[CANCER RESEARCH 44, 4276–4280, October 1984]

Effects of Gastrin on Tumor Growth and Cyclic Nucleotide Metabolism in Xenotransplantable Human Gastric and Colonic Carcinomas in Nude Mice[1]

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ABSTRACT

This study deals with the growth effect of gastrin on two xenotransplantable human gastric carcinomas (SC-6-JCK, poorly differentiated adenocarcinoma; and St-15, mucinous adenocarcinoma) and on one colonic carcinoma (Co-3, well-differentiated adenocarcinoma). In SC-6-JCK, the treatment with s.c. injection of pentagastrin at a dose of 10 μg/mouse once daily for 25 days promoted the growth of the tumor transplanted in nude mice, but gastrin had no effect at all on St-15 and Co-3. In SC-6-JCK, the weight, size, and labeling index of [^{3}H]thymidine of the tumor were significantly increased in comparison with those of the control ($p < 0.05$).

In SC-6-JCK, cyclic adenosine 3′:5′-monophosphate (cAMP) in the tumor was increased by a single i.p. injection of pentagastrin at a dose of 20 μg/mouse in nude mice, but such an increase was not observed in St-15 and Co-3. Cyclic guanosine 3′:5′-monophosphate in SC-6-JCK was slightly increased by gastrin treatment but was not affected in the other tumors. In SC-6-JCK, at 30 min after gastrin treatment when cAMP showed a maximum increase, the activity ratio of cAMP-dependent protein kinase in the tumor was also elevated. *In vitro* also, gastrin stimulated cAMP production and cAMP-dependent protein kinase activation. The data suggest that some human gastric carcinomas may have receptor for gastrin.

INTRODUCTION

Recently, attention has been focused on gut hormone as "growth hormone." Gastrin is well known to stimulate protein, RNA, and DNA synthesis as well as cell division and growth in the digestive mucosa (3, 9, 10, 16). This trophic action occurs in not only normal epithelium but also experimental tumors induced by chemical carcinogen (12). More recently, we have demonstrated that prolonged administration of pentagastrin enhances the incidence of rat stomach tumors induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (24, 27). There are, however, hardly any detailed reports on the effect of gastrin on the growth of human gastrointestinal carcinomas.

There is much evidence to suggest that cAMP[3] and cGMP are involved in the regulation of cell growth and malignant transformation of the intestinal epithelium (2, 20). However, in the human gastric mucosa, the effect of gastrin on the production of cAMP and cGMP is not consistent. Furthermore, the effect of gastrin on cAMP and cGMP accumulation in human gastric carcinoma remains poorly defined.

The purpose of the present study was to investigate the effect of gastrin on the growth of xenotransplantable human gastric and colonic carcinoma lines in nude mice and to clarify whether gastrin stimulates cAMP and cGMP metabolism in these tumors.

MATERIALS AND METHODS

Three types of xenotransplantable tumors successively transmitted s.c. in nude mice were used, 2 gastric cancers [SC-6-JCK, a poorly differentiated adenocarcinoma from a 21-year-old male (28), and St-15, a mucinous adenocarcinoma from a 48-year-old female (22)] and one colonic cancer [Co-3, a well-differentiated adenocarcinoma from a 39-year-old female (22)].

Six-week-old female nude mice (BALB/c-*nu*/*nu*) purchased from Japan Clea (Osaka, Japan) were used in the experiments. These mice were kept under specific-pathogen-free conditions (room temperature, 24 ± 0.5°; humidity, 55 ± 5%), and feed (CL-2; product of Japan Clea), water, cage, and flooring were sterilized by autoclaving (121° for 30 min) before use.

Tumor Growth

After removing the tumor from nude mice, it was cut into small cubes about 1 cu mm in size in RPMI-1640 medium and inoculated into the mouse back s.c. with a trocar needle. For each tumor, 30 mice were used, and 1 week after transplantation, 20 mice of uniform size were selected and randomly divided into 2 groups. In the first group, pentagastrin (Sumitomo Chemical Industries, Ltd., Tokyo, Japan) was administered s.c. at a single daily dose of 10 μg/mouse, and in the remaining group used as a control, 0.9% NaCl solution was given. The period of gastrin treatment was 25 days for SC-6-JCK, 45 days for St-15, and 28 days for Co-3. Tumor size was determined by measuring the long diameter and the short diameter with a μm twice a week and expressed as their product.

After gastrin treatment, [*methyl*-^{3}H]thymidine (18 to 25 Ci/mmol; Amersham Japan, Ltd., Tokyo, Japan) was administered i.p. at a dose of 1 μCi/g body weight. One hr after administration of [^{3}H]thymidine, the tumor was removed, and its weight and size were measured. A part of the tumor was frozen with liquid nitrogen and stored at −80° for measurement of DNA content. DNA content in the tumor was determined by fluorometric assay using 3,5-diaminobenzoic acid (7). The remaining tumor was fixed in 10% buffered formalin and then embedded in paraffin. Serial sections 4.5 μm in thickness were prepared for microautoradiography and immunohistochemistry. Microautoradiography was conducted by the dipping method using NR-M2 emulsion (Konishiroku Co., Tokyo, Japan). After exposing for 4 weeks, the section was developed in Konidol-X developer (Konishiroku Co.), and staining was made with Kernechtrot. The number of [^{3}H]thymidine-labeled cells was determined for more than 3000 tumor cells. Immunostaining was performed for gastrin, somatostatin, carcinoembryonic antigen, α-fetoprotein, secretory component, and α_1-antitrypsin by the immunohistochemical peroxidase-antiperoxidase technique (24). Antisera to gastrin and somatostatin were purchased from Japan Immunoresearch Laboratories Co. (Takasaki, Japan), and the others were from Dakopatts A/C (Copenhagen, Denmark) and used with an optimal dilution.

[1] Supported in part by grants-in-aid for cancer research from the Ministry of Education, Science, and Culture (58010070, 58015078).

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[3] The abbreviations used are: cAMP, cyclic AMP; cGMP, cyclic GMP; PKA, cyclic AMP-dependent protein kinase; TCA, trichloroacetic acid; PKI, cyclic AMP-dependent protein kinase inhibitor.

Received February 22, 1984; accepted June 14, 1984.

cAMP and PKA

In Vivo. In this experiment, a total of 64 nude mice with the tumor enlarged to approximately 1 cm in size was used. After the mice fasted for 24 hr, pentagastrin was administered i.p. at a dose of 20 μg/mouse to 16 mice of each tumor for a total of 48 mice. Furthermore, for SC-6-JCK, 0.9% NaCl solution at a dose of 0.2 ml/mouse was administered i.p. to 16 mice as a control. At 0, 15, 30, and 60 min after administration, the mice were sacrificed by cervical dislocation, and immediately thereafter, the tumor was removed, frozen with liquid nitrogen, and then preserved at −80°. cAMP and cGMP were determined in the 3 tumors, and in addition, PKA was determined in SC-6-JCK.

In Vitro. After removing the tumor (SC-6-JCK) from nude mice, the tumors were sliced 1 mm in thickness, and a total of 28 slices was used. A tumor slice was placed into a plastic culture dish containing 2 ml of RPMI-1640 medium and preincubated in a humidified 5% CO_2-95% air mixture for 30 min at 37°. After preincubation, to each dish were added 5 μmol of pentagastrin or the same volume of phosphate-buffered saline (100 mM), and then they were incubated for 0, 2, 5, and 10 min. After incubation, slices were taken out and immediately frozen with liquid nitrogen and preserved at −80° for determination of cAMP and PKA.

cAMP and cGMP Assay. cAMP and cGMP were determined by radioimmunoassay with the use of YAMASA cAMP and cGMP kits (Yamasa Shoyu, Ltd., Chousi, Japan). About 50 mg of frozen tissue were homogenized and extracted twice with 1 ml of cold 6% TCA solution. After centrifugation at 3000 rpm for 15 min at 4°, the mixed supernatant was washed 3 times with 5 ml of water-saturated ether. Following succinylation of 40 μl of ether-washed solution, cAMP and cGMP were determined by radioimmunoassay.

PKA Assay. The activity of PKA was determined by the modified method of Corbin and Reimann (1). The frozen tissue was homogenized in a 50-fold volume of 10 mM potassium phosphate buffer (pH 6.8), containing 10 mM EDTA, 20 mM 2-mercaptoethanol, and 150 mM NaCl, and centrifuged at 8000 × *g* for 5 min at 4°, and the supernatant thus obtained was used as the protein kinase fraction. Ten μl of the supernatant were assayed at 30° for 10 min in a total volume of 250 μl containing 5 μmol of Tris-HCl buffer (pH 7.5), 5 μmol of magnesium acetate, 100 μg of H2A histone, and 2.5 nmol of [γ-^{32}P]ATP (15,000 to 25,000 cpm/nmol) in the presence and/or absence of 0.5 nmol cAMP and 0.8 μg of heat-stable PKI obtained by the undermentioned procedure. After terminating the reaction by adding an excessive volume of 10% TCA solution, the reaction solution was filtered with a Whatman glass filter. The precipitate was washed with 10% TCA solution and counted by a liquid scintillation counter. Protein kinase activity was expressed in units, where 1 unit was equal to the amount of enzyme which catalyzed the incorporation of 1 nmol of phosphate into the substrate per min. PKA activity was represented with the activity inhibited by PKI. Basal PKA activity was assayed in the absence of cAMP, and total PKA was in the presence of 0.5 nmol of cAMP. Total protein kinase activity was calculated in the absence of PKI and in the presence of cAMP. The activity ratio of PKA (basal PKA activity per total PKA activity) was computed in accordance with the method of Torphy *et al.* (29).

PKI was partially purified from the rabbit skeletal muscle by the method of McPherson *et al.* (15). Under the foregoing conditions, about 98% of the free catalytic subunit of PKA was specifically inhibited. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (5). Calf thymus H2A histone was purified by the method of Oliver *et al.* (19). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO), and the other reagents were obtained from commercial sources. In all experiments, protein was measured by the method of Lowry *et al.* (13).

RESULTS

Effects of Gastrin on Tumor Growth. The effects of gastrin on 3 xenotransplantable tumors are shown in Table 1. In SC-6-JCK (poorly differentiated gastric adenocarcinoma), gastrin promoted the growth of tumor, and following treatment for 2 weeks, the size was significantly larger than that of the control group (Chart 1). However, in the other tumors, *i.e.*, St-15 and Co-3, no difference in tumor size could be demonstrated between the gastrin-treated group and the control group. In SC-6-JCK, in examining the tumor removed following gastrin treatment, the weight of the tumor of the control group was 445 ± 225 (S.D.) mg, and the size was 0.73 ± 0.32 cu cm, whereas the weight of the gastrin-treated group was 1037 ± 475 mg, and the size was 1.67 ± 0.83 cu cm, indicating a significant increase ($p < 0.05$), and the ^{3}H-labeling index, mitotic index, and DNA contents also presented an increasing tendency. However, in St-15 and Co-3, no significant difference was observed between the gastrin-treated group and the control group as shown in Table 1.

By immunohistochemistry, carcinoembryonic antigen immunoreactivity was demonstrated in all 3 tumors. Moreover, in SC-6-JCK, secretory component and α_1-antitrypsin immunoreactivity were observed, while in Co-3, α-fetoprotein immunoreactivity was seen. Gastrin and somatostatin immunoreactivity could not be observed in all the tumors. The effect of gastrin on these functions of tumor cells could not be detected within the scope of our immunohistochemical observation.

Effect of Gastrin on cAMP, cGMP, and PKA. The effect of gastrin *in vivo* on the cAMP and cGMP content in the 3 tumors is shown in Charts 2 and 3. In SC-6-JCK, increase in cAMP content in the tumor was observed by a single i.p. administration of gastrin, the maximum increase of about 150% of the control

Table 1

Effect of gastrin on xenotransplantable gastric and colonic carcinoma in nude mice

Tumor	Treatment[a]	Tumor size (cu cm)	Tumor wt (mg)	Labeling index[b]	Mitotic index[c]	DNA content (μg/mg protein)
Gastric carcinoma (SC-6-JCK)	Gastrin	1.67 ± 0.83[d]	1037 ± 475	13.2 ± 3.5	31.9 ± 9.8	22.3 ± 4.5
	Control	0.73 ± 0.32[e]	445 ± 225[e]	8.7 ± 2.4[e]	22.2 ± 10.7	18.9 ± 8.0
Gastric carcinoma (St-15)	Gastrin	1.06 ± 0.53	644 ± 340	16.2 ± 3.9	17.2 ± 3.9	46.1 ± 4.8
	Control	1.12 ± 0.65	661 ± 384	15.9 ± 3.3	15.6 ± 4.2	42.6 ± 4.5
Colonic carcinoma (Co-3)	Gastrin	1.69 ± 1.08	973 ± 622	25.4 ± 2.3	4.7 ± 2.1	44.0 ± 7.3
	Control	1.35 ± 0.78	782 ± 454	24.4 ± 2.3	6.7 ± 3.0	44.2 ± 5.2

[a] Gastrin (10 μg/mouse) or 0.9% NaCl solution as control was injected s.c. every day for 25 days on SC-6-JCK, 45 days on St-15, and 28 days on Co-3.
[b] Labeling index was represented by the number of labeled nuclei per 100 nuclei.
[c] Mitotic index was represented by the number of mitotic figures per 1000 nuclei.
[d] Mean ± S.D. of 10 tumors from 10 nude mice.
[e] Significant difference between gastrin-treated and control group ($p < 0.05$).

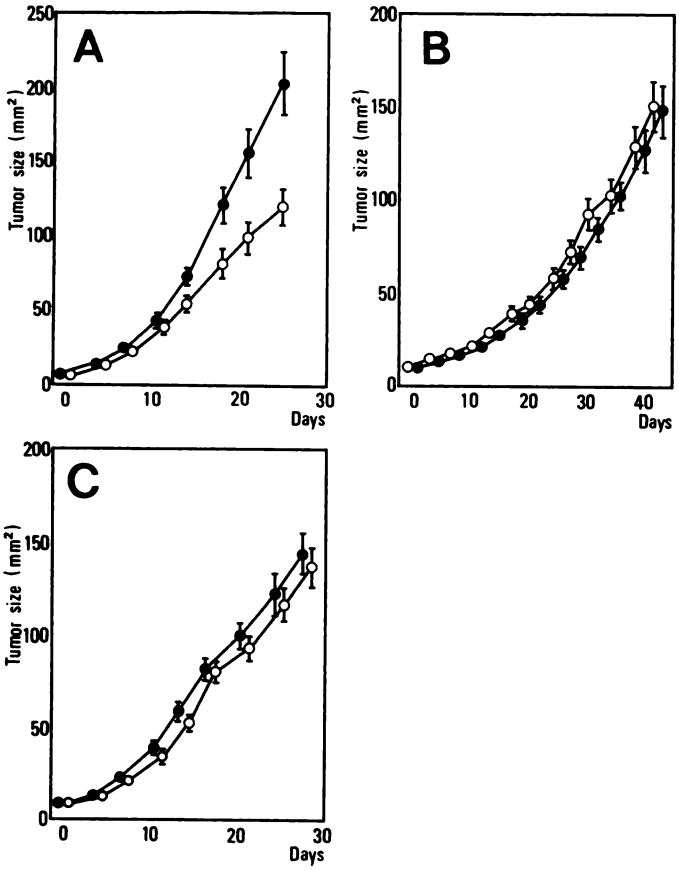

Chart 1. Effect of gastrin on the growth of xenotransplantable gastric carcinomas, SC-6-JCK (*A*) and St-15 (*B*), and colonic carcinoma, Co-3 (*C*), in nude mice. Gastrin (10 μg/mouse) was injected s.c. every day (●); the same volume of 0.9% NaCl solution as control was injected (○). *Points*, mean of 10 tumors; *bars*, S.E.

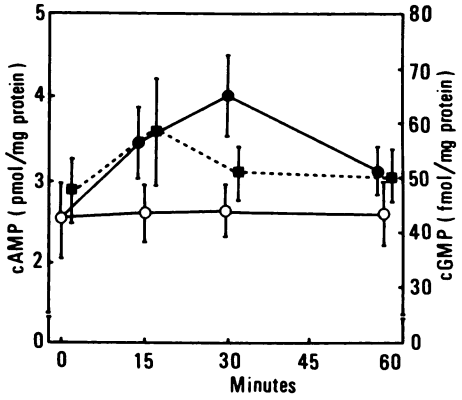

Chart 2. Effect of gastrin on cAMP and cGMP in the gastric carcinoma, SC-6-JCK, in nude mice. Gastrin (20 μg/mouse) was injected i.p. (cAMP, ●; cGMP, ■); the same volume of 0.9% NaCl solution was injected as control (cAMP, ○). *Points*, mean of 4 experiments; *bars*, S.D.

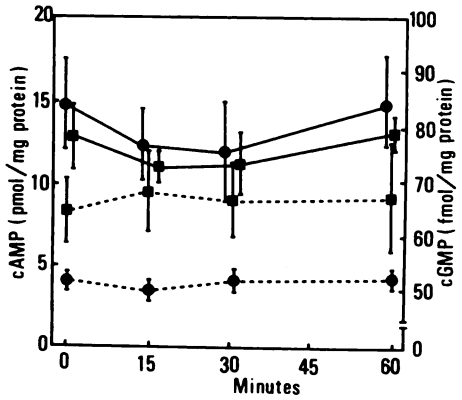

Chart 3. Effect of gastrin on cAMP and cGMP in gastric carcinoma, St-15 (- - - -), and colonic carcinoma, Co-3 (——), in nude mice. Gastrin (20 μg/mouse) was injected i.p. ●, cAMP; ■, cGMP. *Points*, mean of 4 experiments; *bars*, S.D.

being seen 30 min after gastrin treatment. However, in St-15, there was no change in cAMP content in the tumor, but in Co-3, a slight decrease was noted.

cGMP content in SC-6-JCK was increased 15 min after gastrin treatment, but not significant. In the other 2 tumors, cGMP content was not affected by gastrin treatment.

Shown in Table 2 are the effects of gastrin *in vivo* on PKA within the tumor of SC-6-JCK. PKA activity was determined 30 min after gastrin treatment, when the cAMP content in the tumor showed the maximum increase. The PKA activity in the tumor did not present any change by gastrin treatment, but the free catalytic subunit of PKA within the tumor made a significant increase ($p < 0.05$) following gastrin treatment. The specific activity ratio of PKA in the gastrin-treated group was 0.219, which is a significant increase over 0.041 observed in the control group ($p < 0.05$).

The effect of gastrin *in vitro* on cAMP and PKA in SC-6-JCK is shown in Chart 4. The cAMP and PKA activity ratio in tumor slices were increased by gastrin treatment. Both increases were observed 2 and 5 min after gastrin addition. Further in the control (phosphate-buffered saline), the cAMP and PKA activity ratio were not changed during incubation.

DISCUSSION

The results of many *in vivo* and *in vitro* studies indicate that the trophic action of gastrin, independent of gastric acid secretion, is due to a direct interaction of the hormone with receptors on its target cells, such as epithelial cells of the gastric body, duodenum, and pancreas (3, 9, 10, 16). The present study has shown that, of the 3 xenotransplantable tumors, only SC-6-JCK, a poorly differentiated gastric adenocarcinoma line with carcinoembryonic antigen, secretory component, and α_1-antitrypsin immunoreactivity were affected by the trophic action of gastrin. Ohkura *et al.* (18) also reported that COOH-terminal tetrapeptide of gastrin promoted tumor growth of a human gastric well-differentiated adenocarcinoma line xenotransplanted into nude mice. Moreover, Kobori *et al.* (11) described a significant growth effect of gastrin family peptides on a rat stomach cancer cell line BV9 *in vitro*. These data point to the possibilities that tumor cells of some gastric cancers have receptor for gastrin and that their growth may be regulated by gut hormones.

In our previous study, we have demonstrated production of gastrin, glucagon, and glicentin in carcinomas of the human stomach (8, 25, 26). Among these tumors, gastric carcinomas associated with diffusely productive fibrosis showed an extremely poor prognosis. In view of the present results and the trophic action of these peptide hormones, it may be inferred that gastric carcinomas could also partly show "autocrine secretion" for self-stimulation, whereby a tumor cell secretes a peptide hormone for which the tumor cell itself has functional external

Table 2

Effect of gastrin on cAMP and protein kinase of xenotransplantable gastric carcinoma (SC-6-JCK) in nude mice

Tumors were removed 30 min after pentagastrin (20 μg/mouse) or 0.9% NaCl solution as control i.p. injection in nude mice, and cAMP, protein kinase, PKA, and PKA activity ratios were determined as described in "Materials and Methods."

Treatment	cAMP (pmol/mg protein)	Total protein kinase (milliunits/mg protein)	Total PKA (milliunits/mg protein)	Basal PKA (milliunits/mg protein	PKA activity ratio
Gastrin	3.94 ± 0.38[a]	138.4 ± 7.79	77.1 ± 4.87	17.10 ± 3.12	0.219 ± 0.034
Control	2.70 ± 0.26[b]	128.2 ± 15.16	66.3 ± 2.39	2.56 ± 1.63[b]	0.041 ± 0.026[b]

[a] Mean ± S.E. of 4 experiments.
[b] Significant difference between gastrin-treated and control groups ($p < 0.05$).

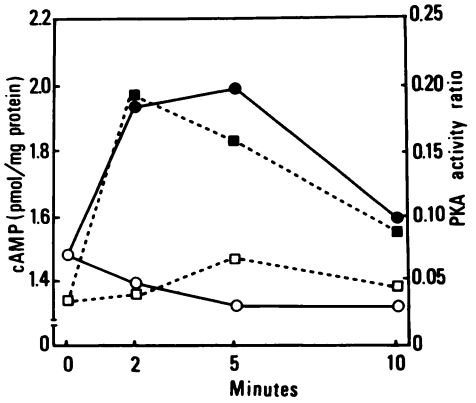

Chart 4. Effect of gastrin on cAMP and PKA in gastric carcinoma, SC-6-JCK, *in vitro*. Gastrin (5 μM) was added after 30-min preincubation (cAMP, ●; PKA, ■); the same volume of phosphate-buffered saline was added as control (cAMP, ○; PKA, □). *Points*, mean of 4 experiments.

receptors as proposed by Sporn and Todaro (23).

The mechanism by which gastrin stimulates the biochemical responses leading to growth has not yet been fully elucidated. Enochs and Johnson (3) have shown one of the earliest responses related to growth following the administration of pentagastrin to be an increase in mRNA. However, it is unknown whether or not gastrin acts upon cell growth through a second messenger, such as one of the cyclic nucleotides. In the present study, only SC-6-JCK, the growth of which was promoted by gastrin, showed a significant increase of cAMP and PKA activity by gastrin *in vivo* as well as *in vitro*. cAMP and PKA seem to mediate the action of gastrin.

There are 2 conflicting hypotheses concerning the role of cAMP in cell growth. The first hypothesis is that cAMP serves as a positive mediator of mitogenesis (14), while the other is that cAMP serves as a negative signal to cell growth (4). Recently, considerable attention has been given to the role of PKA isoenzymes in cell growth and differentiation in several cell systems. Type I isoenzyme of PKA has a positive role in cell proliferation, whereas type II isoenzyme is related to cell differentiation or growth inhibition (17, 21). In human mammary tumor and hepatoma, the activity of type I PKA has been shown to be more increased than that of normal tissues (6, 30). The growth of SC-6-JCK might be promoted by gastrin through the activation of type I PKA. In normal gastric mucosa, the activity of type II is more dominant than that of type I, but in SC-6-JCK, type I and type II are almost equivalent in protein kinase activity.[4] The involvement of selectively activated PKA type I in cancer is of great interest. The effect of gastrin on selective activation of PKA type I in SC-6-JCK is also an important question that needs to be resolved in the future.

[4] H. Sumiyoshi, W. Yasui, A. Ochiai, and E. Tahara, unpublished results.

ACKNOWLEDGMENTS

We are grateful to Professor M. Takeda, Department of Biochemistry, School of Medicine, Hiroshima University, for his helpful suggestions and support.

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