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Triamterene Suppresses Bombesin-enhanced Peritoneal Metastasis of Intestinal Adenocarcinomas Induced by Azoxymethane

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The effects of combined administration of bombesin and the diuretic triamterene on the incidence of peritoneal metastasis of intestinal cancers induced by azoxymethane (AOM) and the labeling index of intestinal cancers were investigated in male inbred Wistar rats. From the start of the experiment, rats were given weekly s.c. injections of AOM (7.4 mg/kg body weight) for 10 weeks and s.c. injections of bombesin (40 μg/kg body weight) every other day, and from week 16, s.c. injections of triamterene (10 and 20 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 cancer metastasis to the peritoneum in week 45. It also significantly increased the labeling index of intestinal cancers. Although administration of both doses of triamterene with bombesin had little or no influence on the enhancement of intestinal carcinogenesis by bombesin, or the location, histologic type, depth of invasion, or labeling index of intestinal cancers, it significantly reduced the incidence of cancer metastasis. These findings indicate that triamterene suppresses cancer metastasis through a mechanism that does not affect the proliferation of intestinal cancers.

Key words: Azoxymethane — Bombesin — Intestinal cancer — Metastasis — Triamterene

We recently reported that the neuropeptide bombesin enhanced the development of colon cancers induced by azoxymethane (AOM) and their metastasis to the peritoneum.[1)] Moreover, we also found that when the diuretic amiloride was administered with bombesin, it significantly reduced the incidence of bombesin-enhanced peritoneal metastasis of intestinal cancers, although it had little or no influence on the location, histologic features, or depth of involvement of intestinal cancers.[2)]

Triamterene, a 6-phenyl-2,4,7-pteridinetriamine, and amiloride are potassium-retaining diuretics of the cycloamidine type.[3)] Both triamterene and amiloride inhibit local transport mechanisms in the distal tubular cells of the kidney which allow the influx of Na^+ and its exchange for K^+ or H^+ ions.[4)] These two drugs are also guanidine analogues.[5)] We anticipated, therefore, that triamterene might also inhibit peritoneal metastasis of intestinal cancers induced by AOM. To investigate this possibility, we examined the effects of triamterene and bombesin on the development of intestinal cancers and their metastasis in Wistar rats.

MATERIALS AND METHODS

Animals Male inbred Wistar rats 6 weeks old were purchased from SLC (Shizuoka). The animals were housed in suspended, wire-bottomed metal cages in our animal quarters at controlled temperature (20 to 22°C) and humidity (30% to 50%), with a 12 h-12 h light-dark cycle. Regular chow pellets (Nihon-Nosan, Tokyo) and tap water were supplied *ad libitum*.

Experimental design All rats were given weekly s.c. injections of 7.4 mg/kg body weight of AOM (Sigma, St. Louis, MO) in 0.9% NaCl solution for 10 weeks. In addition, animals were randomly divided into six groups of 20 rats each which received the following treatments for the duration of the experiment. Group 1, the control group, received injections of the vehicle, plain olive oil, only. Group 2 received injections of 40 μg/kg body weight of bombesin only. Group 3 received injections of 40 μg/kg body weight of bombesin and 10 mg/kg body weight of triamterene. Group 4 received injections of 40 μg/kg body weight of bombesin and 20 mg/kg body weight of triamterene. Groups 5 and 6 received injections of 10 and 20 mg/kg body weight, respectively, of triamterene without bombesin.

Bombesin (Sigma) at 40 μg/kg body weight and triamterene (a gift from Sumitomo Pharmaceuticals Co., Ltd., Osaka) at 10 and 20 mg/kg body weight were prepared as suspensions in olive oil. Injections were given s.c. in a volume of 1 ml/kg body weight between 2 and 3 p.m. every other day, various sites of injection being chosen. Bombesin and triamterene were injected from the start of the experiment and from week 16, respectively.

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Sample collection The first intestinal tumor was found in a rat of group 2 killed in week 35, so rats that survived for more than 35 weeks were included in effective numbers. Rats were killed when they became moribund, and surviving animals 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. Semiserial, 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, while adenocarcinomas were defined as lesions in which neoplastic cells had penetrated the muscularis mucosae to invade the submucosa or deeper layers. As reported previously,[6] adenocarcinomas were further classified as either well-differentiated or mucinous carcinomas. In the former, tumor cells were found in acinar clusters simulating the glandular structures of normal intestinal mucosa. In the latter, mucin secretion was active, resulting in mucinous nodules containing large amounts of extracellular mucin with only a few isolated groups of tumor cells.

Grades of 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,[7] as follows: P_1, metastatic nodules detectable only over the peritoneum near the primary cancer; P_2, a few 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.

Measurement of labeling indices of cancers The labeling indices of large and small intestinal adenocarcinomas were measured in week 45 with an immunohistochemical analysis kit for bromodeoxyuridine (BrdU) incorporation (Becton-Dickinson, Mountain View, CA).[8, 9] For the assay, 10 rats in each group were given only tap water for 12 h. 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 plus 10 mg/kg body weight of triamterene (group 3), 40 μg/kg body weight of bombesin plus 20 mg/kg body weight of triamterene (group 4), 10 mg/kg body weight of triamterene (group 5), or 20 mg/kg body weight of triamterene (group 6). One hour later, the animals received an i.p. injection of BrdU (20 mg/kg body weight) and were killed with ether 1 h later.

To analyze the labeling index 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 number of BrdU-labeled cells per total number of cancer cells.

Statistical analysis Data were analyzed by the χ^2 test, Fisher's exact probability test,[10] or one-way analysis of variance with Dunn's multiple comparison.[11] Data are shown as means$\pm$SE. Differences were considered to be significant at a calculated P value of less than 0.05.

RESULTS

Effects on intestinal tumors Two rats in group 4, 1 in group 5, and 3 in group 6 died before week 32. No intestinal tumors were found in these animals, which

Table I. Body Weight and Incidence and Number of Intestinal Tumors in AOM-treated Rats

Group no.	Treatment	Body weight (g)		Effective no. of rats	No. of rats with intestinal tumors (%)	No. of intestinal tumors per tumor-bearing rat
		Initial	Week 45			
1	Olive oil	152±1	350±17	20	11 (55)	1.3±0.1
2	Bombesin	155±1	303±12	20	20 (100)[b]	2.6±0.3[c]
3	Bombesin + triamterene (10 mg)	152±1	285±13[a]	20	19 (95)[a]	1.8±0.2
4	Bombesin + triamterene (20 mg)	151±1	282±13[c]	18	18 (100)[b]	1.8±0.2
5	Triamterene (10 mg)	153±1	277±15[c]	19	10 (53)	1.9±0.3
6	Triamterene (20 mg)	152±1	293±7[c]	17	9 (53)	1.9±0.3

a, b, c) Significantly different from the value for group 1: *a*) $P<0.05$, *b*) $P<0.02$, *c*) $P<0.01$.

Table II. Location and Histologic Type of Intestinal Tumors in AOM-treated Rats

Group no.	Treatment	No. of intestinal tumors	Location and histology (%)			
			Adenoma		Adenocarcinoma	
			Small intestine	Large intestine	Small intestine	Large intestine
1	Olive oil	14	2 (14)	2 (14)	7 (51)	3 (21)
2	Bombesin	52	5 (10)	21 (40)	11 (21)	15 (29)
3	Bombesin + triamterene (10 mg)	35	2 (16)	12 (34)	8 (23)	13 (37)
4	Bombesin + triamterene (20 mg)	33	3 (9)	11 (34)	12 (36)	7 (21)
5	Triamterene (10 mg)	19	1 (4)	6 (32)	6 (32)	6 (32)
6	Triamterene (20 mg)	17	1 (6)	9 (53)	5 (29)	2 (12)

Table III. Incidence and Grade of Peritoneal Metastasis of Intestinal Adenocarcinomas in AOM-treated Rats

Group no.	Treatment	No. of rats with adenocarcinomas	No. of rats with peritoneal metastasis (%)	Grade of metastasis[a] (%)		
				P_1	P_2	P_3
1	Olive oil	10	0 (0)	0 (0)	0 (0)	(0)
2	Bombesin	17	16 (94)[b]	7 (44)	5 (31)	4 (25)
3	Bombesin + triamterene (10 mg)	19	7 (37)[c]	2 (29)	4 (57)	1 (14)
4	Bombesin + triamterene (20 mg)	18	2 (11)[d]	0 (0)	2 (100)	0 (0)
5	Triamterene (10 mg)	8	1 (13)	0 (0)	0 (0)	1 (100)
6	Triamterene (20 mg)	7	1 (14)	0 (0)	1 (100)	0 (0)

a) Grade of metastasis: P_1, metastatic nodules detectable over the peritoneum near the primary cancer; P_2, a few metastatic nodules detectable over the peritoneum far from the primary cancer; P_3, many metastatic nodules detectable over the peritoneum far from the primary cancer.
b) Significantly different from the value for group 1 at $P<0.001$.
c, d) Significantly different from the value for group 2: *c*) $P<0.01$, *d*) $P<0.001$.

were excluded from the effective numbers. In week 45, rats that had received triamterene with and without bombesin had significantly lower body weights than those in control group 1 (Table I).

The incidences of intestinal tumors and their numbers per tumor-bearing rat in each group are summarized in Table I. In group 1 (olive oil), intestinal tumors were found in 11 (55%) of 20 rats examined, and the average number of tumors per tumor-bearing rat was 1.3 ± 0.1. In group 2 (bombesin alone), the incidence of intestinal tumors and their number per tumor-bearing rat were significantly higher than in group 1. Concomitant administration of bombesin and triamterene (groups 3 and 4) had no significant influence on the incidence of intestinal tumors compared with that in group 2. The average numbers of intestinal tumors per tumor-bearing rat in groups 3 and 4 were slightly higher than that in group 1 and slightly lower than that in group 2, but the differences were not statistically significant. The incidence and average number of intestinal tumors per rat in rats treated with triamterene alone (groups 5 and 6) did not differ significantly from those of rats in group 1.

The locations and histologic types of intestinal tumors in each group are summarized in Table II. There were no significant differences in the locations of intestinal tumors or in the distributions of adenomas and adenocarcinomas in the six groups.

Effects on peritoneal metastasis The incidences and grades of peritoneal metastasis of intestinal adenocarcinomas are summarized in Table III. In group 1 (olive oil), no metastases were found in 10 rats with intestinal adenocarcinomas. In group 2 (bombesin alone), the incidence of peritoneal metastasis was significantly higher than in group 1: peritoneal metastasis was found in 16 (94%) of 17 cancer-bearing rats. Concomitant administration of bombesin and triamterene at both dosages

Table IV. Histologic Type, Depth of Involvement and Labeling Index of Intestinal Adenocarcinomas in AOM-treated Rats

Group no.	Treatment	No. of adeno-carcinomas	Histology (%)		Depth of involvement (%)		Labeling index (%)
			Well-differ-entiated	Muci-nous	sub-mucosa	Muscle layer or deeper	
1	Olive oil	10	5 (50)	5 (50)	4 (40)	6 (60)	34.2±1.7
2	Bombesin	26	16 (62)	10 (38)	10 (38)	16 (62)	53.6±4.4[a]
3	Bombesin + triamterene (10 mg)	21	13 (62)	8 (38)	6 (29)	15 (71)	54.6±3.0[a]
4	Bombesin + triamterene (20 mg)	19	12 (63)	7 (37)	3 (16)	16 (84)	53.8±2.3[a]
5	Triamterene (10 mg)	12	9 (75)	3 (25)	4 (33)	8 (67)	33.8±2.8
6	Triamterene (20 mg)	7	6 (86)	1 (14)	4 (57)	3 (43)	34.6±2.1

a) Significantly different from the value for group 1 at $P<0.001$.

(groups 3 and 4) significantly reduced the incidence of peritoneal metastasis compared with that in group 2. Treatment with both doses of triamterene alone (groups 5 and 6) had no significant influence on the incidence of metastasis compared with that in group 1.

In group 2 (bombesin), many metastatic nodules were found far from the primary adenocarcinomas in 4 (25%) of 16 rats with peritoneal metastasis. However, in groups 3 and 4 (bombesin and triamterene at both dosages), a few metastatic nodules were detected far from or near the primary cancers. No metastases to the liver or lung were found macroscopically or microscopically.

Effects on histologic features and labeling index of cancers The effects of bombesin and triamterene on the histologic type, depth of involvement and labeling index of intestinal adenocarcinomas are summarized in Table IV. Although treatment with bombesin alone (group 2) had no significant influence on the histologic features of intestinal cancers, it significantly increased their labeling index. Administration of bombesin and both dosages of triamterene (groups 3 and 4) had no significant influence on either the histologic features or labeling index of intestinal adenocarcinomas compared with those in group 2.

DISCUSSION

Our present study shows that bombesin enhances the development and peritoneal metastasis of intestinal adenocarcinomas and that triamterene attenuates the enhancement of peritoneal metastasis induced by bombesin, although it has little or no influence on the incidence, histologic features, or labeling index of intestinal cancers. These results suggest that triamterene has an antimetastatic effect.

AOM induces tumors in both small and large intestines. Bombesin also enhances both small and large intestinal carcinogenesis. The observed phenomenon of suppression of peritoneal metastasis is thought to be common to small and large intestinal cancers. Previously, we reported that an ornithine decarboxylase inhibitor, 1,3-diaminopropane, attenuated bombesin-enhanced peritoneal metastasis of intestinal adenocarcinomas induced by AOM.[6] In that series, there was no significant difference in the location of intestinal adenocarcinomas which metastasized to the peritoneum between the group treated with bombesin alone and that treated with bombesin and 1,3-diaminopropane.

Although the exact mechanism by which triamterene attenuates peritoneal metastasis of intestinal adenocarcinomas is unclear, two possible explanations may be considered. One is the effect of triamterene on cancer cell proliferation. Corcino *et al.*[12] showed that triamterene interferes with *de novo* DNA synthesis in short-term human bone marrow cultures. They also found that bombesin's mechanism of action was similar to that by which methotrexate inhibits dehydrofolate reductase. However, Chang and Hall[13] showed that triamterene does not suppress ^{3}H-deoxyuridine incorporation into DNA, and minimally inhibits ^{14}C-thymidine incorporation into DNA in L1210 mouse ascites leukemia cells. Furthermore, triamterene does not influence ^{14}C-uridine incorporation into RNA. These results suggest that triamterene has no effect on DNA synthesis. In the present study, we also found that prolonged administration of triamterene did not influence labeling index of intestinal adenocarcinomas.

A second possible explanation is the effect of triamterene on intracellular calcium ($[Ca^{2+}]_i$). The exact mechanism by which bombesin enhances cancer metastasis is unclear. Bombesin increases $[Ca^{2+}]_i$ in various cell types.[14] Elevated $[Ca^{2+}]_i$ is thought to be closely related to cancer cell invasion.[5] Imamura *et al.*[15] found that serum is required for the *in vitro* invasion of a highly

invasive clone, MM1. Addition of serum to MM1 cell suspension induces an increase in the intracellular pH (pH_i) as well as a transient elevation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$ spike), whereas addition of serum to a poorly invasive clone results in no pH_i change or $[Ca^{2+}]_i$ spike.

Loßnitzer *et al.*[16] examined the effects of triamterene on isoproterenol-induced calcium accumulation in the myocardium of cardiomyopathic hamsters. Concomitant administration of triamterene with the standard dose of isoproterenol decreases the isoproterenol-induced calcium accumulation in a dose-dependent manner. Ogawa[5] examined the effect of triamterene on the $[Ca^{2+}]_i$ spike and the invasive capacity of rat ascites hepatoma cells AH130. He found that triamterene suppresses the $[Ca^{2+}]_i$ spike induced by addition of serum and subsequently suppresses the invasive capacity of a highly invasive clone, MM1, in a dose-dependent manner. These results suggest that triamterene may attenuate cancer cell invasion through suppression of the $[Ca^{2+}]_i$ spike.

Previously, we found that the diuretic amiloride attenuated bombesin-enhanced peritoneal metastasis of intestinal cancers induced by AOM.[2] Amiloride and triamterene are guanidine analogues.[5] Furthermore, amiloride is also supposed to inhibit cancer cell invasion by suppression of the $[Ca^{2+}]_i$ spike.[5] Therefore, the possible mechanism for the suppression of cancer metastasis is common in these two diuretic drugs.

Our present work shows that administration of triamterene significantly suppresses the incidence of bombesin-enhanced peritoneal metastasis of intestinal adenocarcinomas, although it does not significantly influence bombesin-enhanced intestinal tumorigenesis. These findings indicate that triamterene has an antimetastatic action, which may be mediated through decreased $[Ca^{2+}]_i$ levels.

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