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Heparin-Binding EGF-like Growth Factor Is an Autocrine Growth Factor for Rat Gastric Epithelial Cells

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We examined the biological action and expression of heparin-binding EGF-like growth factor (HB-EGF) in a rat gastric mucosal cell line, RGM1. HB-EGF stimulated DNA synthesis of RGM1 cells in a dose-dependent manner. Mitogenic effect of HB-EGF was as potent as that of other known mitogens for gastric epithelial cells, such as hapatocyte growth factor (HGF) and transforming growth factor (TGF)-α. Northern blot analysis showed that RGM1 cells as well as rat gastric mucosal tissue expressed a 2.5-kilobase transcript of HB-EGF. Not only HB-EGF and TGF-α but also HGF caused a rapid induction of HB-EGF mRNA in the cells. Treatment with heparitinase which destroys heparan sulfate proteoglycan (HSPG) or with chlorate which inhibits sulfation of HSPG diminished [^{3}H]thymidine incorporation of RGM1 cells in serum-free medium. In addition, a synthetic peptide corresponding to the heparin-binding domain of HB-EGF inhibits the DNA synthesis of RGM1 cells in serum-free medium in a dose-dependent manner. These results suggest that HB-EGF is an autocrine and paracrine growth factor for gastric epithelial cells and may play significant roles in mucosal repair of the stomach in cooperation with other growth factors.

The gastric mucosa is a rapidly proliferating tissue. Several growth factors including epidermal growth factor (EGF) (1,2), transforming growth factor-α (TGF-α) (2,3), insulin, insulin-like growth factor 1 (2), hepatocyte growth factor (HGF) (4,5) and keratinocyte growth factor (6) have been reported to induce a mitogenic response of gastric epithelial cells. Among these factors, the physiological roles of TGF-α and HGF for gastric mucosal repair have been emphasized (7,8). Investigations have suggested that TGF-α is produced by gastric epithelial cells (7) and that it may act as an autocrine and paracrine growth factor. More recently, it was shown that HGF, produced by mesenchymal cells, is a potent mitogens for gastric epithelial cells in primary culture (4,5) and for a rat gastric mucosal cell line, RGM1 (8).

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family initially purified from conditioned medium of the U-937 macrophage-like cell line (9). This growth factor is characterized by its heparin-binding domain, which interacts with cell surface heparan sulfate proteoglucan (HSPG) (10–12). Subsequent investigations suggested that HB-EGF may participate in autocrine and paracrine activation of EGF receptor in normal and cancerous epithelial cells (13,14). Although HB-EGF and its transcript were detected in human gastric mucosa (15), the action of HB-EGF on gastric mucosal epithelial cells has not been well investigated. In the present study, we report that HB-EGF is a potent mitogen for a rat gastric mucosal cell line, RGM1, and that HB-EGF mRNA is expressed in this cell line as well as in rat gastric mucosa, suggesting that HB-EGF may act as an autocrine and paracrine growth factor for gastric epithelial cells.

METHODS

Cell lines and reagents. The rat gastric mucosal cell (RGM1) line was obtained from Riken Cell Bank (Tsukuba, Japan). RGM1 cells are a diploid, non-transformed epithelial line isolated from normal Wister's rat gastric mucosa (Matsui and

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Ohno, 1988) (8,16). The cells were grown in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum. All assays were done before passage 20. Recombinant human HB-EGF and TGF-α were purchased from R&D Systems, Inc. and Upstate Biotechnology, Inc., respectively. Recombinant human HGF was a generous gift from Dr. T. Nakamura, Division of Biochemistry, Biomedical Research Center, Osaka University Medical School.

Mitogenic assays. RGM1 cells were seeded in 96well plates. Cells were placed in serum-free medium for 48 hr as soon as confluence was reached. Thereafter, the cells were stimulated with the indicated dose of HGF, TGF-α and HB-EGF for 18 hr and labeled with 1 mCi/mL [^{3}H]thymidine between the 18th and 22th hr after treatment. After washing with phosphate-buffered saline three times, the cells were harvested by trypsinization and [^{3}H]thymidine incorporated into the DNA was counted with Betaplate System (Pharmacia). To investigate the role of cell surface heparan sulfate proteoglycan (HSPG) in autocrine growth of RGM1 cells, cells were incubated with either 20 mU/mL heparitinase I (EC 4.2.2.8) or 10 mmol/L chlorate in serum free medium. Effects of treatment with a synthetic peptide corresponding to the heparin-binding domain of HB-EGF on DNA synthesis of RGM1 cells were also determined. Cells were incubated in serum free medium containing the indicated dose of a synthetic peptide (P21) corresponding to the putative 21-amino acid heparin-binding sequence KRKKKGKGLGKKRDPCLRKYK (10,17) human HB-EGF (amino acids 93 through 113 of the 208-residue precursor).

Induction of HB-EGF mRNA by TGF-α, HB-EGF and HGF. Cells were grown in 150-mm dishes. After they reached confluence, serum was deprived for 48 hr. Thereafter, cells were restimulated by 10 ng/mL of TGF-α, HGF, or HB-EGF in serum free medium. Total RNA was extracted 0, 2, 4, 8 or 24 hr after treatment by the method described by Chomczynski and Sacchi (18).

Extraction of total RNA from rat gastric mucosa. A male Wister's rat was used in this experiment. After the rat was killed, the stomach was removed, opened along the lesser curvature and gently washed with physiological saline. Then, gastric mucosa was scraped from the underlying submucosa by glass slides and homogenized in Denaturing Solution containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH7.0), 0.5% sarcosyl, and 0.1M 2-mercaptoethanol. Total RNA was extracted by the method described above.

Northern blot analysis. Ten micrograms of total RNA were fractionated on 1.0% agarose/2.2 M formaldehyde denaturing gels, transferred to membrane (Hybond-N+, Amersham), and UV cross-linked (1200mJ). Hybridization was performed using a Rapid-hyb buffer (Amersham) according to the protocol. The cDNA probes used here were 590-bp KpnI-EcoRI fragment of rat HB-EGF cDNA and a 1.1-kb XbaI-HindIII fragment of rat GAPDH. The probes were labeled with [^{32}P]dCTP by random priming using Multiprime DNA Labeling System (Amersham).

Statistics. Data were expressed as mean ± S.E. Statistical comparisons between two groups were made using the Mann-Whitney U test.

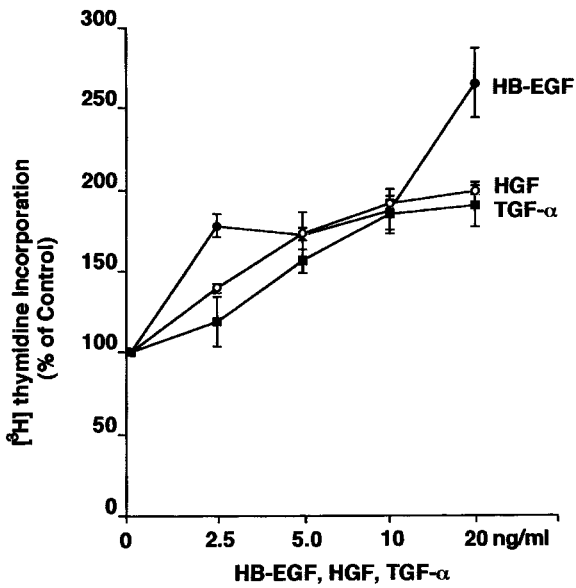

FIG. 1. Effects of HB-EGF, TGF-α, and HGF on DNA synthesis of RGM1 cells. RGM1 cells were seeded on 96 well plates. Following serum starvation for 48 hr, cells were incubated in serum-free medium containing the indicated dose of the reagents for 18 hr. Four hours after addition of [^{3}H]thymidine, radioactivity incorporated into the DNA was counted with the Betaplate system. Values represent the mean ±S.E. from six preparations.

RESULTS

Effects of HB-EGF, TGF-α, and HGF on DNA Synthesis of RGM1 Cells

The DNA synthesis of RGM1 cells was stimulated by HB-EGF in a dose-dependent manner up to 20 ng/mL (Fig. 1). TGF-α and HGF also stimulated DNA synthesis of RGM1 cells in a dose dependent manner. Mitogenic effect of HB-EGF on RGM1 cells was as potent as that of TGF-α and HGF.

Effects of Heparitinase, Chlorate, and P21 on DNA Synthesis of RGM1 Cells

We examined the effects of heparitinase I and chlorate on autocrine growth of RGM1 cells. Both 20 mU/mL heparitinase and 10 mmol/L chlorate diminished the DNA synthesis of RGM1 cells to the level about 60% of the control value in serum free medium (Fig. 2). Then, RGM1 cells were incubated in serum free medium with a synthetic peptide corresponding to the heparin-binding domain of HB-EGF (P21) to clarify the role of HB-EGF on autocrine growth of RGM1 cells. The synthetic peptide P21 inhibited the DNA synthesis of RGM1 cells significantly (Fig. 2) and in a dose-dependent manner (Fig. 3).

Effects of HB-EGF, TGF-α, and HGF on HB-EGF Gene Expression in RGM1 Cells

Northern blot analysis showed a 2.5-kb transcript of HB-EGF mRNA in RGM1 cells (Fig. 4). HB-EGF, TGF-α and HGF induced the expression of HB-EGF mRNA in a similar manner. In every case, maximal expression of HB-EGF mRNA was detected 2–hr later.

HB-EGF Gene Expression in Rat Gastric Mucosa

Northern blot analysis of total RNA extracted from the mucosa of a Wister's rat stomach revealed a 2.5-kb HB-EGF transcript (Fig. 5).

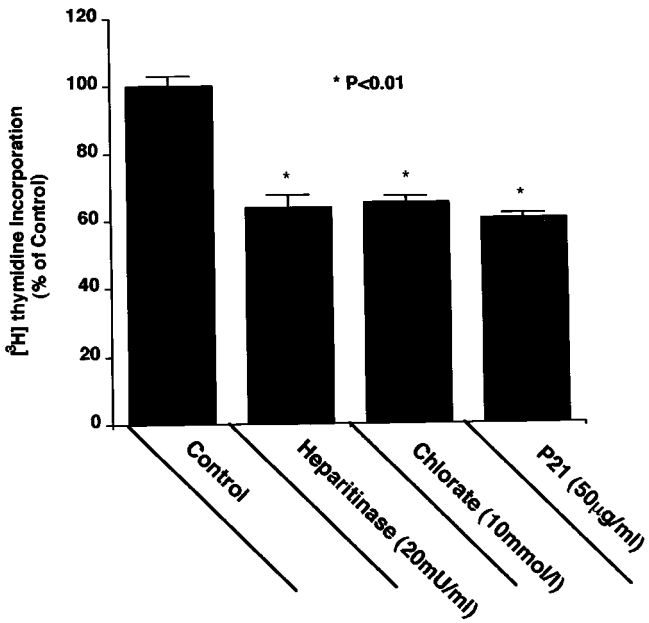

FIG. 2. Effects of heparitinase, chlorate, and a synthetic peptide corresponding to the heparin-binding domain of HB-EGF (P21) on DNA synthesis of RGM1 cells. [^{3}H]thymidine incorporation to the DNA was determined after the cells were incubated with 20 mU/mL heparitinase, with 10 mmol/L chlorate, or with 50 μg/mL P21 for 18 hr. Values represent the mean ±S.E. from six preparations. *P < 0.01 compared with control.

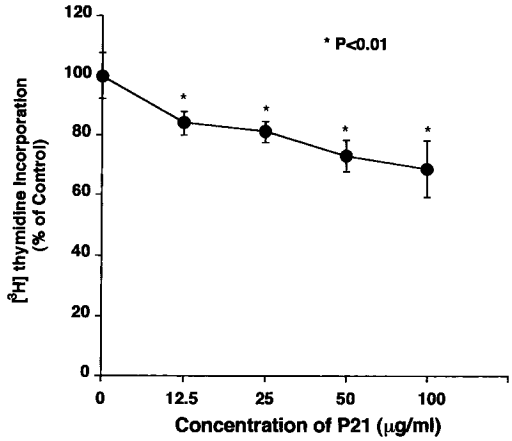

FIG. 3. Dose-dependent inhibition of DNA synthesis of RGM1 cells by a synthetic peptide corresponding to the heparin-binding domain of HB-EGF (P21). [^{3}H]thymidine incorporation to the DNA was determined after the cells were incubated with the indicated dose of a synthetic peptide corresponding to the heparin-binding domain of HB-EGF (P21) for 18 hr. Values represent the mean ±S.E. from six preparations. *P < 0.01 compared with control.

DISCUSSION

The present study showed that HB-EGF, one of the EGF-related polypeptides, is a potent mitogen for a rat gastric mucosal cell line, RGM1. The activity of HB-EGF was as potent as other mitogens for gastric epithelial cells such as TGF-α and HGF. Northern blot analysis clearly demonstrated a 2.5-kb transcript of HB-EGF in RGM1 cells, indicating that the cells produce HB-EGF. HB-EGF is characterized by its interaction with cell surface heparin sulfate proteoglycan (HSPG) (10). It was shown in the literature that the bioactivity of HB-EGF diminished after treatment of cells with heparitinase which destroys cell surface HSPG or with chlorate which

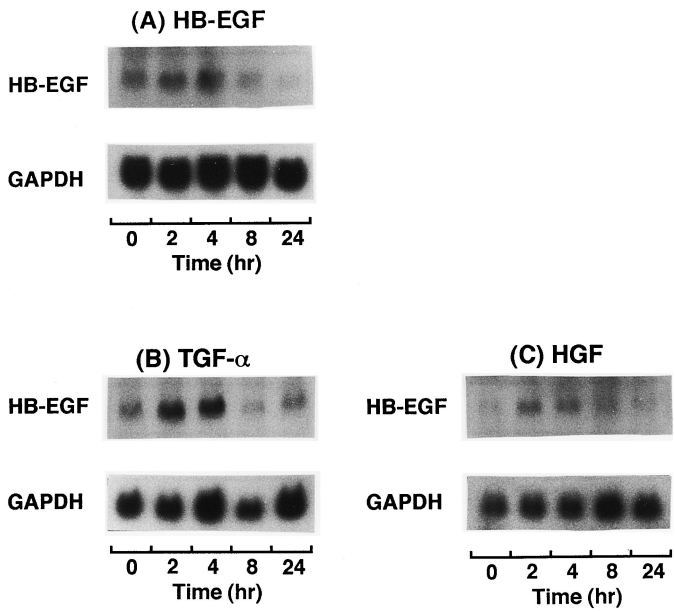

FIG. 4. Effects of HB-EGF (A), TGF-α (B) and HGF (C) on HB-EGF gene expression in RGM1 cells. Following serum starvation for 48 hr, cells were placed in serum-free medium containing 10 ng of HB-EGF, TGF-α or HGF. Total RNA was extracted 0, 2, 4, 8 and 24 hr later. Ten micrograms of total RNA was used for Northern blot analysis.

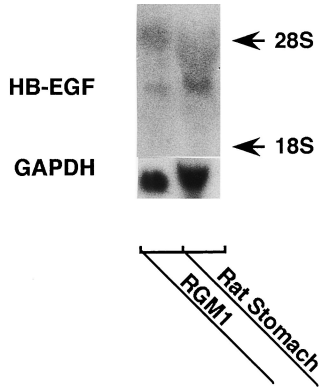

FIG. 5. HB-EGF gene expression in rat gastric mucosa. Total RNA was extracted from the mucosa of a Wister rat stomach. HB-EGF gene expression was assessed by Northern blot analysis.

inhibits sulfation of HSPG (10). In the present study, treatment with heparitinase or with chlorate inhibited the DNA synthesis of RGM1 cells in serum-free medium. Moreover, the synthetic peptide corresponding to the putative heparin-binding domain of HB-EGF which blocks HB-EGF binding to HSPG specifically (10,11), also diminished [^{3}H]thymidine incorporation of the cells in serum free medium in a dose-dependent manner. These results suggest that HB-EGF is an autocrine and paracrine growth factor for the cells.

It was reported that expression of EGF-family polypeptides is auto- and cross-induced within themselves in some epithelial cell lines (13,14). In the present study, both HB-EGF and TGF-α enhanced expression of HB-EGF mRNA in RGM1 cells and maximal expression was observed 2–4 hr after treatment with the reagents. These observations are compatible with the results of the previous investigations. Moreover, HGF which binds to the c-Met receptor but not to the EGF-receptor also enhanced the expression of HB-EGF mRNA. Investigations have indicated that both TGF-α and HGF are potent mitogens for gastric epithelial cells (4,5,8). Our results suggest that HB-EGF may act cooperatively with other mitogens for gastric epithelial cell proliferation.

Northern blot analysis of total RNA from rat gastric mucosa clearly showed the presence of a 2.5-kb transcript of HB-EGF mRNA. Since poly-A RNA selection is not needed for detection of HB-EGF transcript in contrast to the case of TGF-α (7), it is likely that expression of HB-EGF is relatively high in rat gastric mucosa. In conclusion, the present study suggests that HB-EGF is a significant autocrine and paracrine growth factor for rat gastric epithelial cells.

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