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Expression of midkine and receptor-like protein tyrosine phosphatase (RPTP)-β genes in the rat stomach and the influence of rebamipide

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SUMMARY

Background: Midkine has been reported to bind to receptor-like protein tyrosine phosphatase (RPTP)-β and to play important roles in growth and differentiation of various cells. Midkine is expressed in rat stomach during experimental ulcer healing, suggesting that the midkine-RPTP-β system has some physiological functions in the stomach. Rebamipide is a mucoprotective drug used for the treatment of gastric ulcers. We have tested the hypothesis that the ulcer healing mechanism stimulated by rebamipide is linked physiologically to the gastric midkine-RPTP-β system.

Materials and methods: Seven-week-old-male Wistar rats were used. Midkine and RPTP-β gene expression in rat stomach was investigated by laser capture microdissection coupled with the reverse transcription-polymerase chain reaction (RT-PCR). The effects of rebamipide on midkine and RPTP-β expression in rat stomach and the gastric epithelial cell line RGM1 were evaluated by RT-PCR and Northern blot analyses.

Results: Midkine and RPTP-β expression was detected in the gastric mucosal, submucosal and muscle layers. Rebamipide stimulated both midkine and RPTP-β expression in rat stomach and RGM1 cells.

Conclusion: Rebamipide may protect the gastric mucosa by regulating midkine and RPTP-β expression.

INTRODUCTION

Many growth factors and their receptors are known to play important roles in gastric mucosal proliferation. Midkine gene expression during the process of experimental gastric ulcer healing suggests that midkine plays some roles in the stimulation of gastric mucosal proliferation.[1] Midkine was identified as the product of a retinoic acid-responsive gene and it is a member of a new family of heparin-binding growth/differentiation factors.[2, 3] Midkine stimulates the proliferation and migration of several kinds of cells and is mainly involved in neuronal organogenesis.[4–6] However, little is known about the expression and distribution of its receptor in the stomach.

Recently, midkine was demonstrated to bind to a receptor-like protein-tyrosine phosphatase (RPTP)-β[7] a chondroitin sulphate proteoglycan that is expressed abundantly in the brain.[8] The extracellular region of RPTP-β consists of an N-terminal carbonic anhydrase-like domain, a fibronectin type III-like domain and a large cysteine-free, serine-glycine-rich region. It also has two intracellular protein-tyrosine phosphatase (PTP) domains.[8, 9] In the central nervous system, the midkine-RPTP-β system has some physiological functions, such as cellular proliferation, differentiation and adhesion.[5, 7, 10] However, the role of this system in the gastric mucosa is still unknown.

In this study, the distributions of midkine and RPTP-β in rat stomach were investigated using laser capture

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microdissection (LCM) coupled with the reverse transcription-polymerase chain reaction (RT-PCR). Furthermore, we evaluated the effects of rebamipide, a mucoprotective drug used for gastric ulcers and acute gastritis[11] on midkine and RPTP-β expression in rat gastric mucosa and in a gastric epithelial cell line, RGM1.

MATERIALS AND METHODS

Animals

Seven-week-old male Wistar rats, which were cared for and handled in accordance with National Institutes of Health guidelines, were used for all the experiments. They were fasted for 24 h before an experiment but were allowed free access to water. Four rats per group were used for the detection of midkine and RPTP-β mRNA in the gastric mucosa by LCM coupled with the RT-PCR. In order to assess the effects of rebamipide on the expression of midkine and RPTP-β mRNA in the gastric mucosa, the rats were divided into five groups. The first group served as a control and the rats in the other four groups received a single dose of oral rebamipide (100 mg/kg, a gift from Otsuka Pharmaceutical Co, Tokushima, Japan) through a gastric tube and were killed under ether anaesthesia 1, 3, 6, 12 h later. The stomachs were removed, opened along the greater curvature, washed with physiological saline, snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

Cell culture

In order to study the effects of rebamipide *in vitro*, the rat gastric mucosal cell line RGM1, a normal gastric epithelial cell line[12, 13] was used. The cells were cultured in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (1:1, vol:vol) supplemented with 5% heat-inactivated fetal bovine serum. Four groups of cultured cells were inoculated on to collagen type I-coated plastic culture dishes. The first group served as a control and other three were incubated with 0.5 mM rebamipide for 3, 6 and 12 h before the total RNA was extracted.

LCM

Fresh frozen stomach tissues were cut into 5-μm-thick sections in a cryostat, mounted on uncoated glass slides, fixed immediately with 70% ethanol for 30 s and washed with diethylpyrocarbonate (DEPC)-treated water for 30 s. Then, the sections were stained rapidly with haematoxylin for 30 s, dehydrated by passage through an ethanol gradient, counter-stained with eosin Y for 1 min, dehydrated again with an ethanol gradient, cleared with xylene and air-dried. The sections were laser microdissected using an LCM system LM200 (Olympus, Tokyo, Japan), as reported previously.[14, 15] In brief, tissue sections on slides were placed on the stage of a microscope and an area of tissue was selected and covered with LCM transfer film. The focus of the microscope was adjusted so that the field of view of the required specific portion of the tissue was the same size as that of the desired target. Then, under direct microscopic observation, the targeted cells were subjected to brief laser pulses from a laser beam 30 μm in diameter and a laser power of 60 mW directly above. The samples captured by 3000 shots on each transfer film cap were immersed in RNA extraction solution.

RNA extraction and RT-PCR analysis

Total RNA was extracted from frozen tissues of whole stomach, each population of laser-captured cells and cultured cells by a single-step guanidinium thiocyanate-phenol-chloroform method (Isogen; Nippon Gene Co., Tokyo, Japan). Each RNA sample was subjected to RT using a First Strand Synthesis Kit (Stratagene Toyobo, Tokyo, Japan) to prepare cDNA. The resulting first-strand cDNA was amplified by the PCR method. When total RNA was extracted from laser-captured samples, the PCR was performed immediately after RT and RT-PCR amplification of these samples was performed as described previously.[16] The primers used for cDNA amplification were: midkine (499 bp), 5′-GTTGCCCTCT-TGGCTGTCAC-3′ and 5′-TGGTCTCCTGGCACTGGGCA-3′; RPTP-β cDNA (354 bp), 5′-CTGAAAGCCAGCTGGCT-GTA-3′ and 5′-TGGCACCCAAGTCCTGATAG-3′ and β-actin cDNA (560 bp), 5′-TGGGTATGGGTCAGAAG-GAC-3′ and 5′-GCCATCTCTTGCTCGAAGTC-3′. RPTP-β has three alternative splicing isoforms and it was not known which of these was expressed in the stomach. In this study therefore the RPTP-β primer was set in the place of common region. After denaturation of the samples at 95 °C for 10 min, the PCR was carried out in a DNA thermal cycler (Perkin Elmer, Foster City, CA, USA). Thirty amplification cycles each comprising 95 °C for 30 s, 55 °C for 30 s and 72° for 90 s followed

by a final step at 72 °C for 7 min were performed. The PCR products were sequenced directly and RNA extracted from rat brain was used as a positive control.

Northern blot analysis

The total RNA was extracted from each frozen tissue with ISOGEN, separated by electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Shuell, Dassel, Germany). The probes used for Northern blot analysis were a ^{32}P-labelled 0.5-kb EcoRI cDNA fragment of rat midkine and a ^{32}P-labelled 0.8 kb EcoRI cDNA fragment of β-actin. Hybridization was carried out at 42 °C, then the membranes were washed first for 10 min and then for 15 min at room temperature with 2 × saline-sodium citrate containing 0.2% sodium dodecyl sulphate and finally for 20 min at 50 °C in 0.1 × saline-sodium citrate containing 0.2% sodium dodecyl sulphate, as described previously. A bioimage analyser (BAS 2000; Fujix, Tokyo, Japan) was used to detect the radiolabelled DNA probes. The intensity of each signal was measured and standardized with reference to the intensity of the β-actin mRNA signal.

Statistical analysis

All data were expressed as means ± S.E.M. and differences between means were compared by Student's *t*-test, performed using Stat-View 4.0 software (Abacus Concepts, Inc., USA). Differences at $P < 0.05$ were considered significant.

RESULTS

Expression of midkine and RPTP-β genes in the rat stomach and RGM1 cells

Midkine and RPTP-β expression in the stomach was assessed using LCM coupled with the RT-PCR. As shown in Figure 1, LCM with microscopic observation allowed us to collect samples precisely from the mucosal, submucosal and muscle layers. Separate samples from the targeted layers were obtained and bonded to the transfer film. When the film and cap were lifted from the tissue section, untargeted and unimpregnated tissues remained attached to the glass slide. Consequently, the morphology of the transferred cells was preserved and could be readily visualized under a microscope (Figure 1). Figure 2 shows the results of RT-PCR analysis of the LCM-dissected gastric cells. Midkine and RPTP-β expression was detected in the mucosal, submucosal and muscle layers. RGM1 cells also expressed midkine and RPTP-β.

Effects of rebamipide on midkine and RPTP-β expression in rat stomach and RGM1 cells

Expression of midkine and RPTP-β in the stomach after the administration of rebamipide was investigated by both RT-PCR and Northern blot analyses. Rebamipide significantly increased the expression levels of midkine and RPTP-β, with the peak occurring 1 h after administration (Figure 3). Figure 4 shows the effects of rebamipide on the expression of midkine and RPTP-β genes, detected by the RT-PCR, in RGM1 cells. Expression of both genes was stimulated in a time-dependent manner up to 3 h after rebamipide administration.

DISCUSSION

Midkine has been reported to have several functions, including nerve cell proliferation and migration. It also has a growth-promoting effect on fibroblasts[17] and enhances reconstruction of tissues after injury.[18] In a previous study, we showed that midkine expression increased mainly in the gastric submucosal and muscle layers during healing of acetic acid-induced chronic gastric ulcers[1] and suggested that midkine may play several roles in gastric mucosal proliferation, particularly in the formation of granulation tissue.

RPTP-β has been identified as a midkine receptor and it also has some physiological functions, such as cellular proliferation, migration and adhesion.[5, 19] In order to assess the role of the midkine-RPTP-β system in the gastric mucosa, we used LCM coupled with the RT-PCR to investigate the distributions of midkine and RPTP-β in rat stomach. The recent development of LCM allows researchers to obtain pure cell populations using a microscope and analyse gene expression in the microdissected cells. In this study, each tissue section was divided into three layers (mucosa, submucosa and muscle) and cells in each layer were captured separately. Our results showed that both midkine and RPTP-β were expressed in all the layers of the stomach. We also detected midkine and RPTP-β expression in RGM1 cells. These results suggest that midkine can stimulate not only stromal cells, but also gastric

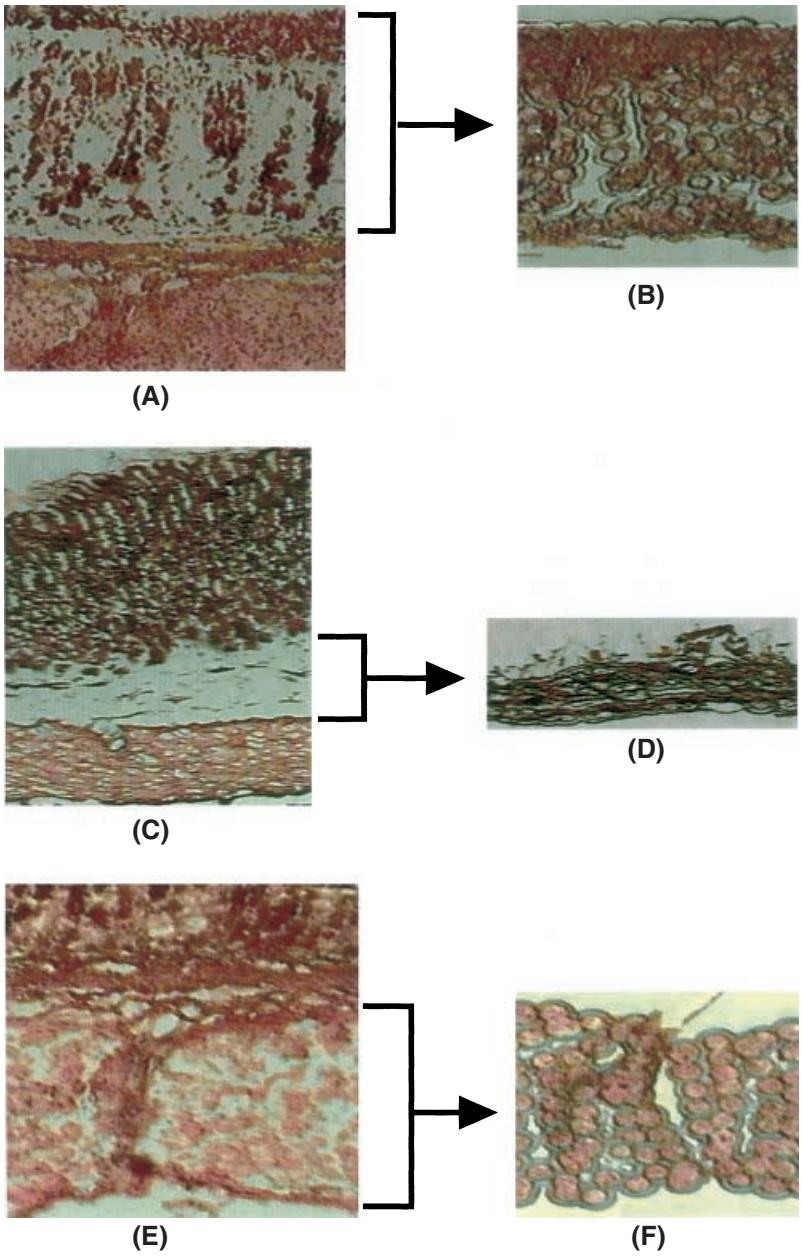

Figure 1. LCM of fresh frozen gastric tissue stained with haematoxylin-eosin. Sections showing remnants after removal of the target cells from mucosal (A), submucosal (C), and muscle (E) layer by LCM. Captured cells from mucosal (B), submucosal (D), and muscle (F) layer are shown on the transfer film.

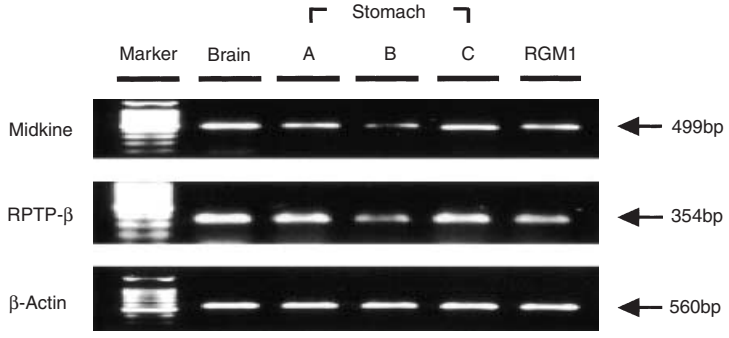

Figure 2. RT-PCR results showing midkine and RPTP-β gene expression in rat stomach and RGM1 cell line. Expression in mucosal (A), submucosal (B), and muscle (C) layer of stomach was analysed by LCM coupled with RT-PCR. As internal control, expression of β-actin was detected.

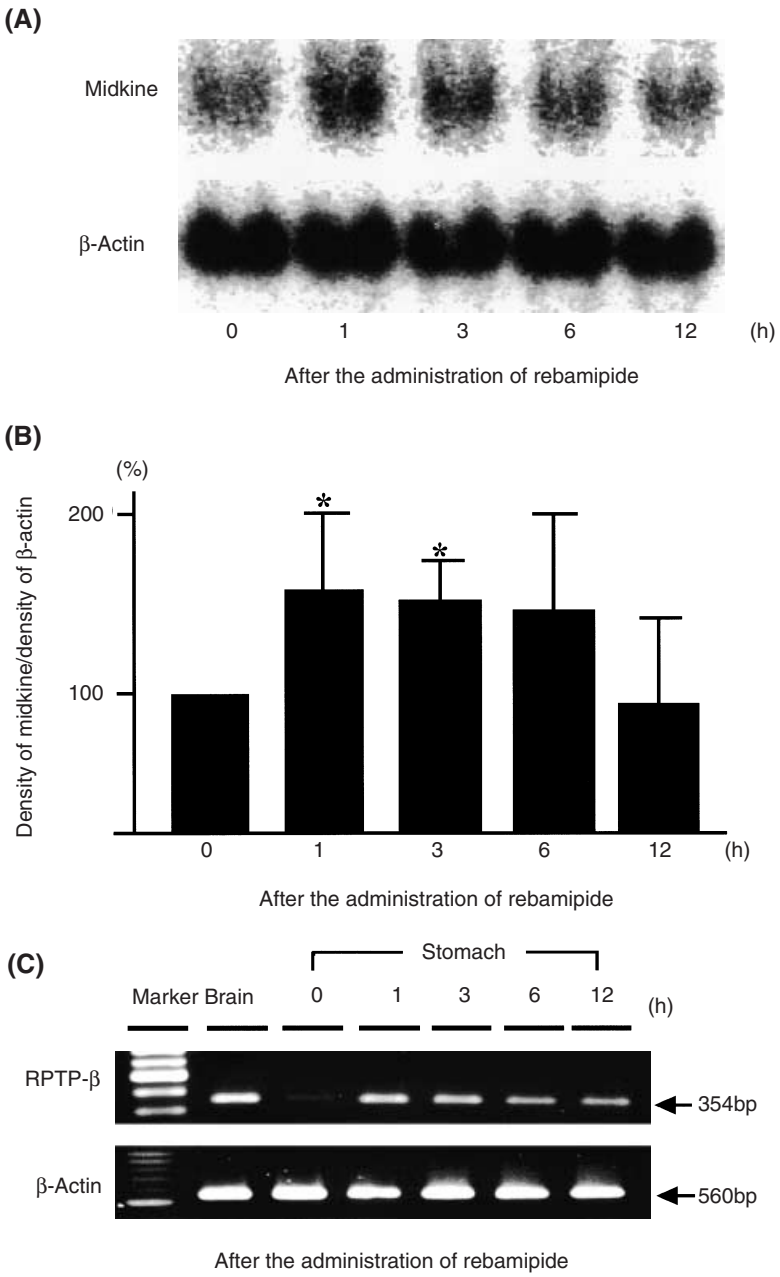

Figure 3. Northen blot (A) and densitometomeric (B) analysis of midkine mRNA expression in rat stomach after rebamipide administration. The signal intensity of midkine was normalized against β-actin. (C) RT-PCR analysis of RPTP-β mRNA in corresponding stomach samples. Rebamipide significantly increased the expression of midkine as well as RPTP- β 1 h after the administration. $^{*}P < 0.05$ ($n = 5$).

epithelial cells, via RPTP-β. Cell-to-cell communication between stromal and epithelial cells is essential to preserve the physiological and immunological functions of the gastrointestinal tract and to enhance mucosal regeneration stimulated by the combination of several kinds of growth factors. The midkine-RPTP-β system may be one of the systems involved in the regulation of gastric mucosal functions, particularly the process of healing damaged gastric mucosa. Previously, we examined the proliferation of RGM1 cells after the stimulation with various concentration of midkine by measuring 3H-tymidine incorporation. However, exogenous midkine did not stimulate the DNA synthesis of RGM1 cells.[1] Therefore, we speculate that midkine may accelerate the epithelial cell migration, without a significant influence on cell proliferation.

Rebamipide, a gastroprotective drug, has been reported to prevent the development of various acute experimental gastric mucosal lesions and to accelerate the healing of chronic gastric ulcers.[11, 20] It also has the

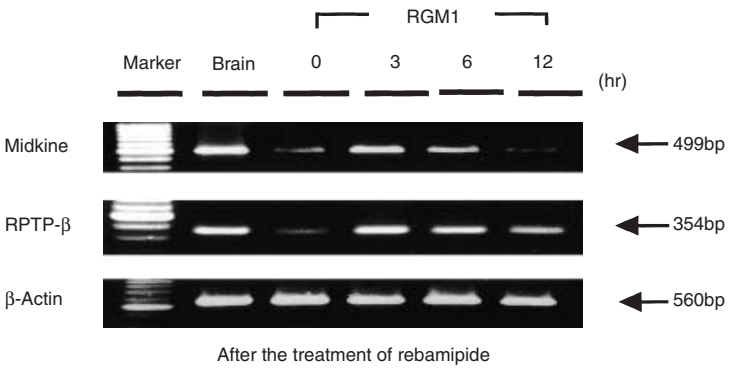

Figure 4. RT-PCR analysis of midkine and RPTP-β gene expression in RGM1 cell line. Expression of β-actin was detected as internal control. Expression of midkine and RPTP-β was stimulated in RGM1 cells up to 3 h after rebamipide treatment.

ability to inhibit neutrophil activity[21] reduce production of inflammatory cytokines[22] scavenge oxygen free radicals[23] and stimulate prostaglandin synthesis.[11] In a previous study, we found that rebamipide stimulated gastric mucus secretion by augmenting prostaglandin EP4 receptor expression in damaged gastric mucosa.[24] However, the gastroprotective mechanism of rebamipide is not completely understood. In this study, we found that rebamipide stimulated midkine and RPTP-β gene expression in rat gastric mucosa and RGM1 cells, suggesting that this system may be one of the mechanisms whereby rebamipide accelerates gastric ulcer healing.

Recently, a vacuolating cytotoxin (VacA) of *Helicobacter pylori* was demonstrated to interact with target cells by binding to RPTP-β.[25] Further studies are necessary to determine the precise roles of the midkine-RPTP-β system in the stomach during *H. pylori* infection.

In conclusion, midkine and RPTP-β gene expression was detected in rat stomach and was augmented by administration of rebamipide. These findings suggest that rebamipide provide gastric mucosal protection by influencing the midkine-RPTP-β system.

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