Water-soluble or Ethanol (20, 50, and 80%) extract of *Dioscorea japonica* (100 μg/ml) was added to A549 cells (A), and the DJE by 50% ethanol (100 μg/ml) was added to Caco-2 (B). mRNA expression of COX-2 (open column) and mPGES-1 (closed column) was measured using real-time PCR. The values are represented as a relative value control cells and represent mean ± S.D. of 5 separate experiments. *p<0.01 compared with the control.

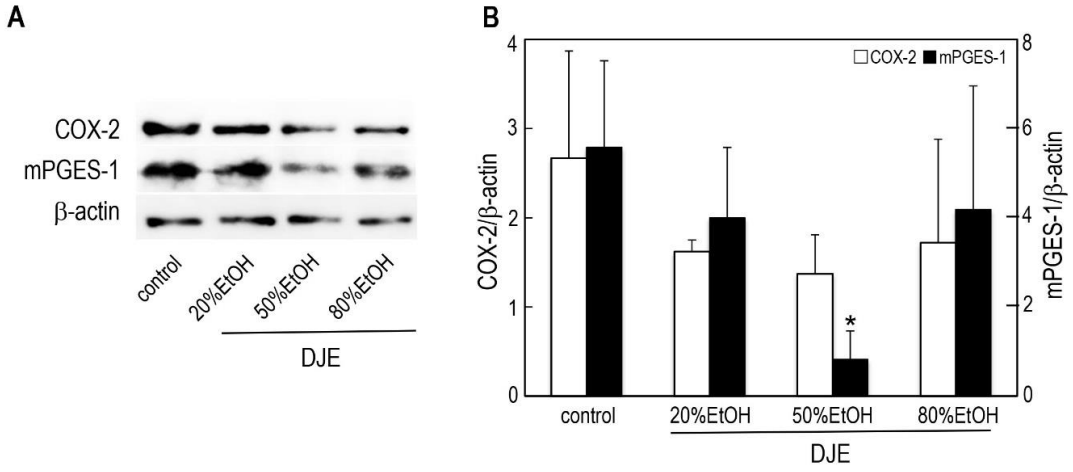


Fig. 4. Changes in protein expression of COX-2 and mPGES-1 after treatment with DJE. The protein expression levels of COX-2 and mPGES-1 were analyzed by Western blotting. The detected COX-2 and mPGES-1 proteins (30 μg total protein/well) were visualized as approximately 70 and 16 kDa bands, respectively (A). The density of each protein band was compared in 3 separate experiments with β-actin (approximately 45 kDa) as a control (B). *p<0.1 compared with the control.

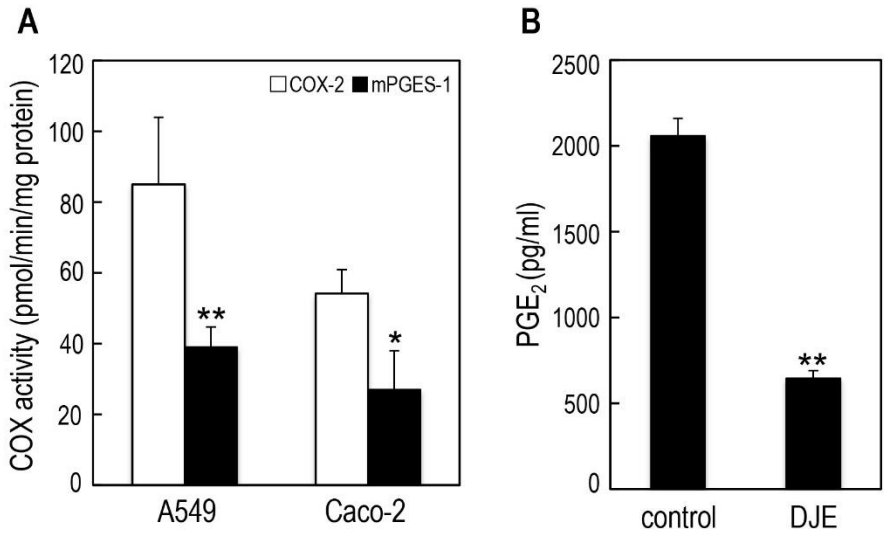


Fig. 5. Decrease in COX activity (A) and PGE_2 production (B) after treatment with DJE. COX activity after the treatment without (open column) or with (closed column) DJE (100 μg/ml) was measured. Supernatants obtained from the centrifugation (10,000 × g) of lysates from A549 and Caco-2 cells were used as enzyme sources for measurement of COX activity. The supernatants were incubated with 25 mM linoleic acid at 24°C for 5 min in the standard COX reaction mixture and the products were quantified using reversed-phase HPLC, as described in the Materials and Methods section. PGE_2 released in the culture medium of A549 cells was quantified by enzyme immunoassay. The values represent mean ± S.D. of 5 separate experiments. *p<0.05 and **p<0.01 compared with the control.

After treatment with DJE by 50% ethanol for 48 h, COX-2 and mPGES-1 protein levels were decreased to 1.37 and 0.82, respectively, compared with the control (2.67 and 5.58) in A549 cells (Fig. 4).

Changes in COX activity and PGE_2 production by DJE treatment

COX activity was assayed by quantifying the metabolism of linoleic acid to HODEs using A549 and Caco-2 cells lysates as enzyme sources, as described in the Materials and Methods section. COX catalyzes the metabolism of arachidonic acid to PGH_2 and linoleic acid to 9- and 13-HODEs[31]. In the present study, linoleic acid was used as the substrate because it presents several advantages over arachidonic acid.

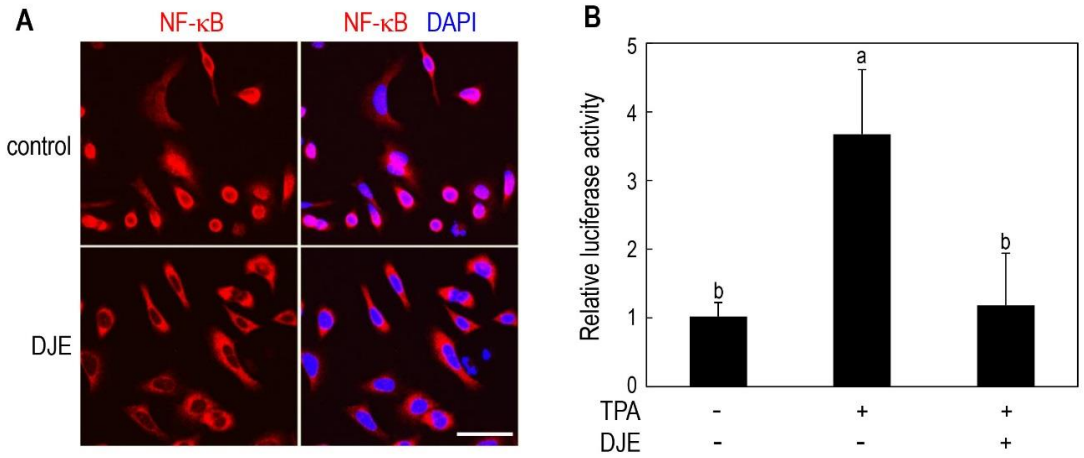


Fig. 6. Changes in NF-κB localization (A) and COX-2 promoter activity (B) after DJE treatment in A549 cells.
Localization of the transcription factor NF-κB (red) and DAPI (blue, nuclear counterstain) was visualized by confocal laser scanning microscopy (A). Scale bar indicates 50 μm. Cluc reporter plasmid with COX-2 promoter was used for the promoter activity assay, as described in the Materials and Methods section (B). TPA (0.1 μM) was used as an inducer of COX-2 expression. The values are represented as a relative value against cells without TPA and DJE and represent mean ± S.D. of 3 separate experiments. [a]$p<0.01$ compared with the control without TPA and DJE and [b]$p<0.01$ compared with TPA treatment without DJE.

HODEs metabolized from linoleic acid were stable and these are quantified by the typical reverse-phase HPLC analysis for low-molecular-weight lipids, although PGH_2 synthesized from arachidonic acid is highly unstable and is immediately and non-enzymatically metabolized to several PGs. After treatment with DJE for 48 h, COX activity was decreased to 46% (39 pmol/min/mg protein) and 50% (27 pmol/min/mg protein) when compared with each control in A549 cells and Caco-2 cells, respectively (Fig. 5A).

PGE_2 production was quantified by enzyme immunoassay. Released PGE_2 in the culture medium of A549 cells was also significantly decreased to 31% (647 pg/ml) by DJE treatment (Fig. 5B).

Translocation of NF-κB and suppression of COX-2 promoter activity by DJE

After treatment with DJE by 50% ethanol (100 μg/ml) for 48 h, localization of the transcription factor NF-κB was analyzed by immunocytochemistry (Fig. 6A). In some pathological conditions such as cancer and inflammation, NF-κB is translocated from the cytosol to the nucleus and plays a key role in regulating COX-2 transcriptional activity. NF-κB immunoreactivity merged with the nuclear counterstain DAPI in control A549 cells (Fig. 6A top panels). The findings indicate that NF-κB is constitutively localized in the nucleus in A549 cancer cells, which exhibit high levels of COX-2. In contrast, DJE treatment induced the translocation of NF-κB from the nucleus to the cytosol (Fig. 6A bottom panels). Additionally, the COX-2 promoter activity was measured using COX-2/Cluc plasmid with Cypridina luciferase reporter gene (Fig. 6B). TPA (0.1 μM), a COX-2 inducer, increased luciferase activity by approximately 4-fold, and addition of DJE

significantly decreased TPA-induced luciferase activity to a level similar to that of the control.

Suppression of Bcl-2 mRNA expression and induction of apoptosis by DJE

mRNA expression of the anti-apoptotic factor Bcl-2 was suppressed after treatment with DJE for 24 h (Fig. 7A). Compared with the control, DJE by 50% ethanol significantly decreased Bcl-2 mRNA levels to 18%.

Further, *in situ* detection of apoptotic cells with TUNEL method revealed a large number of brown-colored positive A549 cells after DJE treatment, although colored cells were hardly observed in the control cells (Fig. 7B).

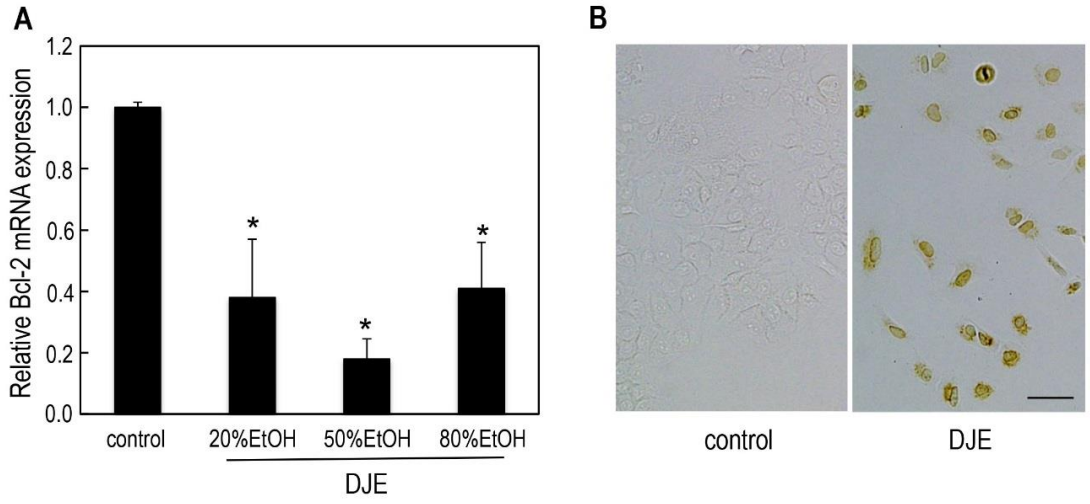


Fig. 7. Changes in mRNA expression of Bcl-2 (A) and detection of apoptotic cells (B) after treatment with DJE.
mRNA expression of the anti-apoptotic factor Bcl-2 was measured by real-time PCR (A). The values are represented as a relative value control cells and represent mean ± S.D. of 5 separate experiments. *p<0.01 compared with the control. *In situ* apoptotic cells were detected by TUNEL method, as described in the Materials and Methods section (B). TUNEL-positive cells were brown-colored. Scale bar indicates 20 μm.

Effect of diosgenin on the expression of COX-2 and mPGES-1 in A549cells

Diosgenin is a major steroidal saponin constituent and is found more abundantly in *Dioscorea japonica* compared with other food substances. Diosgenin suppressed COX-2 expression in A549 cells in a dose-dependent manner (0–10 μM), and 10 μM diosgenin reduced COX-2 expression to 46% compared with the control (Fig. 8). In contrast, diosgenin did not have a significant effect on the expression of mPGES-1.

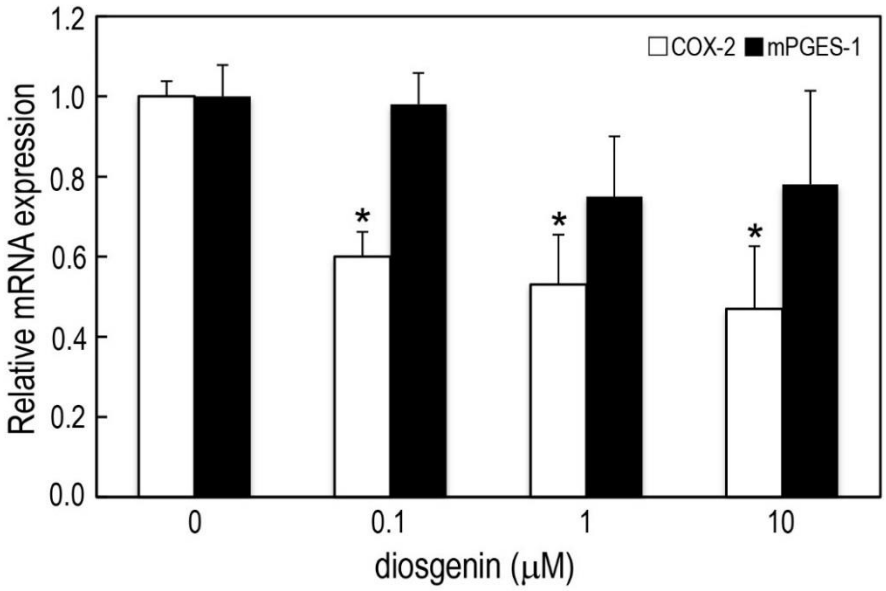


Fig. 8. Effect of diosgenin on the expression of COX-2 and mPGES-1 in A549 cells.
Diosgenin (0.1–10 μM) was added to A549 cells. mRNA expression of COX-2 (open column) and mPGES-1 (closed column) was measured by real-time PCR, as described in the Materials and Methods section. The values are represented as a relative value against 0 μM diosgenin-treated cells and represent mean ± S.D. of 5 separate experiments. *$p<0.05$ *c*ompared with treatment with 0 μM diosgenin.

DISCUSSION

Of all cancers, lung cancer has the highest mortality, and the 5-year survival rate is very low. Therefore, new approaches for the management and prevention of lung cancer are highly essential. In our current study, we attempted to investigate the preventive effects of natural food products on lung cancer by targeting the PGE_2 synthesis pathway. We demonstrated that DJE suppressed the expression of COX-2 and mPGES-1 and exerted anti-carcinogenic effects in human lung carcinoma A549 cells.

Our data show that DJE suppressed mRNA and protein expressions of COX-2 and mPGES-1 and consequently decreased COX activity and PGE_2 production. Furthermore, DJE induced the translocation of NF-κB, the typical transcription factor of COX-2, from the nucleus to the cytosol in A549 cells (Fig. 6A). This finding indicated that DJE regulates COX-2 signal transduction pathway mediating COX-2 induction. Consistent with this hypothesis, the transcriptional activity of COX-2 was significantly decreased by DJE, as shown using an expression plasmid containing COX-2 promoter with a Cluc reporter gene (Fig. 6B). In lung cancer cells, inhibition of COX-2 by trichostatin A[32], a histone deacetylase inhibitor, or celecoxib[33], a NSAID that acts as a selective COX-2 inhibitor, correlates with a decrease in PGE_2 production. Such inhibitors suppress tumor growth with decreasing the levels of Bcl-2 which is the anti-apoptotic factor and is the most important factor associated with a decrease in PGE_2 production[22,34,35]. Our results also showed that DJE suppressed the expression of COX-2 and mPGES-1, which are key enzymes for PGE_2 production, and induced apoptosis with suppression of Bcl-2 in cancer cells.

Several phytochemicals from other natural products, such as resveratrol[36], humulon[37], chrysin[38], and 6-shogaol[39] have been shown to suppress the expression of COX-2. Additionally, the suppressive effect on mPGES-1 expression by phytochemicals are known to sulforaphane[40] and curcumin[41]. Diosgenin, an aglycone of the steroidal saponin dioscin that is abundantly found in *Dioscorea japonica*, may be one of the candidate compounds having the functional effects observed in our study. There are some reports that support our results that diosgenin may have suppressive effects on the signal transduction pathway mediating COX-2 induction. Diosgenin inhibits Akt signaling and its downstream targets, NF-κB and Bcl-2, in human breast cancer cells[42]. In addition, diosgenin has been shown to induce apoptosis in HepG2 and HEL cells[43,44]. In a mouse model for colon cancer, diosgenin was shown to decrease gene expression of inflammatory cytokines such as IL-1β and IL-12β and to prevent colon carcinogenesis[45]. In the present study, the effect of diosgenin (0–10 μM) on the expression of COX-2 and mPGES-1 in A549 cells was investigated. Diosgenin suppressed COX-2 expression in a dose-dependent manner, and COX-2 expression was decreased to 47% by 10 μM diosgenin (Fig. 8). However, the effect of diosgenin on COX-2 expression was lower than that of DJE by 50% ethanol (100 μg/ml DJE), which decreased COX-2 expression to 27% (Fig. 3A). On the other hand, diosgenin did not have a significant effect on mPGES-1 expression. This suggests that multiple components in DJE mediate its ability to inhibit the expression of COX-2 and mPGES-1, leading to anti-inflammation and anti-carcinogenesis, suggesting there may be a novel functional component in DJE that has yet to be identified.

Our result may be important in understanding the preventive effects of *Dioscorea japonica* against the chronic disease that are mediated by PGE_2, although additional *in*

vivo studies are required to address the physiological significance of our findings.

Chapter III

Preventive effect of *Dioscorea japonica* on squamous cell carcinoma of mouse skin involving down-regulation of prostaglandin E_2 synthetic pathway

Skin cancers are caused by any kinds of environmental carcinogens, inflammatory agents, and tumor promoters. It has been known that inflammation associates with development in pre-malignant and malignant transformation of cells and induces tumor development[2,46].

In skin cancers caused by some stimuli such as ultraviolet light and chemicals, induced COX-2 hyperproduces PGE_2[47–49]. Previous reports using COX-2 inhibitor[50] and COX-2 deficient mouse[51] demonstrate the prevention of tumor development in squamous cell carcinoma of the mouse skin. COX-2 is functionally coupled with mPGES-1 for PGE_2 synthesis in pathophysiological conditions[52], and these enzymes are overexpressed in various tumors and inflammation[13,52–54].

Chapter II demonstrated for the first time that DJE suppressed the expression of COX-2 and mPGES-1, resulting PGE_2 decrease induces cancer cell apoptosis in lung cancer A549 cells[55]. The aim of a next study is the verification of the effects of *Dioscorea japonica* on inflammation and carcinogenesis via the suppression of COX-2 and mPGES-1 *in vivo*. In this chapter, therefore, we demonstrate the effects using a two-stage cutaneous chemical carcinogenesis model of mouse skin[56].

MATERIALS AND METHODS

Animal rearing conditions and the method of preparing a model of squamous cell carcinoma

All protocols were approved by the Exclusive Committee on Animal Research at Okayama Prefectural University and the research was conducted in conformity with the Public Health Service (PHS) policy. Seven-week-old male Balb/c mice had free access to drinking water and food. Mice were fed a powder diet (CRF-1; Oriental Yeast Co. Ltd., Tokyo, Japan) with or without (w/w) 1% or 10% *Dioscorea japonica* powder. *Dioscorea japonica* was obtained from Autoraimu Yoshio Ltd. (Niimi, Japan). The outer skin of *Dioscorea japonica* was pared away, and it was dried and pulverized at 40°C. Feed consumption was measured twice a week, and weight was measured every second week. According to the procedure of Modi et al.[56], a squamous cell carcinoma model was induced by topically applying the following to each mouse: firstly, 200 μL of 2 mM 7,12-dimethylbenz[*a*]anthracene (DMBA, as an initiator, Sigma-Aldrich, St. Louis, MO); then, after one week, 200 μL of 80 μM 12-*O*-tetradecanoylphorbol-13 acetate (TPA, as a promoter, Sigma-Aldrich) twice per week for 22 weeks (Fig. 9). Numbers and volumes of cutaneous papillomas were measured. Mice in three experimental groups were applied with 100 μL of 0.05, 0.5 or 5 mg of *Dioscorea japonica* extract eluted from the powder with 50% ethanol 30 min before the application of TPA, and fed a basal diet (CRF-1) without *Dioscorea japonica* powder.

Quantitative reverse transcriptase (RT)-PCR

The gene expression was analyzed by quantitative RT-PCR (iQ5 real-time PCR system, Bio-Rad, Hercules, CA) using cDNA prepared from isolated total RNA. The quantitative PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), and the following PCR primer pairs: *Ptgs1* (COX-1), 5′-CTTTGCA CAACACTTCACCCACC-3′ (forward) and 5′-AGCAACCCAAACACCTCCTGG-3′ (reverse); *Ptgs2* (COX-2), 5′-GCATTCTTTGCCCAGCACTT-3′ (forward) and 5′-AGA CCAGGCACCAGACCAAAGA-3′ (reverse); *Ptges* (mPGES-1), 5′-CTGCTGGTCAT CAAGATGTACG-3′ (forward) and 5′-CCCAGGTAGGCCACGGTGTGT-3′ (reverse); *Ptges2* (membrane-associated PGES-2 (mPGES-2)), 5′-AAGACATGTCCCTTCTGC-3′ (forward) and 5′-CCAAGATGGGCACTTTCC-3′ (reverse); *Ptges3* (cytosolic PGES (cPGES)), 5′-AGTCATGGCCTAGGTTAAC-3′ (forward) and 5′-TGTGAATCA TCATCTGCTCC-3′ (reverse); *Ptgds2* (hematopoietic PGD synthase (H-PGDS)), 5′-CA CGCTGGATGACTTCATGT-3′ (forward) and 5′-AATTCATTGAACATCCGTCTT-3′ (reverse); *Il1b* (interleukin (IL)-1β, 5′-GTCACAAGAAACCATGGCACAT-3′ (forward) and 5′-GCCCATCAGAGGCAAGGA-3′ (reverse); *Il6* (IL-6), 5′-CTGCAAGA GACTTCCATCCAGTT-3′ (forward) and 5′-AGGGAAGGCCGTGGTTGT-3′ (reverse); and *gapdh* (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)), 5′-TGAACGGGA AGCTCACTGG-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). The relative expression levels were shown against normal controls and represent mean ± SD.

Pathohistological and immunohistochemical analyses

Prepared paraffin-embedded sections of mouse skin were used for hematoxylin and eosin (HE) staining and immunohistochemical staining. For single immunolabeling,

sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity, and were blocked with 12.5% Block Ace (DS Pharma Biomedical Co., Ltd., Tokyo, Japan) to block nonspecific bindings. For immunolabeling, we used the following first antibodies: goat anti-COX-2 antibody (1:50, catalogue no. sc-1745, lot no. E102, Santa Cruz Biotechnology, Inc., Santa Cruz CA), rabbit anti-mPGES-1 antibody (1:500, catalogue no. 160140, lot no. 0436398-1, Cayman Chemical Co., Ann Arbor, MI), rabbit anti-H-PGDS antiserum (1:1000, catalogue no. 10004348, lot no. 0451181-1, Cayman Chemical Co.), rat anti-Ly-6G/Ly-6C (Gr-1) antibody (1:500, a marker of neutrophils; catalogue no. 108413, lot no. B141536, BioLegend, San Diego, CA), and mouse anti-Langerin/CD207 antibody (1:1000, a marker of Langerhans cells; catalogue no. DDX0361P-50, lot no. DDX0361-009, DENDRITICS, Lyon, France), and the following second antibodies: biotinylated anti-goat, biotinylated anti-rabbit, Cy3-labeled donkey anti-goat, FITC-labeled donkey anti-rabbit, FITC-labeled donkey anti-goat, FITC-labeled donkey anti-rat, FITC-labeled donkey anti-mouse IgGs (1:400, biotinylated IgGs from Vector Laboratories, Burlingame, CA, and fluorescein-labeled IgGs from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The biotin-labeling was enhanced by ABC Elite kit (Vector Laboratories) and was visualized in 50 mM Tris (pH 7.6) containing 0.1% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide at 37°C. The fluorescein-labeled sections were examined by confocal laser scanning light microscopy (CLSM, Fluoview FV1000, Olympus Co., Tokyo, Japan).

Electrospray ionization mass spectrometry (ESI-MS)

All procedures were performed as described previously[57]. Tissues were homogenized with a Polytron homogenizer in methanol and were incubated overnight.

As internal standards for determination, 100 pmol of *d5*-labeled eicosapentaenoic acid (EPA) and *d8*-labeled 15-hydroxyeicosatetraenoic acid (15-HETE), were added to the samples. The lipids in the sample were extracted using Oasis HLB cartridges (Waters, Milford, MA), and were dried up under nitrogen gas. The analysis was performed using a 4000Q-TRAP quadrupole-linear ion trap hybrid mass spectrometer (AB Sciex, Framingham, MA) with LC (NexeraX2 system; Shimadzu Co., Kyoto, Japan). The sample was applied to a C18 column (Kinetex C18, 2.1 x 150 mm, 1.7 μm, Phenomenex, Inc., Torrance, CA) coupled for ESI-MS/MS. For analyses of fatty acids, the samples are applied to a column and separated by a step gradient with mobile phase A (acetonitrile:MeOH:water = 1:1:1 (v/v/v) containing 5 μM phosphoric acid and 1 mM ammonium formate) and mobile phase B (2-propanol containing 5 μM phosphoric acid and 1 mM ammonium formate) at a flow rate of 0.2 ml/min at 50°C. Whereas, for analyses of oxidized fatty acids, the samples are applied to a column and separated by a step gradient with mobile phase C (water containing 0.1 % acetic acid) and mobile phase D (acetonitrile:MeOH = 4:1; v/v) at a flow rate of 0.2 ml/min at 45°C. Signature ion fragments for each targeted lipid were monitored and quantified by a multiple reaction monitoring (MRM) method. Identification was conducted using MRM transition[58] and retention times. Quantification was performed based on peak area of the MRM transition and the calibration curve obtained with the authentic standard for each compound.

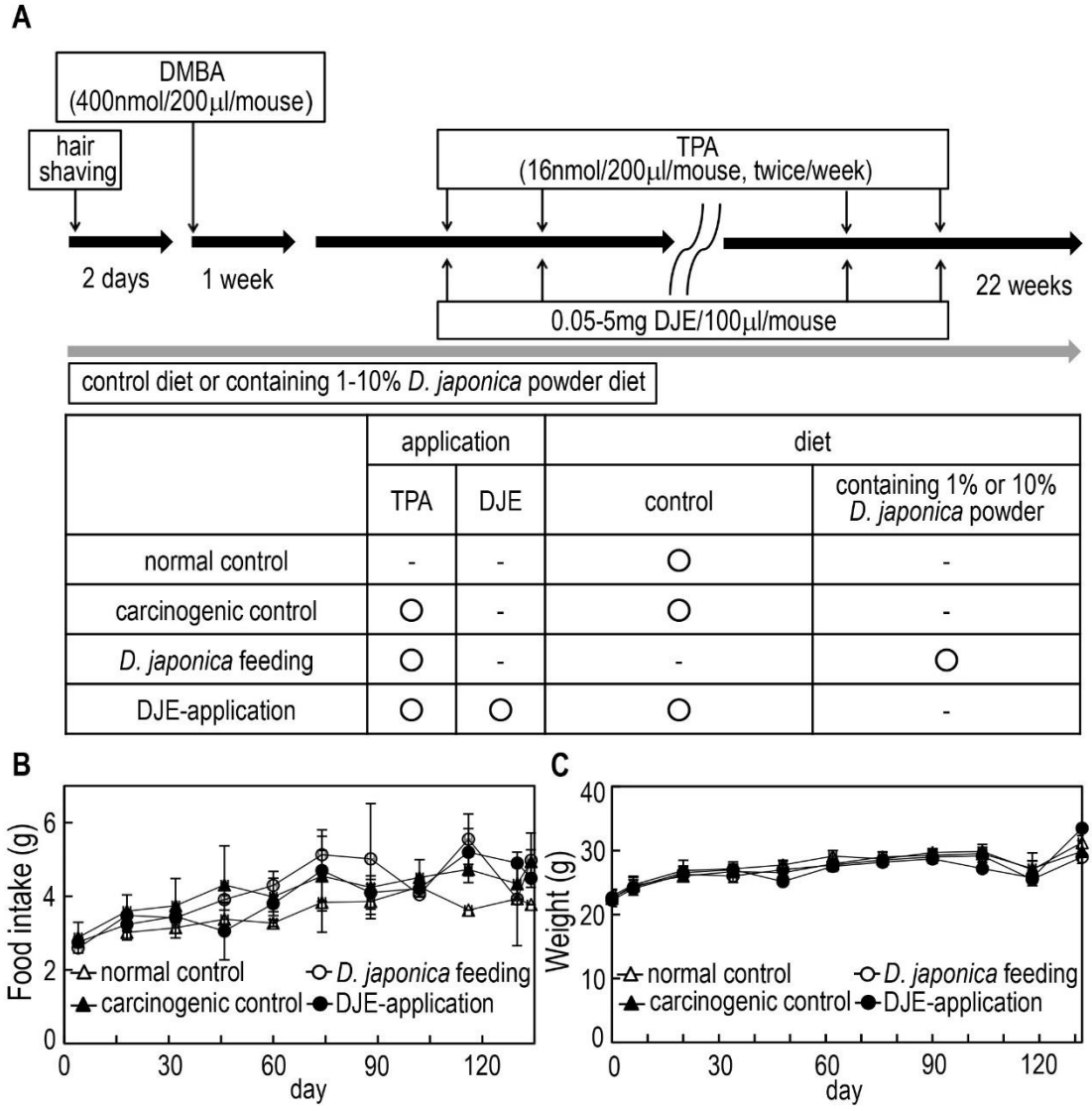
Effect of diosgenin on the expression of COX-2 and mPGES-1 in RAW264 cells

Mouse macrophage-like RAW264 cells were obtained from RIKEN BioResource Center (Tsukuba, Japan). The cells were cultured in Dulbecco's modified

Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/ml streptomycin. RAW264 cells were incubated with or without 100 nM diosgenin. After the incubation for 3h, the cells were stimulated by 2.5 μg/mL lipopolysaccharide (LPS) for 6h. mRNA expression of *Ptgs2* and *Ptges* was measured by quantitative RT-PCR.

Statistics

Data were statistically evaluated by ANOVA with Bonferroni's or Dunnett's post-hoc test at significance level of $p<0.01$ or $p<0.05$.



	application		diet	
	TPA	DJE	control	containing 1% or 10% *D. japonica* powder
normal control	-	-	○	-
carcinogenic control	○	-	○	-
D. japonica feeding	○	-	-	○
DJE-application	○	○	○	-

Fig. 9. Treatment scheme and comparison of food intake and body weight among the experimental groups.

Seven-week-old male Balb/c mice were treated with the respective regimens according to the treatment scheme (A). Animals were divided into four experimental groups: group "normal control" was given control diet and treatment with vehicle; group "carcinogenic control" was given normal diet and treatment with DMBA/TPA; group "*Dioscorea japonica* feeding" (*D. japonica* feeding) was given 1% or 10% *Dioscorea japonic*a powder (w/w) containing diet and treatment with DMBA/TPA; group "*Dioscorea japonica* extract topical application" (DJE-application) was given a normal diet and treatment with DMBA/TPA and 0.05-5 mg of *Dioscorea japonica* extract/100 μl of 50% ethanol. Food intake (B) and body weight (C) were recorded. The values represent mean ± SD.

RESULTS

Comparison of tumor formation among the experimental groups

In the present model of squamous cell carcinoma, DMBA and TPA were used as initiator and promoter, respectively. DMBA is incorporated into Langerhans cells (LCs)

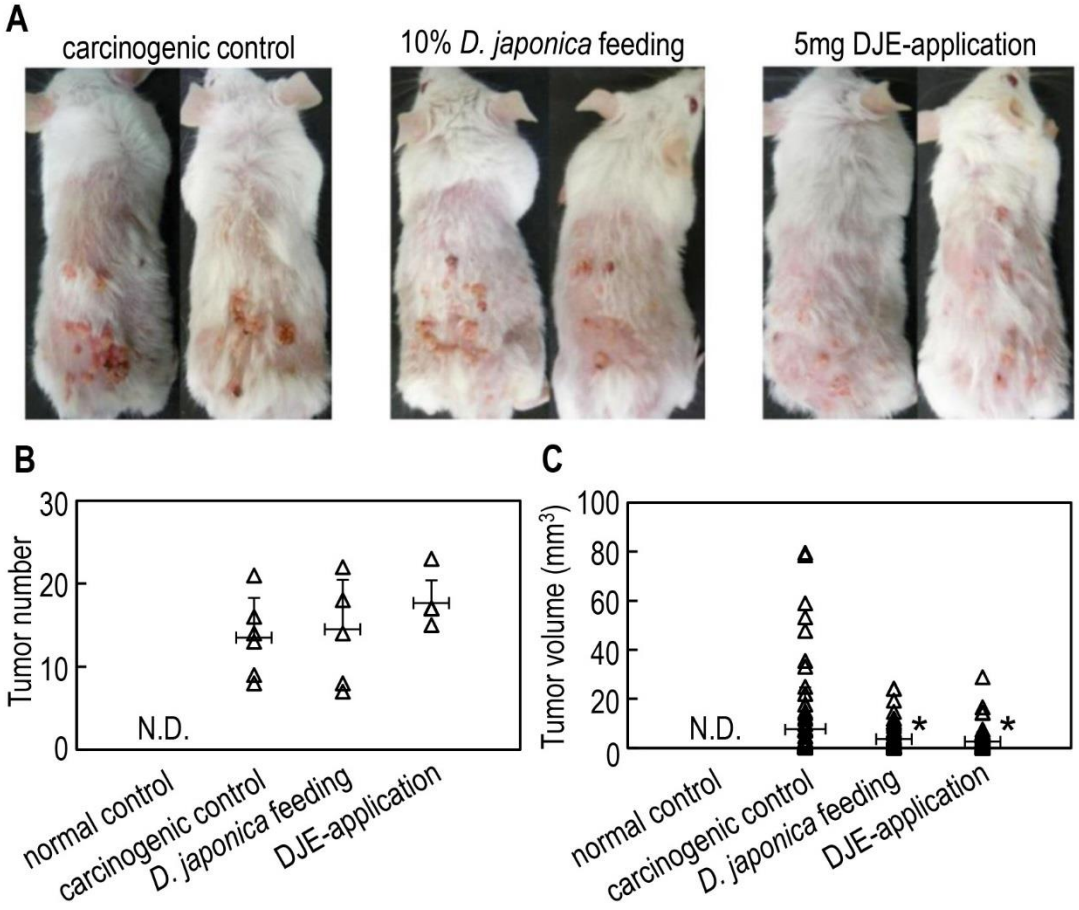


Fig. 10. Comparison of tumor formation among experimental groups.
Representative images of mice from each experimental group after 22 weeks of tumor induction are shown in A. Tumors are identified to enable comparison between normal skin and swelling tissue, and tumor number (B) and volume (C) are indicated. The values represent mean ± SD of 6 mice per group; *p<0.01 compared with the carcinogenic control.

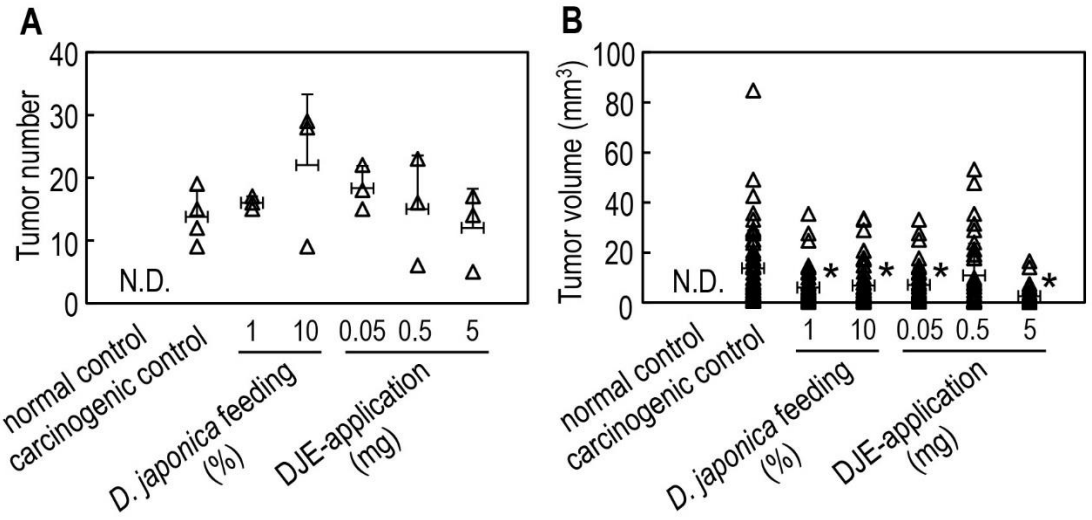


Fig. 11. Dose-dependent effects of *D. japonica* feeding and DJE-application on tumor formation. Tumor number (A) and volume (B) were measured. The values represent mean ± SD of 3-4 mice per group; *p<0.01 compared with the carcinogenic control. N.D.; not detected.

in the epidermis, and is metabolized to DMBA-3,4-diol-1,2-epoxide (DMBADE)[56]. DMBADE is known to strongly induce keratinocytes during carcinogenesis[56,59].

Tumor formation was measured in the experimental groups including *Dioscorea japonica* feeding group and DJE topical application group (Fig. 10). Throughout the experiment, food intake and body weight were not significant differences among the experimental groups (Fig. 9B and C). After 22 weeks of tumor induction, there was no noticeable difference in tumor numbers between the experimental groups with the exception of the normal control (Fig. 10B and Fig. 11A). However, the tumor volumes were significantly decreased in the *Dioscorea japonica* feeding and DJE topical application groups (Fig. 10A, C, and Fig. 11B). These data indicate that *Dioscorea japonica* treatment is more beneficial for tumor volumes than tumor numbers. Thus, *Dioscorea japonica* may inhibit tumor growth.

Suppression of COX-2, mPGES-1, inflammatory cytokines, and decrease of PG products by Dioscorea japonica treatment

To examine the effect of *Dioscorea japonica* administration on mRNA expression in mouse skin (Fig. 12), *Ptgs1* (COX-1), *Ptgs2* (COX-2), *Ptges* (mPGES-1),

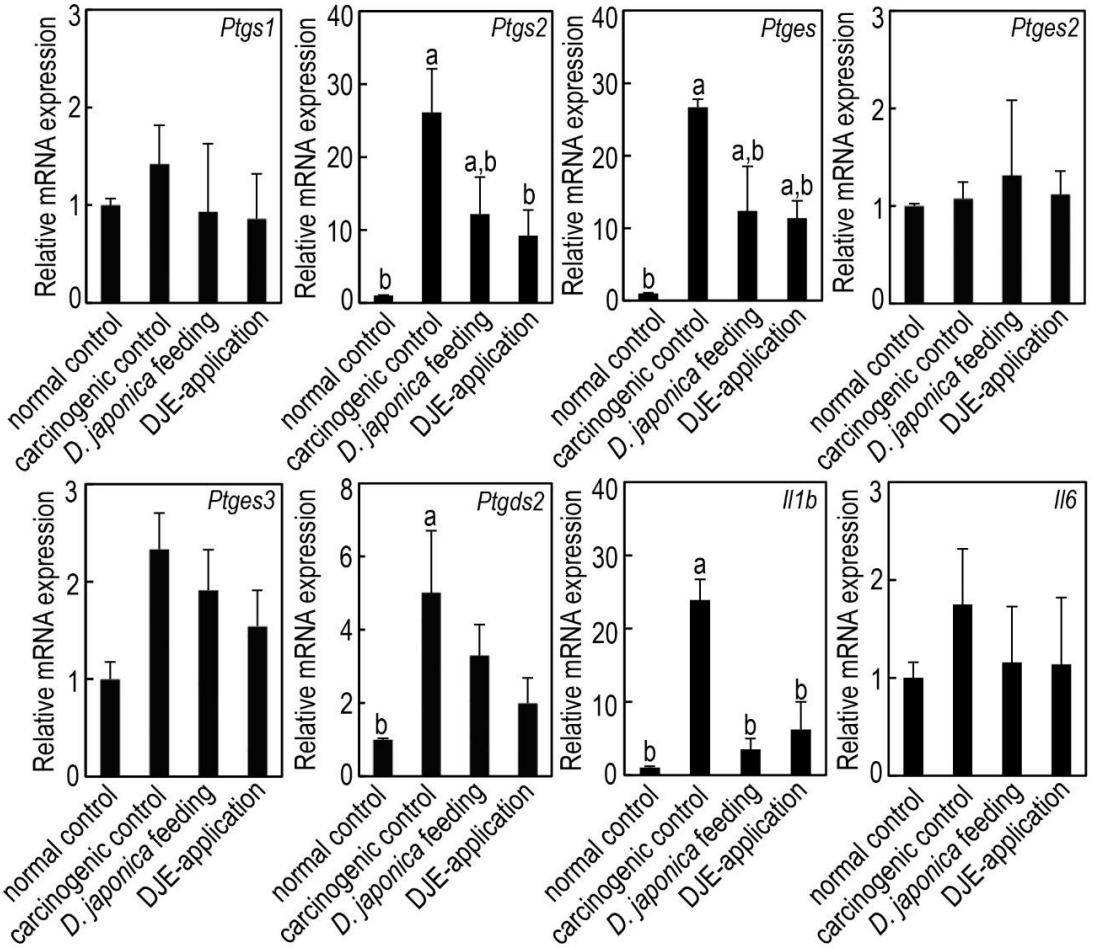


Fig. 12. Changes in gene expression.
In normal or carcinogenic controls, 10% *Dioscorea japnonica* feeding, and 5mg DJE topical application, mRNA expression of *Ptgs1* (COX-1), *Ptgs2* (COX-2), *Ptges* (mPGES-1), *Ptges2* (mPGES-2), *Ptges3* (cPGES), *Ptgds2* (H-PGDS), *Il1b* (IL-1β), and *Il6* (IL-6) was analyzed by quantitative RT-PCR. Expression levels of each mRNA were normalized to that of *gapdh* (GAPDH) mRNA. The relative expression levels are shown against normal control levels and represent mean ± SD of 6 mice per group; $p<0.01$ compared with [a]normal controls and [b]carcinogenic controls.

Ptges2 (mPGES-2), *Ptges3* (another cytosolic PGE_2 synthase, cPGES), *Ptgds2* (PGD_2 synthesizing enzyme, H-PGDS), and inflammatory cytokines *Il1b* (IL-1β) and *Il6* (IL-6) were analyzed by quantitative RT-PCR. In the carcinogenic control mice, *Ptgs2*, *Ptges*, and *Il1b* increased approximately 25-fold compared with the normal control mice. On the other hand, *Dioscorea japonica* feeding led to the suppression of *Ptgs2*, *Ptges*, and *Il1b* mRNA to 47%, 46%, and 15% compared with the carcinogenic control, respectively. In addition, DJE topical application decreased mRNA levels of these genes to 35%, 43%, and 26% compared with the carcinogenic control, respectively. Moreover, mRNA expression of *Il6* in *Dioscorea japonica* treatment was also decreased to almost the same level to that in normal control. Additionally, *Dioscorea japonica* treatment did not affect to the expression of *Ptgds2* and *Ptges3*, although the expression of them was induced to approximately 5-fold and 2.5-fold, respectively in the carcinogenic control mice (Fig. 12). On the other hand, the expression of *Ptgs1* and *Ptges2* was not affected among all

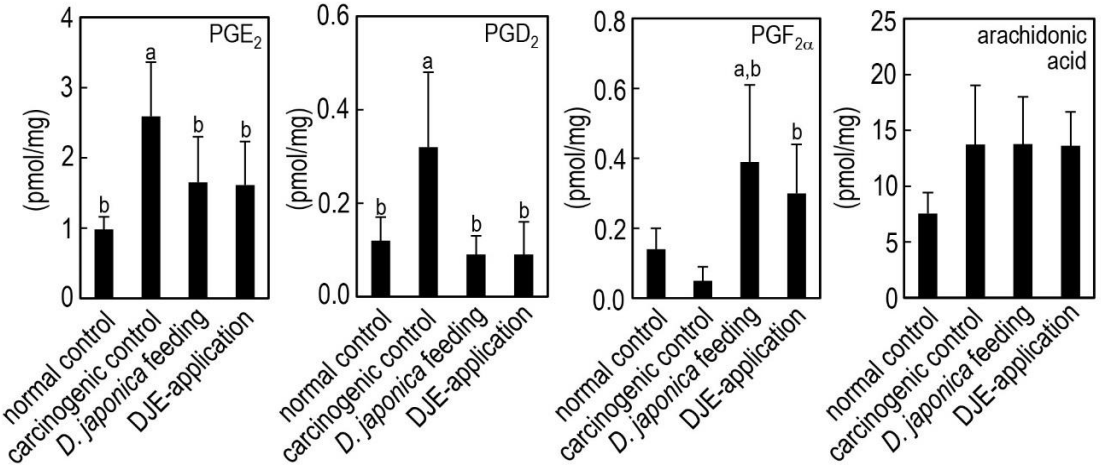


Fig. 13. Changes in amounts of PGs.
The production of PGE_2, PGD_2, $PGF_{2\alpha}$, and arachidonic acid was analyzed by ESI-MS. Amounts indicated represent mean ± SD of 10 samples per group; $p<0.01$ compared with [a]normal controls and [b]carcinogenic controls.

experimental groups (Fig. 12). Furthermore, lipid metabolome analysis in mouse skin was performed by liquid chromatography tandem mass spectrometry (ESI-MS, Fig. 13). We confirmed that the main lipid mediator in mouse skin was PGE_2, and the production of PGE_2 and PGD_2 in *Dioscorea japonica* feeding mice decreased to 63.7% and 28.1%, respectively, compared with the carcinogenic control. DJE topical application also decreased PGE_2 and PGD_2 production to 62.2% and 28.1%, respectively. Whereas, $PGF_{2\alpha}$ increased in *Dioscorea japonica* treatment groups.

Effect of Dioscorea japonica treatment on pathohistological and immunohistochemical analyses

Carcinogenic control mouse epidermis induced exhibited significant epidermal hyperplasia with hyperproliferation of the keratinocytes (upper part of HE staining in Fig. 14). *Dioscorea japonica* treatment markedly suppressed the epidermal hyperplasia.

Moreover, infiltration of a lot of inflammatory cells in the epidermis in carcinogenic control was substantially inhibited by *Dioscorea japonica* feeding and DJE topical application (lower part of HE staining in Fig. 14). Immunohistochemical analyses indicated that COX-2 and mPGES-1 localized in tumor keratinocytes, and, additionally, COX-2 was strongly expressed in epidermal dendritic cells (Langerhans cells, LCs), but mPGES-1 was not observed in LCs (Fig. 14). Similarly, H-PGDS was also highly expressed in LCs, but not in keratinocytes. Interestingly, COX-2 staining was decreased in both tumor keratinocytes and LCs by *Dioscorea japonica* treatment. Additionally, we determined the localization of COX-2 and mPGES-1 in the mouse epidermis with inflammation and tumorigenesis by double immunofluorescent staining. Consistent with immunohistochemical analyses, COX-2 co-localized with CD207 (LC marker) and was

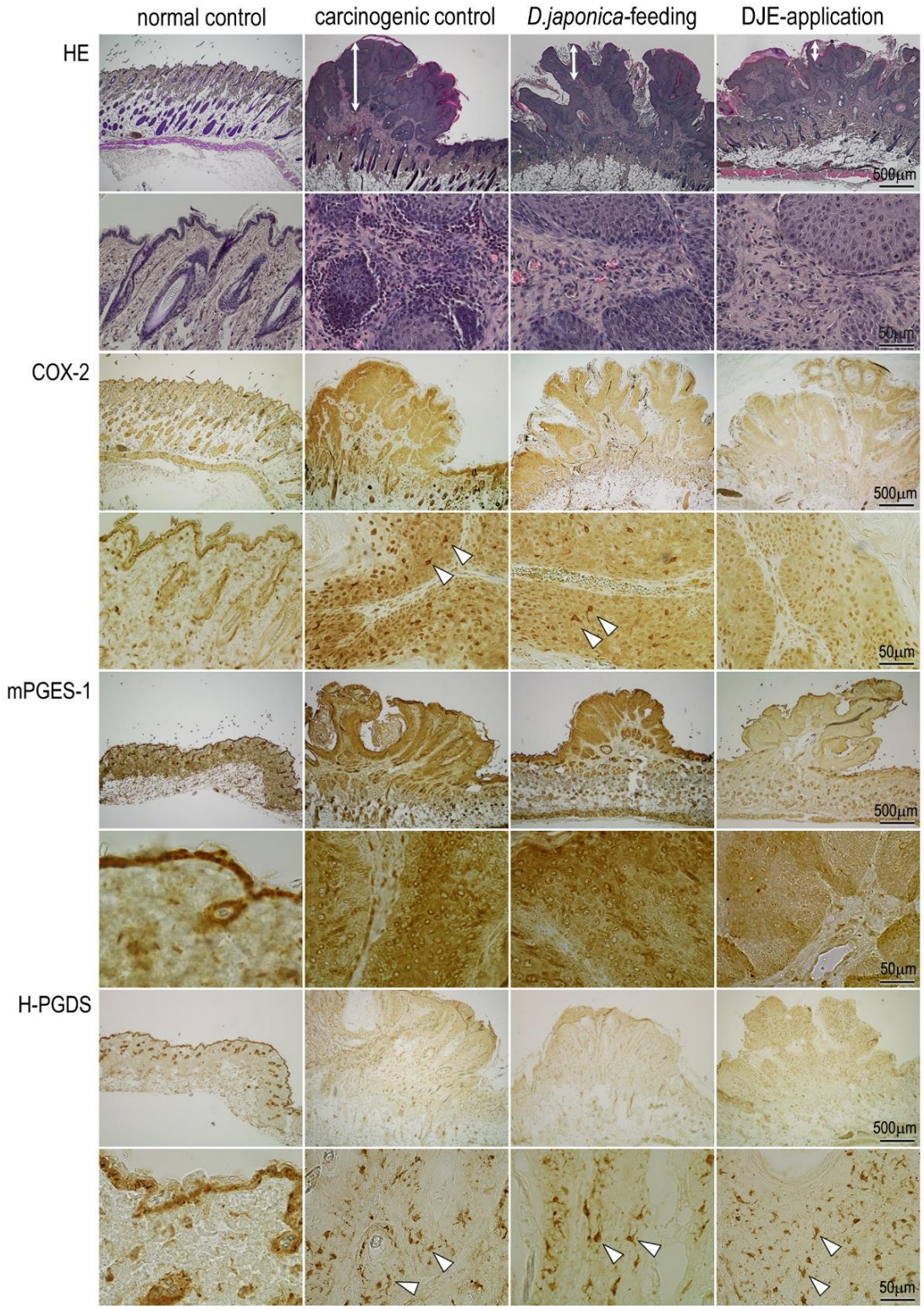


Fig. 14. Histochemical analyses in mouse skin.
Hematoxylin and eosin (HE) staining were pathologically analyzed. Brown-colored immunostaining of COX-2, mPGES-1, and H-PGES indicates each localization. *Double-headed arrows* indicate hyperplasia of accumulated cancer cells and *arrowheads* indicate Langerhans cells.

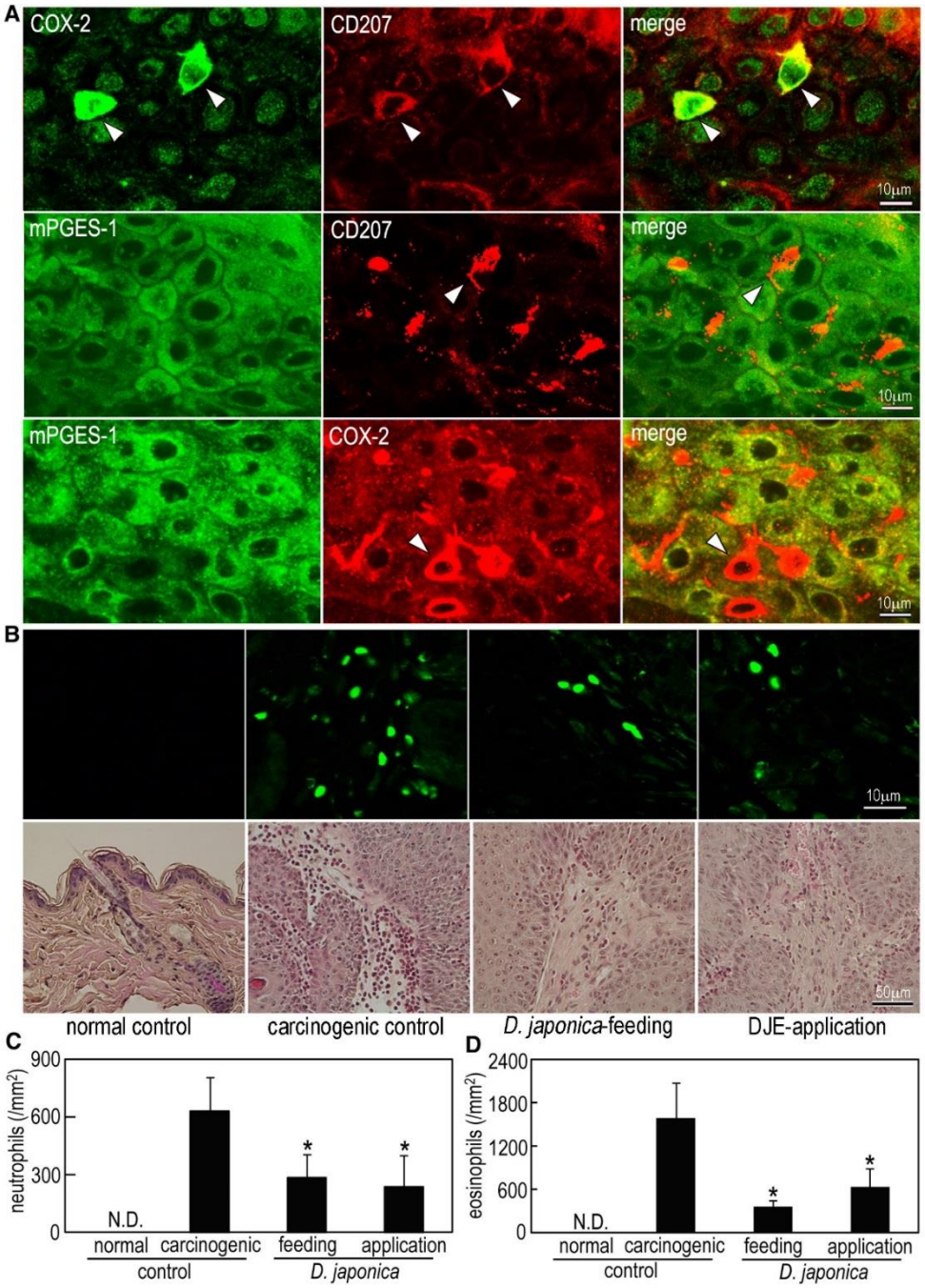


Fig. 15. Double fluorostaining of COX-2, mPGES-1 or CD207 (A), and measurement of infiltrating cells in mouse skin (B-D).

COX-2 and mPGES-1 expressing cells in carcinogenic control mouse epidermis were analyzed by immunofluorescent staining (A). COX-2 and CD207 (marker of Langerhans cells) were co-fluorostained, but mPGES-1 and CD207 were not. mPGES-1 and COX-2 were co-fluorostained in carcinogenic keratinocytes. For double immunofluorescent staining, merged signals are shown in yellow. The number of infiltrating neutrophils was counted in visualized by immunofluorescent staining of Ly-6G/Ly-6C (Gr-1) (green in upper B) and eosinophils were counted after HE staining (bottom B). The comparison of their number was represented as mean ± SD of 10 sections per group (C and D); $^{*}p<0.01$ compared with the carcinogenic control. *Arrowheads* indicate Langerhans cells. N.D.; not detected.

present both in cancer cells and LCs. The immunoreaction of COX-2, especially in LCs, was stronger than that in cancer cells (Fig. 14 and 15A). In contrast, double staining for mPGES-1 and CD207, or for mPGES-1 and COX-2 showed that mPGES-1 co-localized with COX-2 in cancer cells, but not in LCs (Fig. 15A). Furthermore, we conducted immunostaining and HE staining, and counted the number of neutrophils and eosinophils to determine the effect of *Dioscorea japonica* on inflammatory cell infiltration. As shown in Figure 15B, the greatest numbers of neutrophils and eosinophils were observed in carcinogenic controls. Counting the cells showed that *Dioscorea japonica* treatment decreased the infiltrating perilesional neutrophils and eosinophils to less than 50% and 20%-35%, respectively (Fig. 15C and D).

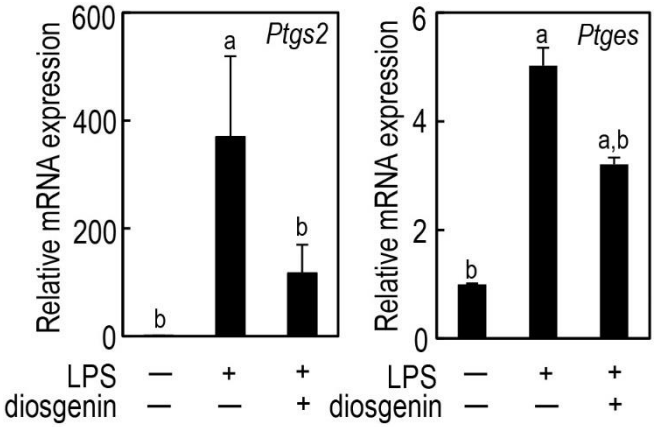


Fig. 16. Effects of diosgenin on the expression of *Ptgs2* and *Ptges* in LPS-stimulated RAW264 cells.

LPS-stimulated RAW264 were cultured with or without 100 nM diosgenin. mRNA expression of *Ptgs2* (COX-2) and *Ptges* (mPGES-1) was measured by quantitative RT-PCR. The expression levels are shown as a relative value against control cells without LPS and diosgenin, and are represented mean ± SD of 3 separate experiments; $p<0.05$ compared with [a]LPS(-)/diosgenin(-) and [b]LPS(+)/diosgenin(-).

DISCUSSION

In this study, we showed that not only COX-2 but also mPGES-1 was overexpressed in squamous cell carcinoma of mouse skin, and they were co-localized in proliferated epidermal cancer cells. In addition, COX-2 was highly expressed, but mPGES-1 was not present in LCs. According to our results, COX-2 may be involved in carcinogenesis, tumor growth, and activation of LCs. On the other hand, main roles of mPGES-1 may be proliferation and development of cancer cells. PGE_2 specific receptors EP1-4 are expressed in normal and tumor epidermis[60,61]. In tumors induced by UVB light exposure and COX-2 overexpression, EPs localize in epidermal hyperplasia[60,61]. The signaling pathways of PGE_2/EP1 and PGE_2/EP3 are involved in immune responses of the skin via T helper type (Th) 1 cells and dendritic cells, respectively[62,63]. Although the presence of EP4 in LCs is immunohistochemically unknown, PGE_2/EP4 signaling promotes migration and maturation of Langerhans cells, and initiates skin immune responses[64]. Taken together with our results, it is suggested that the produced PGE_2 by COX-2 and mPGES-1 induce tumor development and immune responses via the individual receptors in the autocrine or paracrine manner in squamous cell carcinoma of the skin.

In addition, H-PGDS was also predominantly expressed in LCs, and which may indicate a coupling of COX-2 and H-PGDS and the produced PGD_2 regulates LCs activity. LCs play a key role in establishment of cutaneous immunity, and participate in squamous cell carcinoma[56,65]. Human dendritic cells including LCs in the skin express H-PGDS, and produce PGD_2 in response to various stimuli[66]. Thus it is suggested that the produced

PGD_2 in autocrine manner in LCs is involved in cutaneous immune system and inflammatory reactions in the skin. Previous reports indicate the opposing immunomodulatory roles of PGD_2 receptors, DP and CRTH2 (chemoattractant receptor homologous molecule expressed on Th2 cells)[67,68]. In inflammatory processes of the skin, PGD_2 may play a role in the early stages via DP, and in eosinophil migration during the late stages via CRTH2. In each process of developing squamous cell carcinoma, further studies will be needed to determine the roles of H-PGDS and PGD_2 in inflammation and carcinogenesis.

Our recent report demonstrates that *Dioscorea japonica* suppresses the expression of COX-2 and mPGES-1, and has an anti-carcinogenic effect on several model cell lines (reference 55 and unpublished data). In the present study, not only application, but also ingestion of *Dioscorea japonica* affects the expression of COX-2 and mPGES-1, and leads to decreased PGE_2 *in vivo*. *Dioscorea japonica* is rich in a lot of nutrients, and one of them is a plant steroidal saponin such as diosgenin. Diosgenin has been reported to have some preventive effects on mouse colon carcinogenesis[45], mouse squamous cell carcinoma[69] and mouse Alzheimer's disease[70]. Our previous study also showed that diosgenin suppressed COX-2 in A549 cells[55], and additionally our recent experiment showed that it suppressed both of COX-2 and mPGES-1 in LPS-stimulated mouse macrophage-like RAW264 cells (Fig. 16). Therefore, diosgenin is likely one of the effective substances in the present study, and liposoluble and low molecular diosgenin may have effects via the oral and application routes.

In this study, lipid analysis showed that *Dioscorea japonica* did not decrease PGE_2 less than normal control, and therefore it may have no side effects caused by loss of homeostatic PGE_2. It is unclear about effect on kidney and cardiovascular system,

because there are only a few TXB_2 and 6-keto-$PGF_{1\alpha}$, which are stable metabolites of TXA_2 and PGI_2, respectively, in the mouse skin (data not shown). However, mRNA levels of COX-2 and mPGES-1 of *Dioscorea japonica*-treated groups were not lower than that of normal control, which means to keep the production of homeostatic PGE_2 without side-effects. These results suggested that *Dioscorea japonica* has the possibility of a novel preventive agent in inflammation and carcinogenesis.

Chapter IV
Conclusion Remarks

Lipid mediator PGE_2 is widely distributed in various organs, and is involved in several biological phenomena such as inflammation and carcinogenesis. In the PGE_2 synthetic pathway, coupled COX-2 and mPGES-1 are induced at the pathophysiological condition, and hyperproduced PGE_2. NSAIDs are clinically used for symptomatic relief of patient[13]. Most NSAIDs inhibit COX-1 and COX-2, and decrease eicosanoids, and for that reason, they have some side effects such as gastrointestinal injury[14]. Then selective COX-2 inhibitors, such as celecoxib and rofecoxib, had developed as a next-generation NSAIDs. Indeed, the selective COX-2 inhibitors reduce gastrointestinal side effects, but exhibit the other side effects on renal and cardiovascular systems[15,71]. Currently, the terminal enzyme for PGE_2 synthesis, mPGES-1, is expected for a new-generation NSAIDs as a potential drug target[17]. For prevention of chronic disease including cancer, we try to discover functional foods targeting COX-2 and mPGES-1 without side effects.

In this study, we found a novel functionality of *Dioscorea japonica* with suppression of COX-2 and mPGES-1. *Dioscorea japonica* is one of the wild yams and is a relative of the *Dioscoreaceae* family native to Japan. For many years, it is believed that *Dioscorea japonica* has rich nutrition and some functionalities such as gastric mucosal protection and digestive enhancement. Additionally, we demonstrated that *Dioscorea japonica* has the other effects, that is, anti-inflammation and anti-carcinogenesis.

First, we demonstrated DJE suppressed the expression COX-2 and mPGES-1, and also decreased COX activity and PGE_2 production *in vitro*. At least COX-2 expression was regulated at transcriptional level with NF-κB translocation. Decrease of PGE_2 production by the effects of DJE induced carcinoma cells to apoptosis. To confirm the food functionality of *Dioscorea japonica* against the PGE_2-mediated carcinogenesis and

inflammation *in vivo*, we investigated in squamous cell carcinoma of mouse skin exposed to DMBA and TPA. *Dioscorea japonica*-containing feed and DJE-topical application suppressed the expression of COX-2, mPGES-1 and inflammatory cytokines, and inhibited tumor formation, hyperplasia, and inflammatory cells infiltration. Indeed, the lipid analysis also showed that PGE_2 and PGD_2 decreased by *Dioscorea japonica* treatment. Immunohistochemical analyses showed the immunoreactivities of COX-2 and mPGES-1 in tumor keratinocytes and stronger immunoreactivities of COX-2 and H-PGDS in Langerhans cells. Treatment of *Dioscorea japonica* decreased the immunoreactivity of COX-2 and mPGES-1. These results indicate that *Dioscorea japonica* may have preventive effects on inflammation and carcinogenesis via suppression of PGE_2 synthesis pathway. The effects are believed to be due at least in part to relate to the immune system in the squamous cell carcinoma. In addition, our study demonstrated that not only topical application of *Dioscorea japonica*, but also oral intake is effective for anti-inflammatory and anti-carcinogenesis.

These findings show that *Dioscorea japonica* is good functional food and might be utilized in the preventive foods for PGE_2-mediated several pathophysiological functions. Our results on the effects of *Dioscorea japonica* may pave the way for further therapeutic methods. Additionally, it is necessary to identify the effective substances from *Dioscorea japonica*, and find the regulation in signaling pathway of COX-2 and mPGES-1 expression.

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