Preventive effect of *Dioscorea japonica* on squamous cell carcinoma of mouse skin involving down-regulation of prostaglandin E\textsubscript{2} synthetic pathway

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Hyperproduced prostaglandin E\textsubscript{2} by cyclooxygenase-2 and microsomal prostaglandin E synthase-1 evokes several pathophysiological responses such as inflammation and carcinogenesis. Our recent study demonstrated that *Dioscorea japonica* extract suppressed the expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 and induced apoptosis in lung carcinoma A549 cells. In the present study, we investigated the effects of *Dioscorea japonica* on squamous cell carcinoma of mouse skin. *Dioscorea japonica* feeding and *Dioscorea japonica* extract topical application suppressed the expression of cyclooxygenase-2, microsomal prostaglandin E synthase-1, interleukin-1β and interleukin-6 and inhibited tumor formation, hyperplasia and inflammatory cell infiltration. Immunohistochemical analyses showed the immunoreactivities of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 in tumor keratinocytes and stronger immunoreactivities of cyclooxygenase-2 and hematopoietic prostaglandin D synthase in epidermal dendritic cells (Langerhans cells). Treatment with *Dioscorea japonica* decreased the immunoreactivity of cyclooxygenase-2 and microsomal prostaglandin E synthase-1. These results indicate that *Dioscorea japonica* may have inhibitory effects on inflammation and carcinogenesis via suppression of the prostaglandin E\textsubscript{2} synthetic pathway.

Key Words: wild yam, inflammation, carcinogenesis, skin cancer, prostaglandin E\textsubscript{2}

Prostaglandin (PG) E\textsubscript{2}, a lipid mediator, is derived from arachidonic acid, and is involved in several pathophysiological responses such as inflammation, inhibition of gastric acid secretion, pain transmission, neurodegeneration and carcinogenesis.¹ In the PGE\textsubscript{2} synthetic pathway, the isozymes cyclooxygenase (COX)-2 and microsomal PGE synthase-1 (mPGES-1) are induced by the pathophysiological conditions, and the hyperproduced PGE\textsubscript{2} evokes the disease.²,³ Clinically, COX inhibitors, namely non-steroidal anti-inflammatory drugs (NSAIDs), are commonly used for pyretolysis and pain relief,²,³ however, most NSAIDs target constitutive COX-1 and inducible COX-2 and cause side effects such as gastrointestinal toxicity and cardiovascular risk.⁴ On the other hand, a few mPGES-1 inhibitors have been found,⁵ and none have been used clinically. Thus, it would be beneficial to identify natural substances from foods that have safe and functional effects on the regulation of PGE\textsubscript{2} production.

*Dioscorea japonica*, a wild yam, is a relative of the *Dioscoreaeae* family native to Japan. *Dioscoreaeae* yam tubers are usually edible and are rich in many nutrients,⁶ and it has a gastric mucosal protective effect conferred by glycoproteins and digestive enhancement by glycosidase. Recently, we found that *Dioscorea japonica* extract (DJE) suppressed the expression of COX-2 and mPGES-1 in human non-small-cell lung carcinoma A549 cells and colon carcinoma Caco-2 cells, thereby inducing apoptosis in such cells.¹¹ DJE suppresses COX-2 mRNA with translocation of transcriptional factor nuclear factor-κB (NF-κB) to cytosol and a reduction of COX-2 promoter activity.¹¹

In this study, to confirm the effects of *Dioscorea japonica* on inflammation and carcinogenesis via the suppression of COX-2 and mPGES-1 in *vivo* we demonstrate a two-stage cutaneous chemical carcinogenesis model of mouse skin.¹²

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 Auto-rainmu 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.,¹² 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. 1A). 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'-CTTTGACACTTACCACCACC-3' (forward) and 5'-AGCAACACCTCTCTG-3' (reverse); Ptgs2 (COX-2), 5'-GCATTCTTTTCCCCAGC-3T' (forward) and 5'-AGCAACCTCACC-3' (reverse); Ptgds2 [hematopoietic PGD synthase (H-PGDS)], 5'-AGCAAGAGTGGGCTCTCC-3' (forward) and 5'-GCCCATCAGAGGACG-3' (reverse); Ptgds2 [membrane-associated PGD synthase (mPGDS)], 5'-AGCAAGATGGGCTGGTGT-3' (forward) and 5'-GCCCATCAGAGGACG-3' (reverse); Ptgds2 [cytosolic PGD (cPGDS)], 5'-AGCAAGATGGGCTCTCC-3' (forward) and 5'-GCCCATCAGAGGACG-3' (reverse); Ptgds2 [h-PA synthase (H-PA)], 5'-AGCAAGATGGGCTCTCC-3' (forward) and 5'-GCCCATCAGAGGACG-3' (reverse).

Fig. 1. 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 japonica 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. Figure 2 shows tumor formation in the experimental groups including Dioscorea japonica feeding group and DJE topical application group. Throughout the experiment, food intake and body weight were not significant differences among the experimental groups (Fig. 1B 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. 2B and Supplemental Fig. 1A*). However, the tumor volumes were significantly decreased in the Dioscorea japonica feeding and DJE topical application groups (Fig. 2A, C and Supplemental Fig. 1B*). 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. 3), Ptg1 (COX-1), Ptg2 (COX-2), Ptgex (mPGES-1), Ptgex3 (another cytosolic PGE2 synthase, cPGE2), Ptgds2 (PGD2 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, Ptg2, Ptgex and Il1b increased approximately 25-fold compared with the normal control mice. On the other hand, Dioscorea japonica feeding led to the suppression of Ptgex, Ptgex3 and Il1b mRNAs 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 Ptgex3, although the expression of them was induced to approximately 5-fold and 2.5-fold, respectively in the carcinogenic control mice (Fig. 3). On the other hand, the expression of Ptg1 and Ptgex2 was not affected among all experimental groups (Fig. 3). Furthermore, lipid metabolome analysis in mouse skin was performed by liquid chromatography tandem mass spectrometry (ESI-MS, Fig. 4). We confirmed that the main lipid mediator in mouse skin was PGE2, and the production of PGE2 and PGD2 in Dioscorea japonica feeding mice decreased to 63.7% and 28.1%, respectively, compared with the carcinogenic control. DJE topical application also decreased PGE2 and PGD2 production to 62.2% and 28.1%, respectively. Whereas, PGF2α 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. 5). 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. 2. 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. 3. Changes in gene expression. In normal or carcinogenic controls, 10% Dioscorea japonica feeding, and 5 mg 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 *normal controls and *carcinogenic controls.
Counting the cells showed that infiltration. As shown in Fig. 6B, the greatest numbers of neutrophils and eosinophils to determine the effect of 

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. 5). 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 carcinogenesis. Consistent with immunohistochemical analyses, COX-2 co-localized with CD207 (LC marker) and was present both in cancer cells and LCs. The immunoreaction of COX-2, especially in LCs, was stronger than that in cancer cells (Fig. 5 and 6A). In contrast, double immunofluorescent staining for mPGES-1 and CD207, or for mPGES-1 and COX-2 was strongly expressed in epidermal dendritic cells (Langerhans cells, LCs), but mPGES-1 was not observed 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. PGE₂ specific receptors EP1-4 are expressed in normal and tumor epidermis. (24,25) In tumors induced by UVB light exposure and COX-2 overexpression, EPs localize in epidermal hyperplasia. (26,27) The signaling pathways of PGE₂/EP1 and PGE₂/EP3 are involved in immune responses of the skin via T helper type (Th) 1 cells and dendritic cells, respectively. (26,27) Although the presence of EP4 in LCs is immunochemically unknown, PGE₂/EP4 signaling promotes migration and maturation of Langerhance cells, and initiates skin immune responses. (28) Taken together with our results, it is suggested that the produced PGE₂ 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 PGD₂ regulates LCs activity. LCs play a key role in establishment of cutaneous immunity, and participate in squamous cell carcinoma. (12,29) Human dendritic cells including LCs in the skin express H-PGDS, and produce PGD₂ in response to various stimuli. (30) It is thus suggested that the produced PGD₂ 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 PGD₂ receptors, DP and CRTH2 (chemoattractant receptor homologous molecule expressed on Th2 cells). (31,32) In inflammatory processes of the skin, PGD₂ 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 PGD₂ in inflammation and carcinogenesis.

Our recent report demonstrates that Dioscorea japonica suppresses the expression of COX-2 and mPGES-1, and has an anticarcinogenic effect on several model cell lines (reference 11 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 PGE₂ in vivo. Previous studies concerning phytochemical effects on the PGE₂ synthetic pathway report the suppression of COX-2 by resveratrol. (33)
humulon, chrysin, and 6-shogaol and of mPGES-1 by sulforaphane and curcumin. Moreover, we demonstrated that Dioscorea japonica was effective in suppressing both COX-2 and mPGES-1 mRNAs. 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, mouse squamous cell carcinoma and mouse Alzheimer’s disease. Our previous study also showed that diosgenin suppressed COX-2 in A549 cells, 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. 7). Therefore, diosgenin is likely one of

Fig. 5. 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. 6. 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. ND, not detected. See color figure in the on-line version.
the effective substances in the present study, and liposoluble and low molecular diosgenin may have effects via the oral and application routes.

In concluding, our ex vivo and in vivo (present study) results suggest that Dioscorea japonica may have inhibitory effects on inflammation and carcogenesis caused by the hyperexpression of COX-2 and mPGES-1. In the present study, Dioscorea japonica also exerts a preventive effect on squamous cell carcinoma as a refractory skin disease, which has few known specific agents and treatments. Our results on the effects of Dioscorea japonica may pave the way for further therapeutic methods.

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Abbreviations

COX  cyclooxygenase  
cPGES  cytosolic prostaglandin E synthase  
CRTH2  chemotactarctant 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- prostanooid receptor  
EPA  eicosapentaenoic acid  
GAPDH  glyceraldehyde 3-phosphate dehydrogenase  
HE  hematoxylin and eosin  
HETE  hydroxyeicosatetraenoic acid  
H-PGDS  hematopoietic prostaglandin D synthase  
IL  interleukin  
LCs  Langerhans cells  
LPS  lipopolysaccharide  
mPGES-1  microsomal prostaglandin E synthase-1  
mPGES-2  membrane-associated prostaglandin E synthase-2  
MRM  multiple reaction monitoring  
NF-kB  nuclear factor-kB  
NSAIDs  non-steroidal anti-inflammatory drugs  
PG  prostaglandin  
TPA  12-O-tetradecanoylphorbol-13 acetate

Conflict of Interest

No potential conflicts of interest were disclosed.

References

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