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Role of Parathyroid Hormone/Parathyroid Hormone-Related Peptide on Cell Proliferation in the Gastric Mucosa

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Parathyroid hormone-related peptide (PTHrP) is widely expressed in normal tissues and elicits various functions through the PTH/PTHrP receptor. Relaxation effects of PTHrP on gastrointestinal smooth muscle cells were well documented, but the physiological role on mucosal growth and differentiation is little known. The purpose of this study was to evaluate the expression of PTHrP and PTH/PTHrP receptor in the rat gastric mucosa, and the role of PTHrP on mucosal cell proliferation. Male Wistar rats were used in this study. Localization of PTHrP and PTH/PTHrP receptor were observed by immunohistochemistry and in situ hybridization. Expression of PTH/PTHrP receptor mRNA were examined by RNase protection assay in control and stress condition. Double staining with BrDU incorporation was performed to differentiate cell cycle states. Cell proliferative effect by external PTHrP-(1-34) was evaluated by BrDU incorporation. PTHrP immunopositive cells were encountered in and around the mucosal neck area. PTH/PTHrP receptor immunoreactivity was observed in the gastric mucosa broadly. Cells with stronger expression for PTHrP and its cognate receptor were located in the vicinity of generative zone. But BrDU incorporating cells were negative for both PTHrP and PTH/PTHrP receptor. By RNase protection assay, PTH/PTHrP receptor mRNA expression was weak in a steady state, and the receptor expression increased at stress. External PTHrP-(1-34) did not show cell proliferative effect in a steady state. At stress BrDU incorporation was suppressed significantly, and PTHrP-(1-34) increased BrDU incorporation.

These observations suggest that PTHrP and PTH/PTHrP receptor involve maintenance of mucosal growth and differentiation in the stomach.

Key Words: PTHrP, PTH/PTHrP receptor, cell proliferation

Introduction

Parathyroid hormone-related peptide (PTHrP) was first isolated from human tumor cells and was shown to be responsible for producing hypercalcemia in patients with humoral hypercalcemia of malignancy1). PTHrP is the product of a gene that is also expressed in many normal tissues2,3), and the localization of PTHrP in the stomach has been demonstrated in fetal and adult animals4,5). Gene expression of PTH/PTHrP receptor was also demonstrated in many tissues6) and PTHrP elicits physiological effects through PTH/PTHrP receptor in an autocrine/paracrine fashion. Among the various physiological effects of PTHrP, smooth muscle relaxation in visceral and vascular tissues is well known7,8). We previously reported that PTHrP produces a similar response on distended gastric smooth muscle cells in the rat, and concluded that PTHrP might be an important gastrointestinal peptide which regulates gastric contractile activity9). Other physiological roles are the regulation of transepithelial calcium transport and the regulation of cell proliferation and differentiation10,11). A recent report suggested that PTHrP is involved in mucosal differentiation in the rat intestinal mucosa12).

In the current study, we evaluated the expression of PTHrP and PTH/PTHrP receptor in the gastric mucosa and the physiological role of PTHrP on mucosal cell proliferation.

Materials

Animals

Male Wistar-Kyoto rats were purchased from Charles River Japan (Atsugi, Japan) and used at 20-24 weeks of age in this study. The animals were handled according to the guidelines of the NIH Animal Research Committee (Bethesda, MD). Rats were housed...
in groups of 3 to 4 per cage in an air-conditioned room at 24°C (lights on from 7 AM to 7 PM) at the Laboratory Animal Center of Nagasaki University. The rats were allowed free access to food (laboratory chow F2, Japan CLEA, Tokyo) and tap water. RWI stress was applied as described by Takagi and Okabe.18

Localization of PTHrP and PTH/PTHrP receptor by immunohistochemistry and In situ hybridization

Localization of PTHrP and PTH/PTHrP receptor was performed by immunohistochemistry and in situ hybridization. The animals were deeply anesthetized with an overdose of ether after treatment and were perfusion-fixed with a suitable volume of 4% paraformaldehyde via the left ventricle. The stomachs were resected and the fundic portion of the glandular stomach was cut into 3mm thick slices and post-fixed overnight followed by paraffin embedding. The paraffin-embedded tissues were cut into 4 μm sections, deparaffinized with xylene, and rehydrated through a series of ethanols. Deparaffinized sections were preincubated with normal bovine serum to prevent nonspecific binding, and then were incubated overnight at 4°C with an optimal dilution (5 μg/ml) of the primary polyclonal antibody against PTH/PTHrP receptor (Babco, Richmond, CA). Sections were washed in phosphate-buffered saline (PBS), and bound antibodies were localized by the avidin-biotin-peroxidase method using DAB or AEC as the chromogenic substrate. For PTHrP immunohistochemistry, monoclonal antibody was used (Oncogene Science, Inc., Uniondale, NY). The slides were sequentially incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin antibodies. The alkaline phosphatase reaction was revealed using a mixture of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt nitroblue tetrazolium chloride (BCIP/NBT). Negative controls were prepared in each case by replacing the primary antibody with nonimmune mouse serum. Rat kidney served as the positive control for PTH/PTHrP common receptor immunohistochemistry. Human PTHrP-producing lung cancer served as an internal positive control in immunostaining for PTHrP. To examine the topological relation to neck proliferating cells, double staining of PTH/PTHrP receptor and BrDU immunohistochemistry were performed. BrDU immunohistochemistry was performed. BrDU (100 mg/kg) was dissolved with phosphate-buffered saline and injected intraperitoneally two hours before sacrifice.

Using a digoxigenin RNA labeling kit (Boehringer Mannheim, Mannheim, Germany), antisense and sense RNA probes for rat PTH/PTHrP receptors were made. Hybridization was performed as described previously.19

PTH/PTHrP receptor mRNA to be hybridized with digoxigenin-labeled riboprobe was detected by antibody to digoxigenin and stained with BCIP/NBT.

**Localization of PTHrP and PTH/PTHrP receptor by immunohistochemistry and In situ hybridization**

Gene expressions of PTH/PTHrP receptor were examined in a steady state and at RWI stress condition by the RNase protection assay as described previously.18 Rats were divided into the following two groups: group 1, non-stressed control; group 2, RWI stress for 4 h. In group 2, the rats were placed in a restraint cage and immersed up to the xyphoid process for 4 h in water at 23°C. All rats were given food and water ad libitum just before the treatment. Total RNA was extracted from the gastric fundus by the guanidinium thiocyanate method according to the manufacturer's instruction (RNAzol, Tel-Test, Friendswood, TX). Hybridization probes were labeled with [α-32P]CTP using T3 RNA polymerase according to the supplier's guidelines (MAXI script kit, Ambion, Austin, TX). The RNA probes used were as follows: Rat PTH/PTHrP receptor, a 569-bp HindIII/Not I fragment of pTRI-GAPDH (Ambion). Total RNA (30 μg) prepared from rat stomach was hybridized overnight with 1x10^5 cpm of riboprobe and 5 x 10^3 cpm rat GAPDH probe, and then digested with RNase A and RNase T1 (RPAII ribonuclease protection kit, Ambion). The reaction products were resolved on 4% polyacrylamide/8M urea sequencing gels and analyzed after 72 h (PTH/PTHrP receptor) by autoradiography on XAR film with a single intensifying screen. Assays were performed in duplicate, and in each case GAPDH mRNA also was quantified.

**Effects of PTHrP-(1-34) supplementation on cell proliferation**

Effects of PTHrP-(1-34) (Cambridge Research Biochemicals, Cheshire, UK) pretreatment on cell proliferation were evaluated. PTHrP-(1-34) (10 μg/ip) was administered 10 min before the beginning of RWI stress. To assess the cell proliferative state in the neck proliferative zone, BrDU incorporation was examined. BrDU (100 mg/kg) was dissolved with phosphate-buffered saline and injected intraperitoneally two hours before sacrifice in each group of rats. After perfusion-fixation with 4% paraformaldehyde, the stomachs were resected and the fundic portion of the glandular stomach was cut into 3 mm thick slices and post-fixed overnight followed by paraffin embedding. Deparaffinized sections were preincubated with normal
bovine serum to prevent nonspecific binding, and then were incubated overnight at 4°C with an optimal dilution of the primary monoclonal against BrDU (Becton Dickinson, San Jose, CA). Immunohistochemistry of BrDU was performed as described previously. Three arbitrary fields in each rat were scanned at random under 200 X magnification, and the average cell count was used as a representative value for each rat. BrDU incorporation by cells was analyzed with the image analyzer (MCID, Image Research Institute, Ontario, Canada), and the values were expressed as the number of positive cells per 1 mm length of mucosa.

Data analysis
All data are expressed as mean±SD. The ANOVA test was used to determine the statistical significance of the data, and P<0.05 was regarded as significant.

Results

Localization of PTHrP and PTH/PTHrP receptor

Immunoreactivity of PTH/PTHrP receptor was detected broadly in the gastric mucosa (Fig.1A, 1B), in contrast to it PTHrP expressed just vicinity of neck proliferative cells which incorporate BrDU (Fig.1C). Cells around the mucosal generative zone were particularly intensely positive to both antigens. The mucosal surface area and the deepest area were negative or barely positive. Double staining with BrDU demonstrated that BrDU positive cells were negative for both PTHrP and PTH/PTHrP receptor positive cells (Fig. 1B, 1C), although both cells were in close proximity to BrDU positive cells.

PTH/PTHrP receptor mRNA was detected in cells around the mucosal neck by in situ hybridization, localization similar to that with immunohistochemistry (Fig. 2).

Figure 1. Double staining of PTHrP or PTH/PTHrP receptor with BrDU immunohistochemistry in the mucosal layer of the stomach. (A, B): PTH/PTHrP receptor (AEC colorization) was detected widely in the mucosal layer around the generative zone where BrDU-immunopositive cells (arrow heads, BCIP/NBT colorization) were present. PTH/PTHrP receptor expression was intense in and around the generative zone, and sparse or negative in the surface and basal layers. (A; x13, B; x66) (C): PTHrP immunopositive cells (BCIP/NBT colorization) and BrDU-positive cells (arrowheads, DAB colorization) were located close to each other, but existed independently. Nucleolar PTHrP immunoreactivities were partly detected. (x132)

Figure 2. In situ hybridization of PTH/PTHrP receptor mRNA. PTH/PTHrP receptor mRNA was expressed in the lower two-thirds of mucosal layer. Upper- and lower-most areas were negative for PTHrP mRNA. (x33)
**PTH/PTHrP receptor gene expression**

Gene expression of PTH/PTHrP receptor (Fig.3) was analyzed by ribonuclease protection assay in a steady state and at RWI stress. The PTHrP receptor mRNA transcript were detected in the gastric tissue of all groups. PTHrP receptor mRNA levels were weak at steady state. In contrast, the expression of PTH/PTHrP receptor was up-regulated at RWI stress.

![Figure 3. PTH/PTHrP receptor expression analyzed by ribonuclease protection assay. The labeled antisense probes used in these assays are PTH/PTHrP and rat GAPDH. Total RNA (30 μg) from the rat stomach (fundus) was used in the assay. PTH/PTHrP receptor expression was enhanced by RWI stress. Lane 1: control, Lane 2: RWI stress.](image)

**External PTHrP-(1-34) effects on cell proliferation**

Results of BrDU incorporation study were shown in Fig.4 and 5. Fig.4 shows representative finding of BrDU immunohistochemistry. Fig.5 shows the cell number of BrDU incorporation per mm. Cells with BrDU incorporation were encountered in the proliferative zone (Fig.4). In non-stressed rats, no significant differences were present with or without PTHrP-(1-34) pretreatment. BrDU incorporation was significantly decreased in RWI stressed animals. In contrast, PTHrP-(1-34) pretreatment prevented the suppression of BrDU incorporation at stress and this value tended to be higher than that of steady state control.

![Figure 4. BrDU incorporation. Cells with BrDU incorporation were encountered in the proliferative zone (A; steady state control). BrDU incorporation was significantly decreased in RWI stressed animals (B). PTHrP-(1-34) pretreatment prevented the suppression of BrDU incorporation at stress (C). (x13)](image)

![Figure 5. Effect of PTHrP-(1-34) on BrDU incorporation at RWI stress. BrDU incorporation was significantly decreased by RWI stress in non-treated rats. In contrast, PTHrP-(1-34) pretreatment prevented decrease of BrDU incorporation at stress, although cell proliferative effect was not observed by PTHrP-(1-34) pretreatment in the non-stress condition.](image)
Discussion

At present, at least three major secretory forms of the peptide have been shown to exist: an amino-terminal species, a mid-region species, and a carboxyl-terminal species. It is clear that multiple receptors for PTHrP must exist, and these can be divided into two categories: 1) an NH2-terminal PTHrP receptor that binds the PTH-like region of PTHrP, and 2) receptors for midregion and COOH-terminal PTHrP secretory forms. The most well-established effects of PTHrP are mediated via an NH2-terminal PTHrP receptor. NH2-terminal PTHrP binds to the classical PTH receptor and activates both the adenyl cyclase/protein kinase A pathway as well as the cytosolic calcium/inositol phosphate/protein kinase C pathway. However, the role of PTH/PTHrP receptor on mucosal growth and differentiation remains poorly understood now. In this study we focused on PTH/PTHrP receptor expression and its regulation of gastric mucosal proliferation.

Recent reports suggest that PTHrP plays an important role in cell growth and differentiation in many organs or tissues by an autocrine/paracrine mechanism, and the effect of PTHrP on growth or differentiation depends on the tissue and cell type. In the development of neonatal tissues, PTHrP and PTH/PTHrP receptor are essential. PTHrP in breast milk might have a local role in the neonatal gut. In the rat intestine, immunoreactive PTHrP was observed in jejunal epithelial cells all along the villus but not in crypt cells, suggesting a role for PTHrP in differentiating intestinal epithelial cells. Our results suggested that PTHrP and PTH/PTHrP receptor plays some roles in gastric mucosal cell growth and differentiation.

Double staining of BrDU clearly demonstrated that cells with BrDU incorporation were negative for both PTHrP and PTH/PTHrP receptor, although they were closely located in the mucosal generative zone. Furthermore PTH/PTHrP receptor expression and BrDU incorporation fluctuated reciprocally under different conditions (RWI stress and steady state). These findings indicate that S phase cells do not express PTHrP and PTH/PTHrP receptor. The similar pattern was observed in PTH/PTHrP receptor and PCNA expression in benign soft tissue tumor. There have been a limited number of studies on cell cycle and PTHrP. Okano et al indicated a role for PTHrP in the process of smooth muscle cell division. They reported that constitutive immunoreactive levels of PTHrP are low in normally cycling vascular smooth muscle cell and PTHrP-immunoreactive cells were enriched in G2/M.

Immunoreactivity of PTHrP in gastric tissue has been previously reported. Smooth muscle cells in the proper muscle layer and small arteries showed immunoreactivity. PTH/PTHrP receptor expression was reduced abruptly in the most differentiated cells of the gastric mucosa. Similar expression pattern has been reported in the enchondral bone formation.

Cell proliferative effect of PTHrP-(1-34) might be elicited directly through PTH/PTHrP receptor and followed via signal transduction pathways. But there is a possibility to elicit PTHrP effect through cooperation of other cytokines, for instance EGF. PTHrP-(1-34) increases the synthesis of EGF receptors during differentiation process of placenta. In addition, EGF activating the PKC pathway are involved in the up regulation of PTHrP expression in mammary epithelial cells. TGF β is one of up-regulatory factors for PTHrP, and is known to be an apoptotic factor and involved in intestinal mucosal differentiation.

PTH/PTHrP receptor mRNA increased at RWI stress. This up-regulation was induced by hypercorticosteronemia. A previous study demonstrated that dexamethasone dramatically increased steady state levels of PTH/PTHrP receptor mRNA in a time- and dose-dependent manner. In this study serum corticosterone level increased about three times higher than non-stressed control, and the steroid inhibitor (metyrapone, 11b-hydroxylation inhibitor) pretreatment abolished up-regulation of PTH/PTHrP receptor mRNA was abolished by (data not shown). This up-regulation of PTH/PTHrP receptor at RWI stress explains the effective cell proliferative activation by the external PTHrP-(1-34) compared with steady state control. In contrast, PTHrP-(1-34) did not increase BrDU incorporation in a steady state when PTH/PTHrP receptor expression was in a basal level. These findings suggest that PTHrP and its cognate receptor play an important role on maintaining the mucosal integrity rather than simple cell proliferation or differentiation.

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