GROWTH-PROMOTING EFFECT OF GASTRIN ON HUMAN GASTRIC CARCINOMA CELL LINE TMK-1

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A human gastric carcinoma cell line TMK-1 was established in vitro by the soft agar method from SC-6-JCK, a poorly differentiated adenocarcinoma xenotransplanted in nude mice. TMK-1 cells had a doubling time of approximately 35 hr and showed carcinoembryonic antigen (CEA), α1-antitrypsin and secretory component immunoreactivity. Ultrastructurally, the tumor cells were characterized by numerous mitochondria, tubulovesicles and intracytoplasmic canaliculi filled with abundant microvilli. The growth of TMK-1 cells was promoted by 10nM human gastrin (G-17), 2μM tetragastrin or 2μM pentagastrin, among which human gastrin showed the most effective growth promotion. Moreover, incorporation of [3H]thymidine into TMK-1 cells was stimulated by gastrin in a dose-dependent manner. The content of cyclic adenosine 3',5'-monophosphate (cAMP) in TMK-1 cells was increased by gastrin treatment but decreased to the control level within 10 min. cAMP-dependent protein kinase was also activated by gastrin administration.

Key words: Tissue culture — Gastric cancer — Parietal cell differentiation — Gastrin — cAMP-dependent protein kinase

Gastrin, originally isolated as a factor stimulating gastric acid secretion, is divided into several molecular forms which vary in length from 14–34 amino acids. Big gastrin, G-34 which has 34 amino acids, consists of an NH2-terminal pentadecapeptide linked through two lysine residues to a COOH-terminal heptadecapeptide identical to G-17. G-34 is the most abundant circulating gastrin form, whereas G-17 is the predominant gastrin form in pyloric mucosa. However, the COOH-terminal tetra- or pentapeptide amide is responsible for all the biological activities of G-17 or G-34. Gastrin has recently been shown to stimulate cellular proliferation of normal epithelium and tumor cells of the gastrointestinal tract both in vitro and in vivo. We have also demonstrated that prolonged administration of pentagastrin enhances the incidence of rat stomach carcinoma induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Moreover, we have reported that gastrin promotes the growth of xenotransplantable human gastric carcinoma (SC-6-JCK) in nude mice. In the present study, we have established the human gastric carcinoma cell line TMK-1 in culture from SC-6-JCK in order to elucidate the direct effect of gastrin on tumor cells. TMK-1 cell line showed parietal cell differentiation. The influence of gastrin on cell growth, cAMP level, cAMP-dependent protein kinase activity and [3H]thymidine incorporation in TMK-1 cells in serum-free medium is described in this report.

MATERIALS AND METHODS

Establishment of TMK-1 Cell Line A human gastric carcinoma, which was serially transplanted in nude mice (SC-6-JCK, a poorly differentiated adenocarcinoma from a 21-year-old male) was minced into small pieces under sterile conditions. The minced tissue was rinsed with phosphate-buffered saline (PBS(-)) and incubated for 30 min at 37°C in 100 U/ml collagenase (Sigma) and 0.25% trypsin (Difco) in PBS(-). The cell suspension was washed and resuspended in RPMI 1640 (Nissui Seiyaku Co., Ltd., Tokyo) containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Semisolid agar was used for cloning as follows. A 5 ml aliquot of the cultured medium containing 0.5% agar was plated into a 60 mm culture dish as the bottom layer, then 3 ml of cell suspension in the culture medium containing 0.3% agar was plated over the bottom layer. All cultures were incubated at 37°C in 5% CO2 in air.
Light and Electron Microscopic Examination: TMK-1 cells grown on sterile cover glass slides were fixed in 95% ethanol. Cells were stained with Giemsa, hematoxylin and eosin, periodic acid-Schiff (PAS), PAS after diastase digestion, alcian blue and Papanicolaou stain. Immunohistochemical staining was done by the modified method of Tahara et al.13 using antisera for CEA (1:300), α1-antitrypsin (1:400) and secretory component (1:400) (Dakopatts A/C, Copenhagen, Denmark). For electron microscopic examination, the cells were trypsinized and centrifuged. The packed cells were fixed with 2.5% glutaraldehyde dissolved in 0.1M phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide, after which the cells were embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a JEM-100S electron microscope.

Effect of Gastrin on TMK-1 Cells
Gastrin Dose Dependency of DNA Synthesis in TMK-1 Cells: After a 2-hr incubation of the cells in serum-free medium, human gastrin was added to each dish at the concentration of 1pM, 10pM, 100pM, 1nM or 10nM. Then 1 x 10⁶ TMK-1 cells/dish were pulse-labeled with 1 μCi/ml [methyl-3H]thymidine (18 to 25 Ci/mmol, Amersham Japan, Ltd., Tokyo) for 1 hr. After being washed with 10% trichloroacetic acid, cells were dissolved with 0.1M sodium dodecyl sulfate. The radioactivity of a 350 μl aliquot in 10 ml of toluene-containing 1% sodium dodecyl sulfate was counted with a liquid scintillation counter. All determinations were done in triplicate.

In order to determine the time course of the effect of human gastrin, 1 x 10⁶ cells/dish were preincubated for 2 hr and 10nM human gastrin was added. After incubation for 1, 3, 6 and 12 hr, [3H]thymidine 1 μCi/ml was pulsed.

Cell Growth: To exclude the effect of unknown factors in FCS on cell growth, cell growth was examined in serum-free hormone-supplemented medium containing 2 μg/ml insulin (Sigma) and 2 μg/ml transferrin (Sigma). A dose of 2μM pentagastrin (ICI Pharma., Co., Ltd., Osaka), 2μM tetragastrin (San-a Pharma., Ind., Co., Ltd., Tokyo) or 10nM human gastrin (Sigma) was added to each dish. The doses of insulin and transferrin were determined by the method described by Murakami and Masui12 and the doses of pentagastrin and tetragastrin were determined by the method of Sumiyoshi et al.10. The medium was changed every 2 days up to 10 days and the cell numbers were counted at 2, 6, 9 and 13 days after plating.

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Characterization of TMK-1 Cells
Colonies appeared 10 to 14 days after plating in soft agar. Three weeks after plating, some of the growing colonies were recloned by the soft agar method. After cloning twice, the colonies were suspended in the culture medium and plated. Subsequently, the cultures were serially transplanted by treatment with 0.025% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) and maintained in the same medium containing 10% FCS.

TMK-1 cells were oval or cuboidal-shaped in loose contact with the dishes, and grew to form loose clusters with piling-up (Fig. 1). Some cells had irregular processes and were connected to each other. TMK-1 cells had...
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A large nucleus and nucleolus. Some of them had large vacuoles in the cytoplasm. The cytoplasm was stained positive with PAS and negative after diastase treatment. No mucin could be detected. CEA, α1-antitrypsin and secretory component immunoreactivity were demonstrated in some of the cells. The ultrastructure of TMK-1 cells is shown in Fig. 2a, b and c. The border of the cells displayed numerous and fairly uniform microvilli. Junctional complexes between neighboring cells were represented by desmosomes. There were many glycogen granules and many large mitochondria in the cytoplasm. Golgi apparata were observed near the nucleus. Intracytoplasmic canaliculi lined by abundant microvilli and tubulovesicles were frequently seen in some of the cells, but secretory granules could not be found in any of the cells.

TMK-1 cells grew exponentially between the 2nd and 8th days after an initial lag period of 2 days. The mean population doubling time was estimated to be approximately 34.9 hr.

**Effect of Gastrin on Growth of TMK-1 Cells**

Effect of Gastrin on DNA Synthesis in TMK-1 Cells: In the dose-response experiment with human gastrin, the incorporation of [3H]thymidine into the cells was stimulated by human gastrin in a dose-dependent manner up to 10nM (Fig. 3a). The time course of [3H]thymidine incorporation into TMK-1 cells after 10nM human gastrin stimulation is shown in Fig. 3b. Stimulation of DNA synthesis by gastrin was clearly evident 6 hr after gastrin treatment ($P < 0.05$).

The effect of gastrin on TMK-1 cells is shown in Fig. 4. The stimulation of growth by 10nM human gastrin was clearly evident by day 6 ($P < 0.01$) and cell growth was also promoted by addition of 2 μM pentagastrin or 2μM tetragastrin on the 9th day and 13th day in comparison with the control cells ($P < 0.05$). Human gastrin was a more effective growth promoter than pentagastrin or tetragastrin on the 6th and 9th days ($P < 0.05$).

**Effect of Gastrin on cAMP Content and cAMP-dependent Protein Kinase**

An increase of cAMP content in TMK-1 cells was induced by 2μM pentagastrin treatment. The maximum increase of about 180% of the control was observed at 1 min after gastrin treatment. cAMP content declined to the control level within 10 min (Fig. 5a).

The time course of cAMP-dependent protein kinase activity followed the same pattern as that of cAMP (Fig. 5b). The cAMP-dependent protein kinase activity ratios decreased to the control level within 20 min.

**Discussion**

Many gastric cancer cell lines have been established, but there has been no report on gastric cancer cell lines showing parietal cell differentiation. In the present study some of the TMK-1 cells ultrastructurally revealed parietal cell differentiation characterized by abundant mitochondria, tubulovesicles and intracellular canaliculi filled with numerous microvilli. Recently, Capella et al. reported 3 cases of parietal cell carcinoma of the stomach as a newly recognized entity, including 1) intracellular canaliculi with microvilli, 2) tubulovesicles, 3) abundant mitochondria, and 4) absence of secretory granules. TMK-1 cells had these typical parietal cell features. SC-6-JCK, from which TMK-1 cell line was derived, ultrastructurally showed abundant mitochondria and smooth ER in the cytoplasm. Moreover, SC-6-JCK, the growth of which was promoted by gastrin, showed a significant increase of cAMP and cAMP-
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dependent protein kinase activity by gastrin.10 These data point to the possibility that TMK-1 cell line differentiated from a clone of SC-6-JCK tumor cells which carried the receptor for gastrin, like parietal cells. Detailed biological and biochemical examination is necessary to ascertain whether or not this TMK-1 cell line can be designated as parietal cell carcinoma.

Recent studies have shown that gastrin binds to the receptors and stimulates DNA, RNA and protein synthesis in its target cells.

Fig. 2. Electron microscopy of TMK-1 cells. The border of the cell displays numerous microvilli (a). Intracytoplasmic canaliculi around the nucleus are apparent (a, b) (×3000). At high magnification of the same cell (b), intracytoplasmic canaliculi with abundant microvilli, large mitochondria and tubulovesicles are seen (c) (×10000).
such as parietal cells and intestinal epithelial cells, both in vivo and in vitro.\(^2\), \(^4\), \(^6\), \(^17\) - \(^20\) This trophic action occurs not only in normal epithelium of the gastrointestinal tract but also in rat stomach cancer cell line BV9.\(^9\) We have also demonstrated that prolonged gastrin administration enhances the incidence of MNNG-induced rat gastric carcinomas\(^9\) and promotes the growth of xenotransplantable human gastric carcinoma SC-6-JCK.\(^10\) These data suggest that some human gastric carcinomas have a receptor for gastrin, and that their growth may be regulated by gastrin. It is important to ascertain whether gastrin acts upon TMK-1 cell proliferation through cAMP metabolism. In normal parietal cells having separate receptors for histamine, gastrin and acetylcholine, histamine is well known to stimulate acid secretion via H\(_2\) receptors coupled to cAMP production.\(^21\) However, gastrin-induced cAMP production is unlikely to be involved in the mechanism of gastric acid secretion in human parietal cells.\(^22\) Moreover, a number of agents known to increase cAMP, such as cholera toxin and polypeptide hormones, have recently been shown to stimulate multi-

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**Fig. 3.** Gastrin dose-response curve (a) and time course (b) of \([\text{H}]\)thymidine incorporation in TMK-1 cells. (a) 0, 1pM, 10pM, 100pM, 1nM or 10nM human gastrin was added to TMK-1 cells. Bars indicate the SD. (b) 10nM human gastrin was added to TMK-1 cells (○) and the cells were cultured for 1, 3, 6 and 12 hr. Control (●). Each point is the average of triplicate plates.

**Fig. 4.** Effect of gastrin on growth of TMK-1 cells in serum-free medium containing 2 \(\mu\)g/ml insulin and 2 \(\mu\)g/ml transferrin. 10nM human gastrin (○), 2\(\mu\)M pentagastrin (□) or 2\(\mu\)M tetragastrin (△) was added to the plate every 2 days up to the 12th day. Cell numbers were counted on the 2nd, 6th, 9th and 13th day. Control (●).
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In the present study, gastrin raised the cAMP level and cAMP-dependent protein kinase activity, and promoted the growth of TMK-1 cell line. Considering these effects of gastrin on TMK-1 cells, it seems clear that gastrin promotes TMK-1 cell growth through cAMP metabolism. More recently, Kamps et al. reported that cAMP-dependent protein kinase and oncogenic tyrosine kinase might comprise a single divergent gene family, based on the homology of the amino acid sequences. We have also observed that type I cAMP-dependent protein kinase, which is considered to be involved in proliferation and transformation, increased in human gastric adenocarcinomas and SC-6-JCK. More recently, we have demonstrated that type I cAMP-dependent protein kinase can play an important role in the enhanced effect of gastrin on rat stomach carcinogenesis induced by MNNG. The trophic effect of gastrin on TMK-1 cells might also involve this isozyme of cAMP-dependent protein kinase. Further examination will be necessary to learn whether gastrin promotes TMK-1 cell growth through selective activation of type I cAMP-dependent protein kinase.

It is well known that the common COOH-terminal tetrapeptide amide Trp-Met-Asp-Phe-NH2 elicits all the effects of larger molecules, but the biological potency increases with increasing chain length. Katayama et al. have demonstrated that the trophic effect of synthesized human gastrin on cultured rat fundic mucosal cells had a potency approximately 20-fold greater than that of tetragastrin. Johnson and Guthrie have also demonstrated that big gastrin (G-34) and little gastrin (G-17) stimulated DNA synthesis in rat gastric mucosa more effectively than they stimulated acid secretion in vivo. The present study showed that 10nM human gastrin expressed a more significant trophic action than 2µM pentagastrin or 2µM tetragastrin in vitro. These data indicate that the amino acid sequence of gastrin other than C-terminal tetra- or penta-peptide might amplify the growth-trophic

Fig. 5. Time course of cAMP level (a) and cAMP-dependent protein kinase (b) in TMK-1 cells after gastrin treatment. After preincubation for 2 hr in serum-free medium, 2µM pentagastrin (○) was added to TMK-1 cells and cultured for a suitable time. The cells were scraped off with a rubber policeman to determine cAMP level and cAMP-dependent protein kinase activity. Control (●).
action of gastrin. Baldwin suggested that gastrin and the transforming protein of polyoma virus may have evolved from a common ancestor because of their highly homologous amino acid sequences. Further studies seem desirable on the correlation between gastrin and oncoprotein as a tumor growth factor.

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