

Suppressive Effects of Dietary Genistin and Daidzin on Rat Prostate Carcinogenesis

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High intake of phytoestrogens through soybeans and their products is thought to be associated with low incidences of prostate and/or breast cancer in Asian countries. Possible chemopreventive effects of genistin or daidzin on rat prostate carcinogenesis were therefore investigated. Male F344 rats were given 10 biweekly subcutaneous injections of 3,2'-dimethyl-4-aminobiphenyl (DMAB) and then either genistin or daidzin in the diet at a concentration of 0.1% for 40 weeks. Other groups of rats given DMAB were treated with genistin or daidzin together with a high dose of testosterone propionate (TP). Both genistin and daidzin reduced the numbers of ventral prostate carcinomas ($P < 0.05$), with a tendency for decrease in incidence. Invasive carcinomas which developed in the anterior prostate and seminal vesicles with TP were, however, not influenced by the two isoflavones. Thus, the present data suggest that genistin and daidzin possess anti-cancer effects at relatively early stages of prostate cancer development, providing experimental support for epidemiological findings.

Key words: Rats — DMAB — Prostate carcinogenesis — Genistin — Daidzin

While prostate cancer is the most common internal neoplasm and the second leading cause of cancer death in men in the United States,¹⁾ mortality in Japan from this cause is only two-ninths of that in the United States.²⁾ Intake of large amounts of soybeans and their products rich in isoflavones, such as genistin and daidzin, is believed to play a role in this difference.³⁾ The daily consumption of isoflavones in the diet by Japanese people is estimated to be approximately 18 mg,⁴⁾ and geometric mean plasma individual isoflavonoid concentrations are 7 to 110 times higher in Japanese than in Finnish men.⁵⁾ Animal studies have shown that these two isoflavones inhibit carcinogen-induced tumors of mammary glands^{6,7)} and skin.⁸⁾ Recently, Onozawa *et al.*⁹⁾ reported that a soybean isoflavone mixture consisting of 74% genistein and 21% daidzein suppressed the development of invasive carcinomas of the rat prostate/seminal vesicles.

In vitro studies have revealed that genistein is an inhibitor of protein tyrosine kinase, which modulates several cellular activities and plays an important role in cell proliferation and cell transformation,^{10,11)} and also inhibits mammalian DNA topoisomerase II¹²⁾ as well as angiogenesis.¹³⁾ Genistein and daidzein both have a heterocyclic phenol structure that closely resembles estrogens and enables them to bind to estrogen receptors and to exert weak estrogenic activities.^{14–17)} Normally soybeans and their products

contain glycosides of the isoflavones, genistin and daidzin, and these are deglycosylated by intestinal β -glucuronidase to afford the active forms, genistein and daidzein.

We have established an animal model of prostate cancer using 3,2'-dimethyl-4-aminobiphenyl (DMAB).^{18,19)} This carcinogen induces non-invasive prostate adenocarcinomas limited to the ventral lobe, but invasive and metastasizing adenocarcinomas originating from the dorsolateral, anterior lobes or seminal vesicles also arise when pharmacological doses of testosterone are additionally applied.^{20,21)} In the present experiment, we evaluated the chemopreventive effects of genistin and daidzin on both non-invasive and invasive adenocarcinomas of the prostate in rats given DMAB with/without testosterone.

MATERIALS AND METHODS

Animals Male F344 rats, 5 weeks old, were purchased from Charles River Japan (Atsugi) and maintained in accordance with institutional guidelines in compliance with national and international law. They were housed in plastic cages on hardwood chip bedding in an air-conditioned room with a 12-h light/dark cycle and given food (Oriental MF; Oriental Yeast Co., Ltd., Tokyo) and water *ad libitum*.

Chemicals DMAB was purchased from the NARD Institute (Osaka) and testosterone propionate (TP) was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Genistin and daidzin were products of Fujicco Co., Ltd. (Kobe). Their

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purity was of the order of 95%, the remaining 5% being genistein and daidzein, respectively.

Animal experiments

Long-term experiment: The animals were divided into 10 groups (Fig. 1), 6 groups of 20 rats each and another 4 groups of 10 or 11 each. Rats in groups 1 to 6 were given DMAB subcutaneously at a dose of 50 mg/kg b.w. 10 times at 2-week intervals and those in groups 7 to 10 were given a subcutaneous injection of corn oil, instead of DMAB. From week 20, groups 4, 5, 6, 9 and 10 underwent exogenous hormonal administration for 40 weeks, and 0.1% genistin in diet was given to groups 2, 5, 7 and 9, and 0.1% daidzin in diet to groups 3, 6, 8 and 10. The two isoflavones were stored at room temperature and incorporation was performed every 3 to 4 weeks. TP was introduced into 2-cm-long Silastic tubes (Dow Corning Co., Midland, MI, inner diameter, 2 mm; outer diameter, 3 mm, approximately 40 mg) which were sealed at both ends with Silastic medical grade adhesive (Dow Corning Co.) and implanted into the subcutis of the interscapular region under anesthesia with ethyl ether. The TP-filled implants were replaced at 6-week intervals. All surviving rats were killed at experimental week 60 and subjected to complete autopsy. Before this, blood was collected from the aorta under ether anesthesia from 5 animals of each group for measurement of serum levels of testosterone and estradiol. Animals that died earlier or became moribund were also autopsied. The prostates and seminal vesicles were fixed in cold acetone, and the other organs were examined for gross abnormalities and fixed in 10% buffered formalin. After fixation, the ventral prostate removed from the base of the bladder neck and the entire organ were weighed. For tissue preparation of the accessory sex

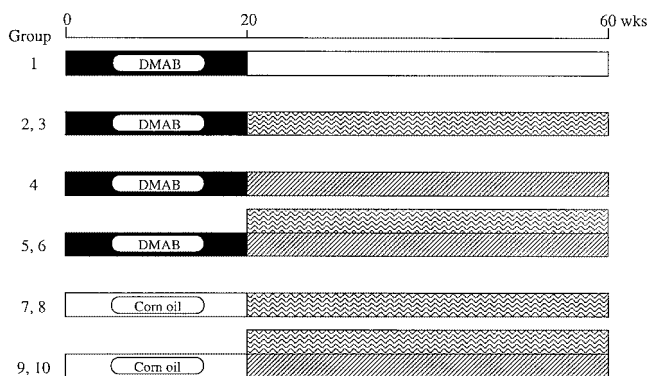


Fig. 1. Experimental protocol I (long-term experiment). Animals used were male F344 rats 6 weeks old at the commencement. ■, 3,2'-dimethyl-4-aminobiphenyl (DMAB) 50 mg/kg b.w. s.c., biweekly; ▨, testosterone propionate (TP), 40 mg in a 2.0 cm long Silastic tube, s.c.; ▩, genistin or daidzin, 0.1% in diet.

organs, two sagittal slices through the ventral prostate, 3 sagittal samples of the dorsolateral prostate including the urethra, and 4 transverse slices from each side of the seminal vesicles including the anterior prostate (coagulating glands) were embedded in paraffin. Single sections (4 μ m) through all tissues were cut and stained with hematoxylin and eosin for histological examination.

For analysis of multiplicity of the ventral carcinomas, areas of ventral prostate epithelium were quantitatively measured with an Image Processor for Analytical Pathology (IPAP, Sumika Technos Co., Osaka).

Differences in body and organ weights and serum levels of hormones were analyzed by means of Student's *t* test. Incidences of tumors and other histopathological lesions were analyzed by means of Fisher's exact probability test (two tailed).

Short-term experiment: Three groups of 5 male F344 rats at 6 weeks of age were given 0.1% genistin or daidzin in the diet for 4 weeks. Paraffin sections of the ventral prostates fixed in 10% buffered formalin were provided for immunohistochemical staining of proliferating cell nuclear antigen (PCNA) for assessment of cell proliferative activity. The number of cells with positively stained nuclei per 1000 epithelial cells was counted and labeling indices were expressed as percentage values. Arterial blood was collected from the aorta under ether anesthesia from animals of each group for determination of serum levels of genistein and daidzein, by radioimmunoassay.

Cell culture and cell proliferation assay The rat prostate carcinoma cell line, PLS10,²²⁾ was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and incubated at 37°C under a humidified 5% CO₂ atmosphere. Cells were seeded into 6-multiwell plates at a density of 5 × 10⁴ cells/2 ml medium, and incubated for 3 days with addition of genistein or daidzein. For assessment of live cells by 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)2H-tetrazolium (WST-1) assay (similar to the 3-(4,5-dimethylthiazol-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay), the medium was changed to 1 ml of phosphate-buffered saline (PBS) containing 100 μ l of WST-1 dye solution (5 mM WST-1, 0.2 mM 1-methoxy-5-methylphenazinium methylsulfate) (Cell counting kit, Wako Pure Chemicals, Co., Osaka) and the plates were incubated for 3 h. The absorbance at 450 nm was measured.

RESULTS

Long-term experiment Ten animals in group 10 died by week 58 without clear reasons. Therefore, the data for this group were excluded from assessment of the results. Administration of DMAB slightly suppressed body weight gain by about 7% at 20 weeks after the start of the study as compared with non-DMAB-treated controls. Treatment

with TP markedly decreased body weights soon after starting the administration and the final body weights were about 27% less than the control values (Table I). Administration of genistin or daidzin did not alter the growth rates of the animals or the final weights of the ventral prostate, liver and kidney. TP significantly increased prostate weights to approximately 3 to 4 times those in the non-TP-treated groups.

Serum testosterone and estradiol data are given in Fig. 2. Subcutaneous implantation of TP-containing Silastic tubes increased the testosterone level to about 5.2 to 8.9 ng/ml while values for non-TP-treated groups were 0.2 to 0.4 ng/ml. No effects of the isoflavones on testosterone levels were evident. Estradiol levels in each group were similar, with no apparent effects of TP or the isoflavones.

As shown in Table II, development of carcinomas of the accessory sex organs in rats treated with DMAB alone was confined to the ventral prostate. TP administration did not

induce tumors in the dorsolateral and anterior prostate or seminal vesicles in the present study. However, with genistin or daidzin, carcinomas of the anterior prostate and seminal vesicles were found at low incidence. The ventral tumors were non-invasive carcinomas while those of the anterior prostate and seminal vesicles were invasive, as observed in previous experiments.^{20, 21} Table III summarizes data for numbers and areas of ventral prostate carcinomas of rats in groups 1 to 3 which survived for the entire experimental period. The incidences in groups 2 and 3 were decreased to about one-third of the control value, but without statistical significance. However, a significant decrease in the number and area of ventral carcinomas in group 2 and the number in group 3 were noted when compared with the control values ($P < 0.05$).

In the groups given DMAB plus TP, no modifying influence of isoflavones was noted (Table II). The isoflavones alone did not induce any proliferative lesions in the

Table I. Final Body and Relative Organ Weights

Group	Treatment	No. of rats	Body weight (g)	Relative organ weights (%)		
				Ventral prostate	Liver	Kidneys
1	DMAB	13	433.5±47.1	0.052±0.015	2.71±0.28	0.60±0.07
2	DMAB → 0.1% genistin	14	452.2±28.1	0.063±0.013	2.72±0.15	0.57±0.03
3	DMAB → 0.1% daidzin	14	427.1±49.5	0.058±0.013	2.68±0.27	0.59±0.05
4	DMAB → TP	5	319.9±43.7	0.204±0.048	2.75±0.17	0.85±0.08
5	DMAB → TP+0.1% genistin	7	321.1±32.8	0.186±0.081	2.95±0.17	0.87±0.07
6	DMAB → TP+0.1% daidzin	5	324.9±48.7	0.208±0.057	2.86±0.17	0.82±0.08
7	Corn oil → 0.1% genistin	10	473.9±15.8	0.062±0.014	2.92±0.16	0.56±0.02
8	Corn oil → 0.1% daidzin	10	465.8±30.5	0.061±0.010	2.79±0.13	0.56±0.04
9	Corn oil → TP+0.1% genistin	5	363.3±33.1	0.193±0.065	3.06±0.39	0.83±0.07

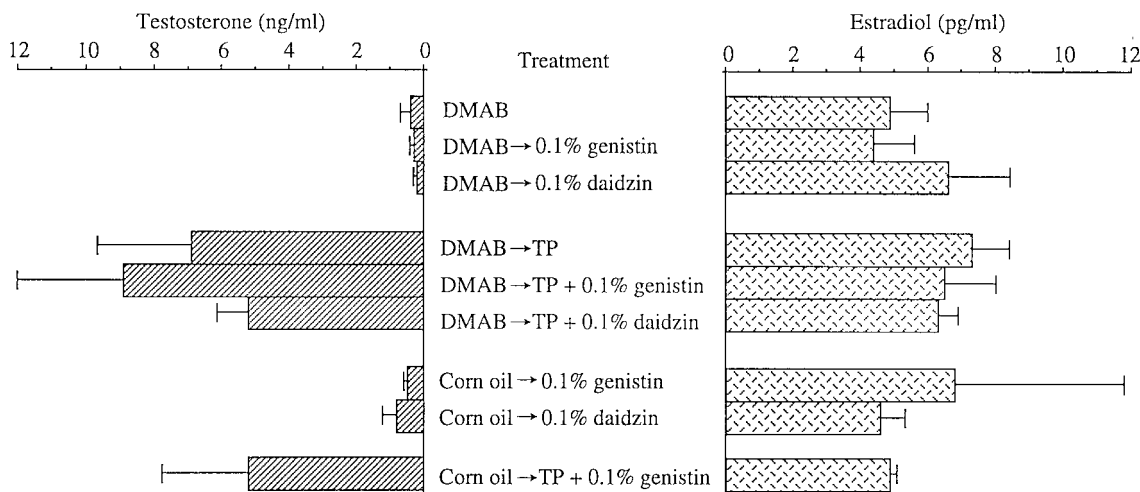


Fig. 2. Effects of genistin and daidzin on serum testosterone and estradiol levels. Data are means±SD.

accessory sex organs. Tumor development was also observed in the small and large intestine, lung, Zymbal's glands and subcutis (data not shown), without modification by the isoflavone administration.

Short-term experiment Administration of the isoflavones for 4 weeks resulted in increased serum levels. However, no significant effect on PCNA labeling indices

in the rat ventral prostate was noted. Similarly, no induction of apoptosis was apparent (data not shown).

Cell proliferation assay Treatment of PLS10 with genistein or daidzein resulted in a significant inhibition of growth in a dose-dependent manner (Fig. 3), genistein showing inhibitory effects at an especially low concentration (3.125 $\mu\text{M}/\text{ml}$).

Table II. Incidences of Neoplastic Lesions in the Prostate and Seminal Vesicles

Group	Treatment	No. of rats	Prostate						Seminal vesicles	
			Ventral		Dorsolateral		Anterior		AH	CA
			AH ^{a)}	CA ^{b)}	AH	CA	AH	CA		
1	DMAB	13	11 (85) ^{c)}	6 (46)	0	0	0	0	10 (77)	0
2	DMAB → 0.1% genistin	14	7 (50)	2 (14)	0	0	0	0	11 (79)	1 (7)
3	DMAB → 0.1% daidzin	14	9 (64)	2 (14)	0	0	0	0	13 (93)	0
4	DMAB → TP	5	1 (20)	0	1 (20)	0	0	0	3 (60)	0
5	DMAB → TP+0.1% genistin	7	2 (29)	0	0	0	1 (14)	1 (14)	6 (86)	0
6	DMAB → TP+0.1% daidzin	5	2 (40)	0	0	0	2 (40)	2 (40)	3 (60)	2 (40)
7	Corn oil → 0.1% genistin	10	0	0	0	0	0	0	0	0
8	Corn oil → 0.1% daidzin	10	0	0	0	0	0	0	0	0
9	Corn oil → TP+0.1% genistin	5	0	0	0	0	0	0	0	0

a) AH: atypical hyperplasia.

b) CA: carcinoma.

c) Percentages in parentheses.

Table III. Multiplicity of the Ventral Prostate Carcinomas

Group	Treatment	No. of rats	Carcinomas	
			No. (/cm ²)	Area (mm ² /cm ²)
1	DMAB	13	3.2±5.3	0.81±1.53
2	DMAB → 0.1% genistin	14	0.5±1.3*	0.08±0.20
3	DMAB → 0.1% daidzin	14	0.4±1.0*	0.18±0.45

* $P < 0.05$ vs. group 1.

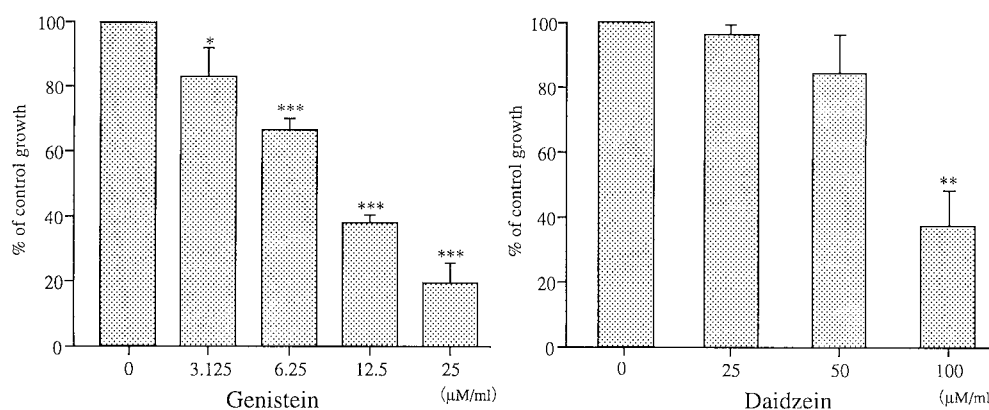


Fig. 3. Effects of genistein or daidzein on growth of PLS10 cells. Cell number was determined by WST-1 assay. Values are means±SD of data from four independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control.

DISCUSSION

In the present experiment, both genistin and daidzin reduced the numbers of ventral carcinomas and tended to depress their incidence. These data on inhibitory effects of genistin and daidzin offer partial support for the epidemiological finding of an inverse association between intake of soybean products and the risk of prostate cancer in Japanese.

However, no effects of the isoflavones were evident against the development of invasive carcinomas with testosterone exposure in other than the ventral prostate. We have previously reported that non-invasive ventral prostate carcinomas are androgen-dependent while invasive carcinomas of the anterior prostate or seminal vesicles are androgen-independent.^{23,24)} On the other hand, genistein and daidzein exhibited dose-dependent inhibition of the growth of androgen-independent PLS10 rat prostate cancer cells *in vitro*, and this is in keeping with previous reports on human prostate cancer cell lines.²⁵⁻²⁸⁾ The data suggest that the mechanism of action of genistin or daidzin depends on the androgen receptor signaling pathway at the low concentrations prevailing under *in vivo* conditions, while an androgen receptor-independent pathway may operate at the higher concentrations possible *in vitro*. A similar phenomenon has been reported with genistein in breast cancer cells.²⁹⁾ Possible reasons for the discrepancy with the finding by Onozawa *et al.*, that a number of isoflavones in combination inhibited rat prostate invasive carcinomas with a similar protocol to that in the present

experiment,⁹⁾ are that their mixture contained genistein, daidzein and glycitein among others, and that they were given throughout the experimental period, including the administration period of DMAB and TP. Genistein and daidzein both exert inhibitory effects, presumably in a competitive manner, on metabolic activation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) by CYP1A2,³⁰⁾ which is thought to activate aromatic amines such as DMAB.³¹⁾ Therefore these isoflavones may inhibit the metabolic activation of DMAB in the initiating stage.

Since no reduction in prostate weights was observed in rats given genistin or daidzin alone for a long period, their estrogenic action must be very weak, because administration of estrogen to male rats act as a chemical castration and markedly reduces the weights of accessory sex organs.³²⁾ The lack of changes in cell kinetic parameters also suggests that genistin and daidzin are not toxic to the prostate or seminal vesicles of male rats. If they affect only tumor cells, these isoflavones might be ideal chemopreventors.

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