Original article

Induction by bombesin of peritoneal metastasis of gastric cancers induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in Wistar rats

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Abstract

Background. We investigated the effect of the gastrointestinal regulatory peptide, bombesin, on the development of peritoneal metastasis from gastric cancers induced in rats by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and on Rho activity in the gastric cancers.

Methods. Rats were allocated to three groups. All groups received MNNG (100 µg/ml) solution for 25 weeks from the start of the experiment. Group 1 (controls) received olive oil injections from the start of MNNG treatment; group 2 animals received alternate-day s.c. injections of bombesin (40 µg/kg body weight) in olive oil from the start of the experiment until the end of the experiment at week 52; and group 3 received the s.c. bombesin injection on alternate days from week 26 until week 52. The effect of bombesin on Rho activity in gastric cancer was examined by Western blotting.

Results. Bombesin given from the start of the experiment (group 2) and after the MNNG treatment (group 3) both significantly increased the incidence of gastric cancer metastasis, compared with controls, at week 52: The incidence of metastasis was significantly higher in group 2 than in group 3. Bombesin from the start of the experiment (group 2) significantly increased the incidence of tumors with deeper invasion or more infiltrative growth pattern, or lymphatic vessel tumor invasion, while bombesin after MNNG treatment (group 3) significantly increased the incidence of lymphatic vessel invasion. Bombesin also increased the activity of Rho protein in the tumors.

Conclusion. Bombesin significantly increased the incidence of peritoneal metastasis from gastric cancers through the activation of Rho protein.

Key words Peritoneal metastasis · Gastric cancer · Bombesin · *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine · Animal model

Introduction

In chemically induced cancers, distant metastasis to lymph nodes, lungs, liver, or peritoneum is very rare, and, therefore, metastatic models have been created in animals by the transplantation of cancer cells. However, the modes of metastasis in these experimental models in animals are quite different from the modes of metastasis in humans.

We previously reported a new metastatic model in animals [1]. Administration of the gastrointestinal regulatory peptide, bombesin, significantly increased the incidence of metastasis to the peritoneum from intestinal adenocarcinomas induced by azoxymethane. This bombesin-induced peritoneal metastasis was very similar to metastasis in humans.

Bombesin is a 14-amino-acid peptide, originally isolated from the skin of the frog *Bombina bombina* [2]. In mammals, bombesin-like activity has been reported in the gastrointestinal tract, and bombesin-like peptides are considered to be putative neurotransmitters [3–5]. Bombesin has important roles in the physiology and pathology of the gastrointestinal system [5–7]. Previously, we [8] found that bombesin enhanced gastric carcinogenesis in Wistar rats after the administration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was stopped. These findings suggested that bombesin might induce the development of peritoneal metastasis from gastric cancers. To investigate this possibility, we examined the effect of bombesin, administered from the start of the experiment, or after MNNG treatment for 25 weeks, on the development of peritoneal metastasis from gastric cancers induced by MNNG in Wistar rats.

Materials and methods

Animals

Seventy-five inbred male Wistar rats (6 weeks old), purchased from Japan SLC (Shizuoka, Japan), were housed in suspended, wire-bottomed cages in the animal quarters at a controlled temperature (20°C to 22°C) and humidity (30% to 50%), with a 12-h 12-h light-dark cycle. Regular chow pellets (Nihon-Nosan, Yokohama, Japan) were available ad libitum.

Experimental design

The animals were randomly divided into three groups of 25 rats each. Each group was given drinking water containing MNNG (100 µg/ml; Aldrich Chemical, Milwaukee, WI) for 25 weeks. The MNNG was dissolved in deionized water at a concentration of 1 mg/ml and kept in a cool, dark place. The stock solution was diluted to 100 µg/ml with tap water immediately before use. Each rat was given MNNG solution from a bottle covered with aluminum foil to prevent MNNG photolysis, and the solution was replenished every other day.

Until the end of the experiment at week 52, the groups received the following treatments: group 1 (the control group) received injections of olive oil only, from the start of MNNG treatment; group 2 received injections of bombesin from the start of the MNNG treatment; and group 3 received injections of bombesin given after 25 weeks of MNNG treatment.

Bombesin (Sigma Chemical, St. Louis, MO, USA), at 40 µg/kg body weight, was prepared as a suspension in olive oil. Injections were given s.c. at various sites, in a volume of 1 ml/kg body weight, between 2 and 3 p.m. every other day.

Histologic sampling

One rat in group 1 was killed at week 30. No gastric tumor was found in this rat and, therefore, this rat was excluded from the analysis of results. All surviving animals were killed at the end of week 52. The internal organs of all animals killed at week 52 were carefully examined. The stomach was opened along the greater curvature and pinned flat on a cork mat. When tumors were observed, the upper half of the tumor was excised for Rho examination and frozen in liquid nitrogen. The stomach was then fixed with a buffered picric acid-formaldehyde solution for histologic examination. The fixed stomach was cut into 3-mm-wide longitudinal strips. The specimens were embedded in paraffin, and semiserial, 5-µm-thick, sections were stained with hematoxylin and eosin. All sections were examined without knowledge of the groups to which they belonged.

Definition and classification of gastric cancers

Histologically, adenocarcinomas were defined as lesions in which neoplastic cells had penetrated the muscularis mucosae to invade the submucosa or deeper layers. As previously reported [9], adenocarcinomas were classified as very well differentiated, well differentiated, or poorly differentiated.

Definition and grades of peritoneal metastasis

Peritoneal metastasis was defined as the presence of cancer cells in the extra-gastric peritoneum, but no cancer cells in the gastric peritoneum (serosa). Grades of peritoneal metastasis from gastric adenocarcinomas were classified as follows [10]: P₁, metastases to the adjacent peritoneum but not the distant peritoneum; P₂, a few metastases to the distant peritoneum; and P₃, numerous metastases to the distant peritoneum.

Patterns of infiltrating growth of gastric cancers

The predominant patterns of infiltrating growth into the surrounding tissue were classified as follows [10]: α, the tumor showed expanding growth and a distinct border from the surrounding tissue; β, this category was between types α and γ; and γ, the tumor showed infiltrating growth and an indistinct border from the surrounding tissue.

Assay for guanosine triphosphate (GTP)-bound cellular Rho

The effect of bombesin on the activity of Rho in the gastric cancer tissue was examined using the method of Ren et al. [11]. Five cancer tissue specimens from each of groups 1, 2, and 3 were homogenized with RIPA buffer (50mM Tris, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate [SDS], 500mM NaCl, 10mM MgCl₂, 10 µg/ml each of leupeptin and aprotinin, and 1mM phenylmethylsulfonylfluoride [PMSF]). Tissue lysates were clarified by centrifugation at 13000 g at 4°C for 10 min, and were incubated for 45 min at 4°C with glutathione beads (Amersham Pharmacia Biotech, Uppsala, Sweden) coupled with bacterially expressed GST-RBD (Rho-binding domain of Rhotekin) fusion protein (provided by Martin A. Schwartz, Scripps Research Institute, La Jolla, CA, USA). The beads were washed four times with buffer B (Tris buffer containing 1% Triton X-100, 150mM NaCl, 10mM MgCl₂, 10 µg/ml each of leupeptin, and 0.1mM PMSF). Bound Rho proteins were detected by Western blotting, using a monoclonal antibody against Rho A (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein extracted from group 1

Table 1. Incidence and number of gastric cancers in MNNG-treated rats

Group no.	Treatment ^a	No. of rats analyzed	No. of rats with gastric cancers (%)	No. of gastric cancers per cancer-bearing rat
1	Olive oil	24	23 (96)	1.6 ± 0.2
2	Bombesin from the start of MNNG treatment	25	25 (100)	2.1 ± 0.3
3	Bombesin after MNNG treatment	25	22 (88)	1.6 ± 0.5

^aTreatment; rats were given alternate-day s.c. injections of 1 ml/kg of olive oil from the start of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment (group 1), or alternate-day s.c. injection of 40 µg/kg body weight of bombesin from the start of MNNG treatment (group 2); or alternate-day s.c. injection of 40 µg/kg body weight of bombesin after 25 weeks of MNNG treatment (group 3) until the end of the experiment at week 52

Table 2. Incidence and grade of peritoneal metastasis of gastric adenocarcinomas in MNNG-treated rats

Group no.	Treatment ^a	No. of rats with gastric cancers	No. of rats with peritoneal metastasis (%)	Grade ^b (%)		
				P1	P2	P3
1	Olive oil	23	0 (0)	0 (0)	0 (0)	0 (0)
2	Bombesin from the start of MNNG treatment	25	17 (68) ^{c,e}	9 (53)	5 (29)	3 (18)
3	Bombesin after MNNG treatment	22	7 (32) ^d	4 (57)	2 (29)	1 (14)

^aFor explanation of treatments, see Table 1

^bGrade of metastasis; P1, metastatic nodules detectable over the peritoneum near the primary cancer; P2, a few metastatic nodules detectable over the peritoneum distant from the primary cancer; P3, many metastatic nodules detectable over the peritoneum distant from the primary cancer

^{c,d}Significantly different from the value for group 1: ^c $P < 0.001$; ^d $P < 0.02$

^eSignificantly different from the value for group 3: $P < 0.05$

cancer tissue with RIPA buffer, but not coupled with GST-RBD fusion protein was used as control Rho A.

Statistical analysis

Results were analyzed using the χ^2 test, Fisher's exact probability test [12], or one-way analysis of variance with Dunn's multiple comparison [13]. Values are shown as means ± SE. Differences were considered significant at a calculated P value of less than 0.05.

Results

Incidence and number of gastric cancers

At week 52, bombesin administration had no significant effect on the body weight of rats.

In the controls (group 1; olive oil alone), gastric cancers were found in 23 (96%) of the 24 rats examined. However, the administration of bombesin in groups 2 and 3 had no significant effect on the incidence of gastric cancers compared with that in group 1. The number of gastric cancers per tumor-bearing rat in group 2 (bombesin from the start of MNNG treatment) was slightly, but not significantly, greater than that in group 1 (Table 1).

Incidence and grade of peritoneal metastasis

In the controls (group 1; olive oil alone), no peritoneal metastasis of gastric cancer was found in the 23 rats bearing gastric cancers (Table 2). In groups 2 (bombesin from the start of MNNG treatment) and 3 (bombesin after MNNG treatment), peritoneal metastasis was found in 17 (68%) of the 25 cancer-bearing rats and in 7 (32%) of the 22 cancer-bearing rats, respectively (Table 2; Fig. 1). The incidence of peritoneal metastasis in groups 2 and 3 was significantly higher than that in group 1. There was also a significant difference in the incidence of peritoneal metastasis between groups 2 and 3. However, no significant difference was found in the grades of peritoneal metastasis between groups 2 and 3.

Effects of bombesin on histologic features of gastric cancers and Rho activity

There was no significant difference in the histologic type of gastric cancers among the three groups (Table 3). However, prolonged administration of bombesin from the start of MNNG treatment (group 2) significantly increased the incidence of muscle layer or deeper cancers and lymphatic vessel invasion, and significantly decreased the incidence of infiltrating growth pattern type

Fig. 1a–c. Induction of peritoneal metastasis by bombesin from gastric cancers. **a** Microphotograph shows many metastatic nodules on the omentum. **b,c** Microphotographs of peritoneal metastasis. **a** $\times 5$; **b** $\times 20$; **c** $\times 100$

α compared with group 1. Bombesin administration after MNNG (group 3) also significantly increased the incidence of lymphatic vessel invasion.

Administration of bombesin increased the activity of Rho in gastric cancer (Fig. 2). However, there were no differences in Rho activity between groups 2 and 3.

Discussion

Previously, we reported that the administration of bombesin, a 14-amino-acid peptide, significantly increased the incidence of metastasis to the peritoneum from intestinal adenocarcinomas induced by azoxymethane [1]. In another study, we found that long-term administration of bombesin after MNNG treatment significantly increased the incidence of gastric cancers induced by MNNG [8]. However, in a gastric cancer model, peritoneal metastasis from the gastric cancers was observed in only one (7%) of 15 tumor-bearing rats (data not shown). In that study series, the drinking water contained 50 $\mu\text{g/ml}$ MNNG, and most of the induced gastric cancers were submucosal, but not deeper. In the present study, we used 100 $\mu\text{g/ml}$ MNNG solution and we found that bombesin, whether given from the start of the experiment or after the MNNG treatment, significantly increased the incidence of peritoneal metastasis from the gastric cancers: the incidence being 68% with the former treatment and 32% with the latter. This bombesin-induced peritoneal metastasis was similar to metastasis in humans.

Although the precise mechanisms by which bombesin induces peritoneal metastasis of gastric cancers are unclear, at least two possible explanations may be considered. One possibility is the effect of bombesin on intracellular calcium. Bombesin induced a transient rise in cytoplasmic calcium levels in various normal and tumor cells [14–20]. Elevated intracellular calcium is thought to be closely related to tumor cell invasion. Imamura et al. [21] found that the addition of serum to a suspension of highly invasive tumor cells induced an increase in the intracellular pH, as well as a transient elevation of intracellular calcium, whereas the addition of serum to a poorly invasive clone did not result in changes in intracellular pH or calcium. Moreover, we [22] found that the addition of 1-oleoyl-lysophosphatidic acid to rat ascites hepatoma cells resulted in an instantaneous increase in intracellular calcium, but when these cells were pretreated with the antimetastatic agent, ginsenoside Rg3, the intracellular calcium spike induced by 1-oleoyl-lysophosphatidic acid was completely abolished. Hellmich et al. [23] found that bombesin induced either intracellular calcium oscillations or biphasic elevation in intracellular calcium, and that the activation of mitogen-activated protein kinase (MAPK) kinase (MEK) was important for maintaining the elevated intracellular calcium levels induced by bombesin. The MAPK-MEK cascade was closely related to the tumor cell invasion [24]. However, we did not examine the intracellular calcium level induced by bombesin in this animal model.

The second possibility is the effect of bombesin on the low-molecular-weight G protein, Rho. The Rho

Table 3. Effects of bombesin on histologic type, depth of involvement, infiltrating growth pattern, and vessel invasion of gastric cancer

Group no.	1	2	3
Treatment ^a	Olive oil	Bombesin from the start of MNNG treatment	Bombesin after MNNG treatment
No. of gastric cancers	37	53	36
Histologic type (%)			
Very well differentiated	6 (16)	16 (30)	5 (14)
Well differentiated	31 (84)	37 (70)	31 (86)
Depth of involvement (%)			
Submucosa	31 (84)	33 (62)	25 (69)
Muscle layer or deeper	6 (16)	20 (38) ^c	11 (31)
Infiltrating growth ^b (%)			
α	29 (78)	20 (38) ^{d,f}	25 (69)
β	7 (19)	22 (42)	10 (28)
γ	1 (3)	10 (28)	1 (3)
Vessel invasion (%)			
Venous	0 (0)	0 (0)	0 (0)
Lymphatic	0 (0)	24 (45) ^{c,g}	8 (22) ^d

^aFor explanation of treatments, see Table 1

^bPattern of infiltrating growth: α , cancer shows expanding growth and a distinct border from the surrounding tissue; β , this category is between types α and γ ; γ , cancer shows infiltrating growth and an indistinct border from the surrounding tissue

^{c,d,e}Significantly different from the value for group 1: ^c $P < 0.001$; ^d $P < 0.01$; ^e $P < 0.05$

^{f,g}Significantly different from the value for group 3: ^f $P < 0.02$; ^g $P < 0.05$

Fig. 2. Rho activation by bombesin in gastric cancer (Western blot). Samples were obtained from each rat in group 1 (olive oil) and group 2 (bombesin from the start of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment). The *fifth* line shows control Rho activity

family of guanosine triphosphatases (GTPases) play an important role in the control of cell shape, adhesion, movement, and growth [25]. Bombesin rapidly stimulates the formation of focal adhesions and actin stress fibers [26,27], and stimulates the tyrosine phosphorylation of multiple proteins, including p125 focal adhesion kinase (FAK) and paxillin [28–30]. These processes were regulated by the small GTP binding protein Rho; *Clostridium botulinum* C₃ exoenzyme treatment of cells caused a marked decrease in bombesin-stimulated tyrosine phosphorylation of p125 FAK and paxillin [28]. Imamura et al. [31] reported that the Rho/ROCK (Rho-associated coiled-coiled forming protein kinase) pathway played central roles in the invasion and phagokinetic movement of rat ascites hepatoma (MM1) cells, in cooperation with integrin. In the present study, we examined the effect of bombesin on Rho activity in gastric cancer tissue, and found that bombesin enhanced the activity of Rho protein.

In conclusion, bombesin significantly increased the incidence of peritoneal metastasis from gastric cancers through the activation of Rho protein. This animal model of peritoneal metastasis should be useful for the investigation of cancer metastasis.

Acknowledgments This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for the Second-Term Comprehensive 10-Year Strategy for Japan.

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