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Effects of a Soybean Isoflavone Mixture on Carcinogenesis in Prostate and Seminal Vesicles of F344 Rats

Mizuki Onozawa,[1, 2] Toshihiko Kawamori,[1] Masaki Baba,[1] Kazunori Fukuda,[1] Toshiya Toda,[3] Hidetaka Sato,[4] Mikinobu Ohtani,[2] Hideyuki Akaza,[2] Takashi Sugimura[1] and Keiji Wakabayashi[1, 5]

[1]Cancer Prevention Division, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, [2]Department of Urology, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-0006, [3]Research and Development Laboratory, Fujicco Co., Ltd., 6-13-4 Minatojimanakamachi, Chuo-ku, Kobe, Hyogo 650-0046 and [4]Tama Laboratory, Japan Food Research Laboratories, 6-11-10 Nagayama, Tama City, Tokyo 206-0025

Several epidemiological studies have suggested an inverse association between the risk of prostate cancer and intake of soybeans and their products. *In vitro* data pointing to possible anti-carcinogenic properties of the soybean isoflavone, genistein, led us to investigate the chemopreventive potential of soybean isoflavones in a rat carcinogenesis model induced by 3,2′-dimethyl-4-aminobiphenyl (DMAB) and testosterone propionate (TP). Animals received DMAB s.c. injections at 2-week intervals for the first 20 weeks and implanted silicon tubes containing 40 mg of TP, replaced at 6-week intervals throughout the experiment. The soybean isoflavone mixture consisting of 74% genistein and 21% daidzein was mixed in basal diet (AIN-76A) at concentrations of 100 and 400 ppm and fed to F344 male rats throughout the experiment. Rats treated with carcinogens and administered isoflavone mixture at 100 and 400 ppm developed adenocarcinomas at incidences of 35% and 29%, respectively, in the prostate and seminal vesicles, whereas the figure was 60% for those maintained on control diet. Feeding of the isoflavone mixture at 100 and 400 ppm significantly inhibited the number of argyrophilic nucleolar organizer regions (AgNORs) in adenocarcinomas of the accessory sex glands as compared to those of rats fed control diet. No influence on the development of neoplastic lesions originating in other organs was noted. The results of this study provide evidence that soybean isoflavones may have potential as chemopreventive agents against carcinogenesis in the prostate.

Key words: Prostate cancer — Seminal vesicle cancer — Genistein — Isoflavone — Chemoprevention

Prostate cancer is one of the leading causes of cancer death in males in Western countries, including North America. Over the last 25 years, despite extensive efforts toward earlier detection and treatment, the mortality rate for prostate cancer has steadily increased.[1)] The identification of androgens as the major regulators of epithelial cell proliferation, both in normal and malignant prostate tissues, was originally expected to offer a target for therapeutic intervention.[2, 3)] However, in practice, the concept of "total androgen ablation" therapy has achieved only limited success.[2, 4)] As treatment failures for advanced carcinomas continue to frustrate, more emphasis has recently been focused on possible strategies to prevent the development of invasive prostate cancer.[5)] Several reports concerning chemoprevention of prostate carcinogenesis are available. For example, finasteride, a 5-α reductase inhibitor, which inhibits the conversion of testosterone to dihydrotestosterone, and a casodex, a pure anti-androgen agent, inhibited the development of rat prostate carcinogenesis induced by treatment with a combination of 3,2′-dimethyl-4-aminobiphenyl (DMAB) and testosterone propionate (TP).[6, 7)]

Epidemiological studies suggest that consumption of diets containing soybeans and soybean-related products may reduce the risk of certain types of cancer, including that in prostate.[8)] Soybeans and soybean products are good sources of several phytochemicals, including genistein, that probably reduce cancer in man.[9–11)] Laboratory animal assays have also provided evidence that soybean-related products can inhibit chemically induced carcinogenesis.[10–12)] Genistein is a specific inhibitor of protein tyrosine kinase, which modulates several cellular activities and plays an important role in cell proliferation and cell transformation,[13, 14)] and also has inhibitory effects on mammalian DNA topoisomerase II[15)] and anti-oxidant potential.[16)] Its chemical structure resembles that of estrogenic steroids, and it can bind to the estrogen receptor,

[5] To whom correspondence should be addressed.
E-mail: kwakabay@gan2.ncc.go.jp

although its estrogenic activity is very weak.[12] Our recent study indicated that genistein inhibits the growth of a human prostate cancer cell line (LNCaP) due to suppression of DNA synthesis and induction of apoptosis.[17] Soybean isoflavones, especially genistein, might be expected to play an important role in the prevention of cancer.

In the present study, we evaluated the chemopreventive efficacy of a soybean isoflavone mixture including genistein on carcinogenesis in the prostate and seminal vesicles induced by a combination of DMAB plus TP in rats. Argyrophilic nucleolar organizer regions (AgNORs) have been found to be associated with histologic grade of differentiation,[18, 19] tumor stage,[20] and survival[21] in patients with prostate carcinoma. Therefore, AgNOR counts in cancer tissues were also investigated to elucidate the mechanisms of influence of the isoflavone mixture on carcinogenesis in the prostate and seminal vesicles.

MATERIALS AND METHODS

Chemicals and animals DMAB was purchased from the Nard Institute (Osaka) and TP was from Tokyo Kasei Kogyo (Tokyo). Corn oil was obtained from Nacalai Tesque (Kyoto). Soybean isoflavone mixture was prepared as follows: first, defatted soybeans were extracted with boiled water. The extract was applied to a Sepabeads SP207 column (Mitsubishi Kasei Co., Tokyo), and eluted with methanol. The methanol solution was evaporated and then hydrolyzed with hydrochloric acid to remove glucose from the coexisting glucoside-conjugated form. The soybean isoflavone mixture thus prepared consisted of genistein and daidzein (approximately 74% and 21%, respectively) (Table I). A total of 216 male F344 rats (purchased from Charles River Japan Inc., Atsugi), 5 weeks old and weighing approximately 92 g at the beginning of the experiment, were housed in plastic cages with wood chips in an air-conditioned room with a 12-h light/dark cycle, 24±2°C room temperature, and 55% relative humidity. Rats were given AIN-76A basal diet (Dyets Inc., Bethlehem, PA) or basal diet containing 100 and 400 ppm soybean isoflavone mixture and water *ad libitum*.

Experimental procedure The experiment was performed according to the protocol established by Shirai *et al.*,[22] as shown in Fig. 1. Animals were divided into 6 groups, and s.c. implanted with silicon tubes (Kaneka Medix Co., Osaka) containing 0 and 40 mg of TP under ether anesthesia. The initial implantation of the tubes was carried out at the second week of the experiment, and they were replaced at 6-week intervals thereafter, throughout the experiment. From 1 week after the initial implantation, DMAB dissolved in corn oil was injected into the subcutis of the TP-treated animals at a dose of 50 mg/kg body weight, 10 times at 2-week intervals. Rats receiving tubes without TP were injected with the corn oil vehicle alone. The rats were maintained on control or experimental diets containing 100 and 400 ppm isoflavone mixture until the termination of the experiment. In total, 66 rats were employed for each carcinogen-treatment and 6 rats for the non-carcinogen-treatment groups. Body weights were recorded weekly for the first 8 weeks and then every 4 weeks. Animals were monitored daily for general health and rats in a moribund state were killed before the end of the experiment. At the 50th week, all animals were killed under ether anesthesia and the major organs were examined for gross abnormalities. After laparotomy, the male accessory sex glands including prostate and seminal vesicles were resected and tissues were fixed in 10% buffered formalin. Then, sagittal slices of the ventral prostate, a transverse section of the dorso-lateral prostate, and longitudinal sections of the anterior prostate and the seminal vesicles were embedded in paraffin blocks, cut and stained with hematoxylin and eosin for histological examination. The lesions were diagnosed following the classification of Ito and Shirai.[23]

AgNOR count AgNOR staining was carried out according to the method described previously.[24] The numbers of AgNORs in neoplastic lesions of the prostate and seminal vesicles in each carcinogen-treated group were determined by counting 100 cells in randomly chosen fields and data were expressed as numbers of AgNORs/nucleus.

Statistical analysis Data on body and organ weights were analyzed by one-way factorial ANOVA, and the incidences of neoplastic lesions were analyzed using Armitage's χ^2 test. The numbers of AgNORs/nucleus were analyzed using the unpaired Student's *t* test. Differences were considered statistically significant at $P<0.05$.

RESULTS

General observations Body weights of rats in each group at different time points are shown in Table II. Body weights of rats given the carcinogen were lower than those of animals without carcinogen treatment throughout the experiment. However, no significant influence of the isoflavone mixture was apparent. A total of 124 rats were found dead before the termination of the experiment. There was no significant difference in survival rates among the groups maintained on different concentrations of the isoflavone mixture. These rats were necropsied for gross abnormalities and one, treated with DMAB and TP and maintained on control diet, moribund on the 246th day, was found to have a prostate adenocarcinoma. Therefore, the rats alive after that day were counted in the effective numbers. The mean weights of the prostate and seminal vesicles in each group are shown in Table III. The relative weights of the prostate and the seminal vesicles in rats given carcinogens were greater than the val-

ues in rats without carcinogen treatment. Relative weight gain of the accessory organs due to the TP implantation was evident for the ventral and dorso-lateral prostate.

Tumor incidence in the prostate and seminal vesicles Table IV summarizes data for incidences of neoplastic lesions in the prostate and seminal vesicles. All neoplastic lesions in the prostate and seminal vesicles were diagnosed as atypical hyperplasias or adenocarcinomas. All adenocarcinomas were classified as moderately or well differentiated. Atypical hyperplasias were recognized as very early epithelial lesions showing cellular and structural atypia. The incidences of the neoplastic lesions in the control diet group were comparable to those previously reported.[6, 7, 22]

In the ventral prostate, no adenocarcinomas were found. In the dorso-lateral and anterior prostate, the isoflavone mixture supplement decreased the incidence of adenocarcinomas, but the differences were not statistically significant. In the seminal vesicles, isoflavone mixture at both doses significantly suppressed the development of adenocarcinomas as compared to that of the control diet group. Overall, the incidence of adenocarcinomas in the prostate and seminal vesicles of rats fed control diet was 60%, and those in rats fed isoflavone mixture at doses of 100 and

Table I. Percentage Composition of Isoflavone Mixture

	% composition
Genistein	74.2
Daidzein	20.7
Glycitein	1.6
Genistin	0.4
Others	3.1
Total	100.0

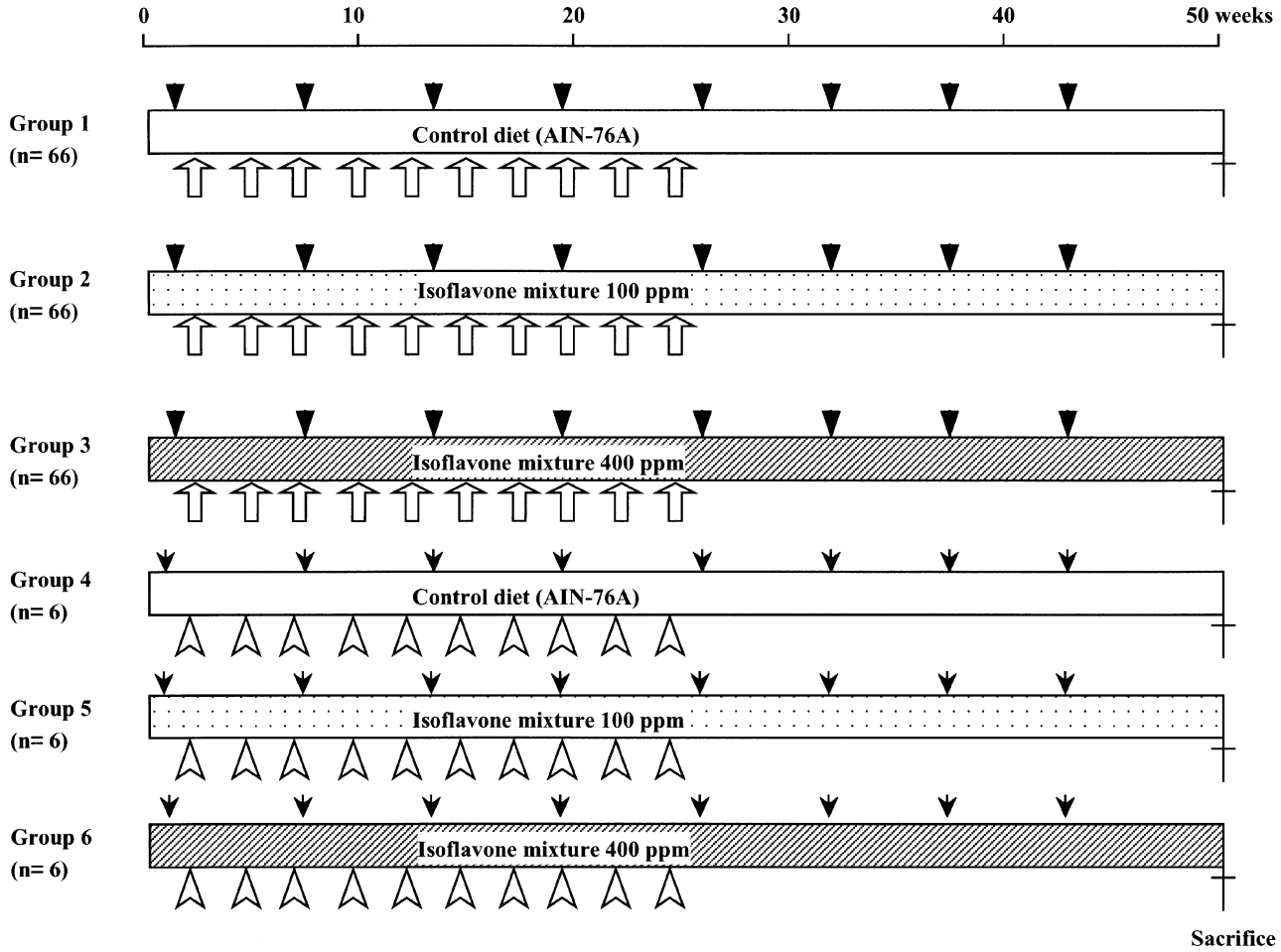

Fig. 1. Experimental protocol. Silicon tube implantation containing 40 mg (⇓) and 0 mg (⇓) of TP. ⇧ 3,2′-dimethyl-4-aminobiphenyl (DMAB) 50 mg/kg b.w. s.c. injection, ⇧ vehicle (corn oil) s.c. injection.

Table II. Body Weights of Rats

Group	Body weight[a] (g) at week					
	0	4	12	24	40	50
Carcinogen-treatment						
Control diet	92±6	184±10	231±28	272±29	294±35	313±26
Isoflavone mixture (100 ppm)	91±5	186±10	236±25	275±26	297±38	314±32
Isoflavone mixture (400 ppm)	92±5	185±10	234±26	276±27	292±30	319±31
Vehicle-treatment						
Control diet	92±5	204±11	288±19	346±26	402±28	418±35
Isoflavone mixture (100 ppm)	92±4	198± 7	294±14	354±27	422±36	445±35
Isoflavone mixture (400 ppm)	92±4	208± 7	308± 7	369±22	415±21	428±20

a) Figures represent mean±SD.

Table III. Effects of the Soybean Isoflavone Mixture on Prostate and Seminal Vesicle Weight in F344 Male Rats

Group	Effective no. of rats	Prostate (g)			Seminal vesicles (g)
		Ventral	Dorso-lateral	Anterior	
Carcinogen-treatment					
Control diet	35	0.53±0.11[a] (0.17)[b]	0.73±0.13 (0.24)	0.20±0.05 (0.07)	1.23±0.50 (0.40)
Isoflavone mixture (100 ppm)	49	0.56±0.13 (0.18)	0.73±0.22 (0.24)	0.19±0.07 (0.06)	1.26±0.68 (0.42)
Isoflavone mixture (400 ppm)	45	0.58±0.12 (0.18)	0.76±0.14 (0.23)	0.21±0.06 (0.07)	1.07±0.37 (0.34)
Vehicle-treatment					
Control diet	6	0.42±0.12 (0.10)	0.70±0.15 (0.17)	0.25±0.05 (0.06)	1.22±0.25 (0.29)
Isoflavone mixture (100 ppm)	6	0.46±0.11 (0.11)	0.66±0.08 (0.15)	0.24±0.06 (0.06)	1.13±0.18 (0.26)
Isoflavone mixture (400 ppm)	6	0.43±0.06 (0.10)	0.60±0.06 (0.15)	0.21±0.04 (0.05)	1.00±0.16 (0.24)

a) Figures represent mean±SD.
b) Figures in parentheses represent percentages of body weights.

Table IV. Effects of the Dietary Soybean Isoflavone Mixture on the Incidences of Neoplastic Lesions in the Prostate and Seminal Vesicles of F344 Rats

Group	Effective no. of rats	Number of animals with neoplastic lesions								
		Prostate						Seminal vesicles		Carcinoma in the prostate and seminal vesicles
		Ventral		Dorso-lateral		Anterior				
		AH[a]	Carcinoma	AH	Carcinoma	AH	Carcinoma	AH	Carcinoma	
Carcinogen-treatment										
Control diet	35	20 (57)[b]	0 (0)	3 (9)	5 (14)	27 (77)	8 (23)	33 (94)	13 (37)	21 (60)
Isoflavone mixture (100 ppm)	49	31 (63)	0 (0)	0 (0)	5 (10)	37 (76)	9 (18)	47 (96)	8 (16)[c]	17 (35)[c]
Isoflavone mixture (400 ppm)	45	13 (29)[c]	0 (0)	1 (2)	1 (2)	36 (80)	6 (13)	45 (100)	6 (13)[c]	13 (29)[d]

a) AH represents atypical hyperplasia.
b) Figures in parentheses represent percentages of tumor incidence.
c, *d*) Significantly different from the control diet group by χ^2 test: *c*) $P<0.05$ and *d*) $P<0.01$.

400 ppm were 35% and 29%, respectively, being significantly lower. The incidence of atypical hyperplasia in the ventral prostate was significantly decreased in rats administered 400 ppm of the isoflavone mixture. No significant differences in the incidences of atypical hyperplasia in dorso-lateral and anterior prostate and seminal vesicles were observed between control and isoflavone mixture diet groups.

able V. Effect of the Soybean Isoflavone Mixture on Numbers f AgNORs in Neoplastic Cells of the Prostate and Seminal Vescles

Group	No. of AgNORs/nucleus[a)]
Carcinogen-treatment	
Control diet	1.82±0.26
Isoflavone mixture (100 ppm)	1.51±0.14[b)]
Isoflavone mixture (400 ppm)	1.35±0.18[c)]

a) Figures represent mean±SD.
b, *c*) Significantly different from the control diet group by Student's *t* test: *b*) $P<0.05$ and *c*) $P<0.01$.

No neoplastic lesions were found in any rats without carcinogen treatment.

AgNOR analysis The data for AgNORs are shown in Table V. Administration of the isoflavone mixture at 100 and 400 ppm significantly decreased the number of AgNORs/nucleus in adenocarcinomas of the prostate and seminal vesicles as compared to the value for the control diet case ($P<0.05$ and $P<0.01$, respectively).

Tumor incidence in other organs Treatment with DMAB plus TP also induced neoplastic lesions in the colon (adenoma/adenocarcinoma), the subcutis (malignant schwannoma), and the mammary glands (fibroma/fibroadenoma/adenoma). The incidences of colonic adenoma and adenocarcinoma were 53% and 22%, respectively, and that of malignant schwannoma of the subcutis was 40%. Mammary gland tumors, including fibroma, fibroadenoma and adenoma, developed with an incidence of 9%. No significant alteration in the incidence of these lesions was caused by the treatment with the soybean isoflavone mixture.

DISCUSSION

In the present study, the soybean isoflavone mixture suppressed the development of adenocarcinoma in the prostate and seminal vesicles, and atypical hyperplasia in the ventral prostate as compared to those of rats administered control diet.

The soybean isoflavone mixture used in the present study was purified from soybeans and consisted mainly of genistein and daidzein. The observed inhibitory effects provide experimental support for the epidemiological finding that high consumption of soybeans and related products may partly account for the lower risk of prostate cancer in Japanese.[12)] Genistein exerts its potential as an anti-estrogen not only by binding to estrogen receptor, but also by stimulating sex hormone-binding globulin production to decrease the free and active hormone in the blood.[12)] Genistein has chemopreventive efficacy against mammary carcinogenesis.[25)] Whether the anti-estrogenic potential of genistein is involved in its suppression of carcinogenesis in the prostate and seminal vesicles of rats remains unclear, but the results of the present study suggest that the isoflavone mixture suppresses cell proliferation in tumors. AgNOR counts per cell correlate well with cell proliferation in several cancers in rodent models[24, 26)] and in humans.[21, 27)] Especially, in prostate cancer, AgNOR has been employed as an indicator of histological grade and prognosis. In our previous study, genistein was shown to inhibit the growth of human prostatic cancer cells (LNCaP) due to suppression of DNA synthesis and induction of apoptosis.[17)] This was also associated with reduced prostate-specific antigen (PSA) expression.[17)] Many other known biological actions of genistein, such as antioxidant effects,[16)] inhibition of tyrosine-specific protein kinase,[13, 14)] inhibition of topoisomerase II[15)] and induction of apoptosis[17)] may also be related to the suppression of neoplastic lesion development in the prostate and seminal vesicles. It is noteworthy that the isoflavone mixture did not appear to cause any toxicity. The available data thus suggest that isoflavone compounds should be evaluated in chemopreventive trials in humans.

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