Int. J. Cancer: **86,** 416–420 (2000)

Publication of the International Union Against Cancer

GENISTEIN ATTENUATES PERITONEAL METASTASIS OF AZOXYMETHANE-INDUCED INTESTINAL ADENOCARCINOMAS IN WISTAR RATS

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The effects of the soybean isoflavonoid genistein on the development of bombesin-enhanced peritoneal metastasis from intestinal adenocarcinomas induced by azoxymethane (AOM) were investigated in male inbred Wistar rats. From the beginning of the experiment, rats were given 10 weekly s.c. injections of AOM (7.4 mg/kg body weight) and s.c. injections of bombesin (40 μg/kg body weight) every other day, and from week 16, s.c. injections of genistein (5 or 10 mg/kg body weight) every other day until the end of the experiment in week 45. Bombesin significantly increased the incidence of intestinal tumors and of cancer metastasis to the peritoneum. Although genistein administered at either dose had little or no effect on the enhancement of intestinal carcinogenesis by bombesin or on the location, histologic type, depth of involvement, labeling index, or growth pattern of intestinal cancers, it significantly decreased the incidence of cancer metastasis. Genistein also significantly decreased the incidence of lymphatic vessel invasion of adenocarcinomas, which was enhanced by bombesin. Our findings indicate that genistein attenuates cancer metastasis by inhibiting cancer cell invasion into lymphatic vessels through activities that do not affect the growth of intestinal cancers. *Int. J. Cancer* 86: 416–420, 2000.

Genistein, an isoflavonoid found in soybeans and soy-based products, is a potent inhibitor of protein kinases (Walter, 1941; Eldridge, 1982) which might be responsible for the lower incidence of prostate cancer in countries where soybeans are a dietary staple (Adlercreutz, 1990). Moreover, consumption of genistein is associated with a lower incidence of metastasis despite a sustained high incidence of organ-confined prostate cancer (Bergan *et al.*, 1996). In experimental studies, genistein inhibits the motility of bladder cancer cells (Theodorescu *et al.*, 1998) and the metastasis to the lung of melanoma cells (Menon *et al.*, 1998). These findings suggest that genistein may be an antimetastatic agent.

We have developed a new metastatic model in rats (Iishi *et al.*, 1992) in which bombesin, a 14-amino-acid peptide originally isolated from the skin of the frog *Bombina bombina*, enhances the peritoneal metastasis of intestinal adenocarcinomas induced by AOM. Using this model, we have investigated the mechanism of cancer metastasis *in vivo* (Iishi *et al.*, 1994, 1995). In the present study, we used this bombesin-induced metastasis model to investigate the effects of genistein on the development of intestinal carcinomas induced by AOM and their metastasis to the peritoneum.

MATERIAL AND METHODS

Animals

One hundred twenty 6 week-old male inbred Wistar rats were purchased from Japan SLC (Shizuoka, Japan). They were housed in suspended, wire-bottomed metal cages in our animal quarters at controlled temperature (20 to 22±°C), humidity (30% to 50%), and light (12-hr cycle), with free access to normal tap water and regular chow pellets (Nihon-Nosan, Yokohama, Japan).

Carcinogen and treatment

Animals were randomly divided into 6 groups of 20 rats each and given weekly s.c. injections of 7.4 mg/kg body weight of AOM (Sigma, St. Louis, MO) in 0.9% NaCl solution for the first 10 weeks and received the following treatments: group 1, the control group, olive oil (vehicle) only; group 2, bombesin only; groups 3 and 4, bombesin and 5 and 10 mg/kg body weight of genistein, respectively; groups 5 and 6, 5 and 10 mg/kg body weight of genistein without bombesin, respectively.

Bombesin (Peptide Institute, Osaka, Japan) at 40 μg/kg body weight and genistein (Sigma) at 5 and 10 mg/kg body weight were prepared as suspensions in olive oil and injected s.c. in a volume of 1 ml/kg body weight every other day. Bombesin and genistein were given between 2 and 3 p.m. at various sites from the start of the experiment and from week 16 until the end of the experiment (week 45), respectively.

Histologic examinations

The first tumor was found in the large intestine of a rat of group 2 killed after becoming moribund in week 38, so rats that survived for more than 38 weeks were included in the effective numbers. Rats were killed when they became moribund due to intestinal obstruction, and surviving rats were killed at the end of week 45. The internal organs of all animals killed during the experiment or in week 45 were carefully examined. The large and small intestines were opened, pinned flat on a cork mat, and fixed with buffered picric-acid-formaldehyde solution. Tumor-bearing areas and areas suspected of bearing lesions were excised and embedded in paraffin. Semi-serial, 5-μm-thick sections were cut to expose the central part of the tumor or the stalk, when present, and were stained with hematoxylin and eosin. In addition to tumors, flat mucosa from each segment of the fixed intestine with no visible tumors was cut into 3-mm-wide strips, which were embedded in paraffin. Thin sections were prepared and examined microscopically for tumor foci. All sections were examined without knowledge of their group of origin.

Definition of intestinal tumors

Adenomas were defined histologically as lesions in which neoplastic cells were confined to the mucosal layer, whereas adenocarcinomas were defined as lesions in which neoplastic cells had penetrated the muscularis mucosae to invade the submucosa or deeper layers. As previously reported (Iishi *et al.*, 1994), adenocarcinomas were further classified as either well-differentiated or mucinous carcinomas.

Peritoneal metastasis

Grades of metastasis of intestinal adenocarcinomas to the peritoneum were classified according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus in Japan (Japanese Research Society for Cancer of the Colon and Rectum, 1998), as follows: P_1, metastatic nodules detectable only over the peritoneum near the primary cancer; P_2, a few

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Received 7 August 1999; Revised 12 November 1999

metastatic nodules also detectable over the peritoneum far from the primary cancer; and P_3, many metastatic nodules also detectable over the peritoneum far from the primary cancer.

Patterns of infiltrating growth of adenocarcinomas

The predominant patterns of infiltrating growth into the surrounding tissue were also classified according to the General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus in Japan (Japanese Research Society for Cancer of the Colon and Rectum, 1998), as follows: α, tumor shows expanding growth and a distinct border with the surrounding tissue; β, tumor growth and borders were intermediate between those of types α and γ; and γ, tumor shows infiltrating growth and an indistinct border with the surrounding tissue.

Determination of labeling indices of cancers

The labeling indices of adenocarcinomas in the large and small intestines were determined in week 45 with an immunohistochemical analysis kit for bromodeoxyuridine (BrdU) incorporation (Becton-Dickinson, Mountain View, CA) (Gratzner, 1982; Morstyn *et al.*, 1983). For the assay, 10 rats in each group were given only tap water for 12 hr. Then rats received 1 ml/kg body weight of olive oil (group 1), 40 μg/kg body weight of bombesin (group 2), 40 μg/kg body weight of bombesin and 5 (group 3) or 10 (group 4) mg/kg body weight of genistein, or 5 (group 5) or 10 (group 6) mg/kg body weight of genistein. One hour later, the animals received an i.p. injection of BrdU (20 mg/kg body weight) and were killed with ether 1 hr later. The large and small intestines were removed and fixed in 70% ethanol for 4 hr. Tumor-bearing large and small intestines were then embedded in paraffin. Sections 3-μm thick were immersed in 2 N HCl solution for 30 min and then in 0.1 M $Na_2B_4O_7$. Slides were also immersed in 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity and then treated with 10% horse serum. Specimens were incubated with an anti-BrdU monoclonal antibody (diluted 1:20) for 2 hr, washed, stained with a biotin-conjugated horse anti-mouse antibody (Vector, Burlingame, CA; dilution 1:200) for 30 min, and treated with avidin-biotin-peroxidase complex (Vector) for 30 min. The reaction product was detected with 3,3′-diaminobenzidine tetrahydrochloride. Cells that contained BrdU were identified by the presence of a dark pigment over their nuclei.

To analyze the labeling indices of intestinal adenocarcinomas, we counted the number of BrdU-labeled and -unlabeled cells among 500 to 1000 cancer cells. On the basis of these measurements, we calculated the labeling index as the percentage of BrdU-labeled cells per total number of cancer cells.

Statistical analysis

Results were analyzed with the chi-square test, Fisher's exact probability test (Siegel, 1956), or one-way analysis of variance with Dunn's multiple comparison (Miller, 1966). Data are shown as means ± SE. Differences were considered significant at a calculated p value of less than 0.05.

RESULTS

Intestinal tumors

All rats survived for more than 38 weeks, so all were included in the effective numbers. In week 45, administration of bombesin or genistein or both had little or no effect on the body weights of rats (Table I).

In group 1 (control), intestinal tumors were found in 10 (50%) of the 20 rats examined. In group 2 (bombesin), the incidence of intestinal tumors was significantly higher than in group 1. Concomitant administration of bombesin and genistein at 5 (group 3) or 10 (group 4) mg/kg body weight had no significant effect on the incidence of intestinal tumors compared with that in group 2. The incidences in rats treated with genistein alone (groups 5 and 6) were not significantly different from that in group 1.

The location of intestinal tumors and the distribution of adenomas and adenocarcinomas did not differ significantly among the 6 groups (Table II).

Peritoneal metastasis

In group 1 (control), no peritoneal metastasis was found in 9 rats bearing intestinal adenocarcinomas (Table III). In group 2 (bombesin), the incidence of peritoneal metastasis was significantly higher than in group 1: peritoneal metastases were found in 12 (92%) of the 13 rats with intestinal cancers. Combined treatment with bombesin and genistein at 5 (group 3) or 10 (group 4) mg/kg body weight significantly decreased the incidence of peritoneal metastasis compared with that in group 2, and the percentage of metastases that were P_1-grade was higher in groups 3 and 4, but not significantly, than in group 2. Treatment with genistein alone at either dosage (groups 5 and 6) had no significant effect on the incidence of peritoneal metastasis compared with that in group 1.

Histo-pathologic features of intestinal cancers

The location, histologic type, depth of involvement, infiltrating growth pattern and venous invasion of intestinal adenocarcinomas did not differ significantly among the 6 groups (Table IV).

In group 1 (control), lymphatic vessel invasion was not found in any of the 11 adenocarcinomas. However, administration of bombesin (group 2) significantly increased the incidence of lymphatic vessel invasion: lymphatic vessel invasion was found in 7 (54%) of 13 adenocarcinomas. Genistein at either dosage (groups 3 and 4) significantly decreased the incidence of lymphatic vessel invasion compared with that in group 2.

Labeling index of intestinal cancers

Although treatment with bombesin (group 2) had no significant effect on the histologic features of intestinal adenocarcinomas, it

TABLE I – BODY WEIGHT AND INCIDENCE OF INTESTINAL TUMORS IN AOM-TREATED RATS

Group number	Treatment[1]	Body weight (g)		Effective number	Number of rats with intestinal tumors (%)
		Initial	Week 45		
1	Control	120 ± 3	354 ± 4	20	10 (50)
2	Bombesin	115 ± 2	347 ± 7	20	20 (100)[2]
3	Bombesin + Genistein 5 mg/kg	119 ± 2	348 ± 10	20	18 (90)[2]
4	Bombesin + Genistein 10 mg/kg	118 ± 2	343 ± 5	20	18 (90)[2]
5	Genistein 5 mg/kg	116 ± 1	338 ± 9	20	9 (45)
6	Genistein 10 mg/kg	117 ± 2	349 ± 7	20	10 (50)

[1]Treatment: Groups 1 and 2, rats were given weekly s.c. injections of AOM for 10 weeks and also s.c. injections of 1 ml/kg body weight of olive oil (group 1) or 40 μg/kg body weight of bombesin (group 2) every other day until the end of the experiment; Groups 3 and 4, rats were given weekly s.c. injections of AOM for 10 weeks and also alternate-day s.c. injections of bombesin until the end of the experiment plus alternate-day s.c. injections of 5 (group 3) or 10 (group 4) mg/kg body weight of genistein from week 16 to the end of the experiment; Groups 5 and 6, rats were given weekly s.c. injections of AOM for 10 weeks and also alternate-day s.c. injections of 5 (group 5) or 10 (group 6) mg/kg body weight of genistein from week 16 to the end of the experiment.–[2]Significantly different from the value for group 1 at $p < 0.01$ (chi-square test).

TABLE II – LOCATIONS AND HISTOLOGIC TYPES OF INTESTINAL TUMORS IN AOM-TREATED RATS

Group number	Treatment[1]	Number of tumors	Location (%)		Histologic type (%)	
			Small intestine	Large intestine	Adenoma	Adeno-carcinoma
1	Control	14	2 (14)	12 (86)	3 (21)	11 (79)
2	Bombesin	21	5 (24)	16 (76)	8 (38)	13 (62)
3	Bombesin + Genistein 5 mg/kg	23	4 (17)	19 (83)	7 (30)	16 (70)
4	Bombesin + Genistein 10 mg/kg	22	5 (23)	17 (77)	4 (18)	18 (82)
5	Genistein 5 mg/kg	11	2 (18)	9 (82)	2 (18)	9 (82)
6	Genistein 10 mg/kg	14	2 (14)	12 (86)	6 (43)	8 (57)

[1]For explanation of treatments, see footnote to Table I.

TABLE III – INCIDENCES AND GRADES OF PERITONEAL METASTASIS OF INTESTINAL CARCINOMAS IN AOM-TREATED RATS

Group number	Treatment[1]	Number of rats with adenocarcinoma	Number of rats with peritoneal metastasis (%)	Grade of metastasis (%)		
				P_1	P_2	P_3
1	Control	9	0 (0)	0 (0)	0 (0)	0 (0)
2	Bombesin	13	12 (92)[2]	2 (17)	4 (33)	6 (50)
3	Bombesin + Genistein 5 mg/kg	14	1 (7)[3]	1 (100)	0 (0)	0 (0)
4	Bombesin + Genistein 10 mg/kg	17	2 (12)[3]	2 (100)	0 (0)	0 (0)
5	Genistein 5 mg/kg	9	1 (11)	0 (0)	0 (0)	1 (100)
6	Genistein 10 mg/kg	8	0 (0)	0 (0)	0 (0)	0 (0)

[1]For explanation of treatments, see footnote to Table I.–[2]Significantly different from the value for group 1 at $p < 0.001$ (Fisher's exact probability test).–[3]Significantly different from the value for group 2 at $p < 0.001$ (Fisher's exact probability test).

TABLE IV – LOCATIONS, HISTOLOGIC TYPES, DEPTHS OF INVOLVEMENT, INFILTRATING GROWTH PATTERNS AND VESSEL INVASIONS OF INTESTINAL CARCINOMAS IN AOM-TREATED RATS

Group number	Treatment[1]	Number of adenocarcinoma	Location (%)		Histology (%)		Depth of involvement (%)		Infiltrating pattern (%)			Vessel invasion (%)	
			Small intestine	Large intestine	Well differentiated	Mucinous	Submucosa or muscle layer	Subsesosa or serosa	α	β	γ	venous	lymphatic
1	Control	11	2 (18)	9 (82)	7 (64)	4 (36)	5 (45)	6 (55)	4 (36)	2 (18)	5 (46)	0 (0)	0 (0)
2	Bombesin	13	4 (31)	9 (69)	6 (46)	7 (54)	5 (38)	8 (62)	2 (15)	4 (31)	7 (54)	0 (0)	7 (54)[2]
3	Bombesin + Genistein 5 mg/kg	16	4 (25)	12 (75)	6 (37)	10 (63)	6 (37)	10 (63)	6 (38)	4 (24)	6 (38)	0 (0)	1 (7)[3]
4	Bombesin + Genistein 10 mg/kg	19	6 (32)	13 (68)	10 (53)	9 (47)	8 (42)	11 (58)	7 (37)	3 (16)	9 (47)	0 (0)	1 (5)[4]
5	Genistein 5 mg/kg	9	2 (22)	7 (78)	4 (44)	5 (56)	4 (44)	5 (56)	1 (11)	3 (33)	5 (56)	0 (0)	0 (0)
6	Genistein 10 mg/kg	8	2 (25)	6 (75)	6 (75)	2 (25)	3 (37)	5 (63)	4 (50)	1 (12)	3 (38)	0 (0)	0 (0)

[1]For explanation of treatments, see footnote to Table I.–[2]Significantly different from the value for group 1 at $p < 0.01$ (Fisher's exact probability test).–[3,4]Significantly different from the value for group 2: [3]$p < 0.02$, [4]$p < 0.01$ (Fisher's exact probability test).

significantly ($p < 0.001$, one-way analysis of variance) increased their labeling indices (44.2 ± 2.0) compared with those in group 1 (30.5 ± 1.1). Concomitant administration of bombesin and genistein at either dosage (groups 3 and 4) had no significant effect on the labeling index of intestinal cancers (45.0 ± 1.8 for group 3 and 45.6 ± 2.2 for group 4) compared with that in group 2.

DISCUSSION

Our results show that bombesin enhances the peritoneal metastasis and lymphatic vessel invasion of intestinal adenocarcinomas induced by AOM and that the soybean isoflavonoid genistein attenuates the bombesin-enhanced peritoneal metastasis and lymphatic vessel invasion of cancers but has little or no effect on the incidence, histologic features, depth of involvement, infiltrating growth pattern, venous invasion, or labeling index of intestinal adenocarcinomas. These results suggest that genistein has antimetastatic activity.

Aprikian *et al.* (1997) reported that bombesin stimulates invasion of PC-3 prostate cancer cells using the Boyden chamber assay, and Saurin *et al.* (1999*a*) reported that bombesin induces cell spreading and lamellipodia formation and stimulates cell adhesion to collagen I in the human colon carcinoma Isreco1 cell line. Moreover, Saurin *et al.* (1999*b*) found that high levels of the mRNA of the receptor of gastrin releasing peptide, which is a mammalian homologue of bombesin, are correlated with the presence of lymphatic vessel invasion in human colon cancers. Although the exact mechanism by which bombesin enhances metastasis of intestinal adenocarcinomas is unclear, bombesin has been reported to increase intracellular calcium concentration $[Ca^{2+}]_i$ (Bold *et al.,* 1996; Saurin *et al.,* 1999*a*) and to activate tyrosine phosphorylation of p125 focal adhesion kinase (FAK) and of integrin-associated proteins (Sinnett-Smith *et al.,* 1993; Aprikian *et al.,* 1997).

Elevated $[Ca^{2+}]_i$ is thought to be related to tumor cell invasion. Imamura *et al.* (1991) found that the *in vitro* invasion of highly invasive cancer cells is initiated by addition of serum, which induces an increase in intracellular pH as well as a transient elevation of $[Ca^{2+}]_i$ and that addition of serum to poorly invasive cancer cells does not increase intracellular pH or $[Ca^{2+}]_i$. We have found that ginsenoside Rg_3 inhibits bombesin-induced metastasis

in rats (Iishi *et al.,* 1997). Shinkai *et al.* (1996) observed that pretreatment with ginsenoside Rg_3 abolishes the transient elevation of $[Ca^{2+}]_i$ induced by l-oleoyl lysophosphatidic acid (LPA) in highly metastatic cancer cells and inhibits their invasion *in vitro*. Gould *et al.* (1995) found that pretreatment with genistein attenuates the histamine-induced increase in $[Ca^{2+}]_i$ and myosin regulatory light chain phosphorylation in intact swine carotid media tissues. These results suggest that genistein may attenuate cancer cell invasion through suppression of $[Ca^{2+}]_i$ elevation.

Stetler-Stevenson *et al.* (1993) reported that the interaction between cancer cells and the extracellular matrix at focal adhesion sites may trigger signals for migration of cells. The signal pathway involved in this interaction was shown to be associated with protein tyrosine phosphorylation and to be mediated by integrins (Clark and Brugge, 1995). Yan and Han (1998) found that genistein suppresses protein tyrosine phosphorylation in response to cell adhesion to the extracellular matrix and subsequently inhibits invasion through the extracellular matrix in melanoma cells.

Imamura *et al.* (1996) found that LPA, at concentrations sufficient to induce cancer cell invasion, transiently increases tyrosine phosphorylation, mainly of 110- to 130-kDa proteins in tumor cells. However, that this LPA-induced invasion is inhibited by genistein suggests that a protein tyrosine kinase is involved in the signal pathway of cancer cell invasion. Imamura *et al.* (1996) also found with an immunoprecipitation technique that p125 FAK is a major protein of 110- to 130-kDa proteins phosphorylated in response to LPA. Tyrosine phosphorylation of paxillin by LPA was also detected. These findings suggest that rho p21-mediated tyrosine phosphorylation of such proteins as FAK and paxillin is a strong candidate for the signal pathway.

Fotsis *et al.* (1993) found that genistein inhibits endothelial cell proliferation and *in vitro* angiogenesis. Shao *et al.* (1998) also found that genistein inhibits angiogenesis in the breast cancer cells xenograft. However, we previously found that bombesin has no significant effects on the tumor vascularity of intestinal adenocarcinomas induced by azoxymethane (Iishi *et al.,* 1997).

It is possible that genistein attenuates bombesin-enhanced cancer metastasis through regulating proteolytic activities of cancer cells. Festuccia *et al.* (1998) found that bombesin induces urokinase-type plasminogen activator and stimulates secretion and activation of matrix metalloproteinase-9 in prostatic tumor cells. Fotsis *et al.* (1993) reported that genistein decreases the activity of urokinase-type plasminogen activator in endothelial cells. Moreover, Shao *et al.* (1998) reported that genistein down-regulates matrix metalloproteinase-9 and up-regulates tissue inhibitor of metalloproteinase-1.

In conclusion, our results show that administration of genistein significantly decreases the incidence of bombesin-enhanced peritoneal metastasis of intestinal adenocarcinomas induced by AOM but does not significantly affect their proliferation.

ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid for the Second-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan and by the Special Coordination Funds for Promoting Science and Technology of the Science and Technology Agency of the Japanese Government.

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