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Epiregulin stimulates proliferation of rabbit gastric cells in primary culture through autophosphorylation of the epidermal growth factor receptor

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Abstract

Epiregulin, a growth factor of the epidermal growth factor (EGF) family, was recently purified from conditioned medium of a mouse fibroblast-derived tumor cell line. It was reported that epiregulin exhibited bifunctional properties in the regulation of cell growth. However, the effect of epiregulin on gastric cell proliferation is not known. The aims of this study were to determine whether: (1) epiregulin affects proliferation of rabbit cultured gastric cells, (2) epiregulin-induced stimulation of cell proliferation is mediated by the tyrosine kinase pathway, and (3) epiregulin stimulates autophosphorylation of EGF-receptors. Epiregulin stimulated cell proliferation to a significant extent. This effect was completely blocked by treatment with genistein. Epiregulin stimulated tyrosine phosphorylation of a 170 kDa protein, which represents the EGF receptor, in a dose-dependent fashion. These findings suggest that epiregulin has mitogenic effects on rabbit gastric cultured cells, possibly mediated via the tyrosine kinase pathway through autophosphorylation of EGF receptors.

Keywords: Epiregulin; Growth factor; Cell proliferation; Tyrosine phosphorylation; Epidermal growth factor; Epidermal growth factor receptor; Gastric cell

1. Introduction

Epiregulin, an EGF-related growth-regulating peptide, was purified from conditioned medium of the NIH3T3/clone T7 mouse fibroblast-derived tumor cell line. It is a 46-amino acid single chain peptide, and its amino acid sequence exhibits 24–50% amino acid sequence identity with sequences of other EGF-related growth factors. Epiregulin exhibited bifunctional regulatory properties: it inhibited the growth of epithelial tumor cells such as HeLa, A431, and lung carcinoma A 549 cells, but stimulated the growth of fibroblasts and various other types of cells including Balb/c 3T3 A31 cells, rat primary hepatocytes, and human aortic smooth muscle cells (Toyoda et al., 1995). However, the effect of epiregulin on gastric cell proliferation is not known.

The aims of this study are to determine whether: (1) epiregulin affects proliferation of rabbit cultured gastric cells, (2) epiregulin-induced stimulation of cell proliferation is mediated by the tyrosine kinase pathway and (3) epiregulin stimulates autophosphorylation of EGF-receptors.

2. Materials and methods

2.1. Chemicals

Epiregulin was a gift from Taisho Pharmaceutical (Tokyo, Japan). EGF was purchased from Wako (Osaka, Japan). Genistein (GIBCO, Grand Island, NY, USA) was dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO, USA).

2.2. Gastric monolayer culture

Non-fasted Japanese white rabbits (Nihon Keari Breeding, Osaka, Japan) weighing 2.5–3.0 kg were used in this

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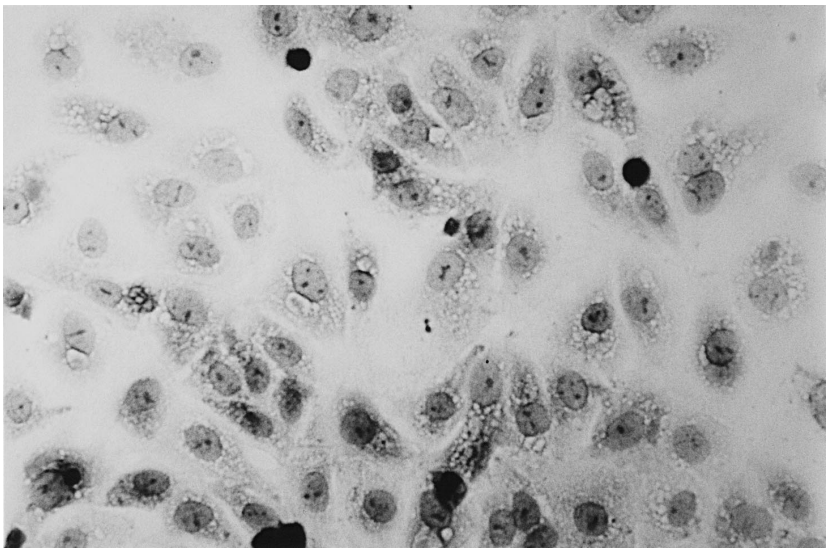

Fig. 1. PAS staining of cultured rabbit gastric cells. The majority of the cells are PAS-positive. (Original magnification: ×400.)

study. They were anesthetized with an intravenous injection of 50 mg/kg of Nembutal (Abbott, North Chicago, IL, USA). The stomach was opened along the greater curvature and the fundic area was excised. The gastric mucosa was then scraped and minced into 2–3 mm^3 pieces, and incubated in Hanks' balanced salt solution (HBSS; Nissui Pharmaceutical, Tokyo, Japan) containing 1.5 mg/ml collagenase (GIBCO) at 37°C for 45 min. Isolated gastric cell clumps were filtered through gauze and washed with sterile HBSS four times. The cells were cultured in Coon's modified Ham's F-12 medium (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS; GIBCO), 15 mM Hepes buffer (Wako), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 μg/ml gentamicin and 0.25 mg/ml amphotericin B (Sigma) at 37°C with 5% CO_2 in air in a humidified atmosphere (Ota et al., 1990). The cells were cultured on collagen type I-coated culture plates (Sumitomo Bakerite Medical, Tokyo, Japan). To identify mucus-secreting cells, cells were cultured on chamber slides (Nunc, Naperville, IL, USA) and periodic acid-Schiff (PAS) staining was performed.

2.3. Evaluation of cell proliferation

Initially, gastric cells (5×10^4 cells/well) were inoculated onto culture plates. After one day of culture, the medium was changed to serum-free medium instead of 10% FBS, and various doses of epiregulin (0–100 ng/ml) or EGF (0–100 ng/ml) were added.

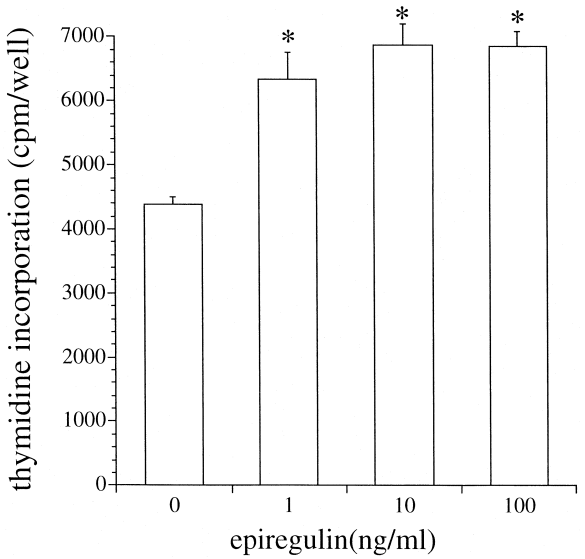

Fig. 2. Effect of epiregulin on proliferation of cultured rabbit gastric cells. Epiregulin stimulated proliferation of gastric cells to a significant extent. Mean ± SEM of six experiments are shown. * $p < 0.01$ versus controls.

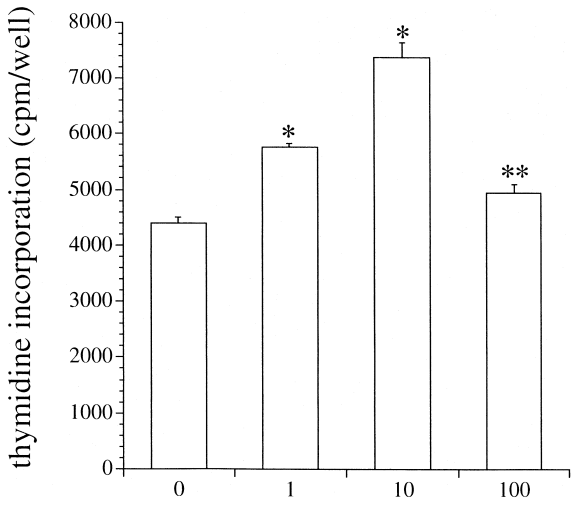

Fig. 3. Effect of EGF on proliferation of cultured rabbit gastric cells. EGF stimulated proliferation of gastric cells to a significant extent. Mean ± SEM of six experiments are shown. * $p < 0.01$, * * $p < 0.05$ versus controls.

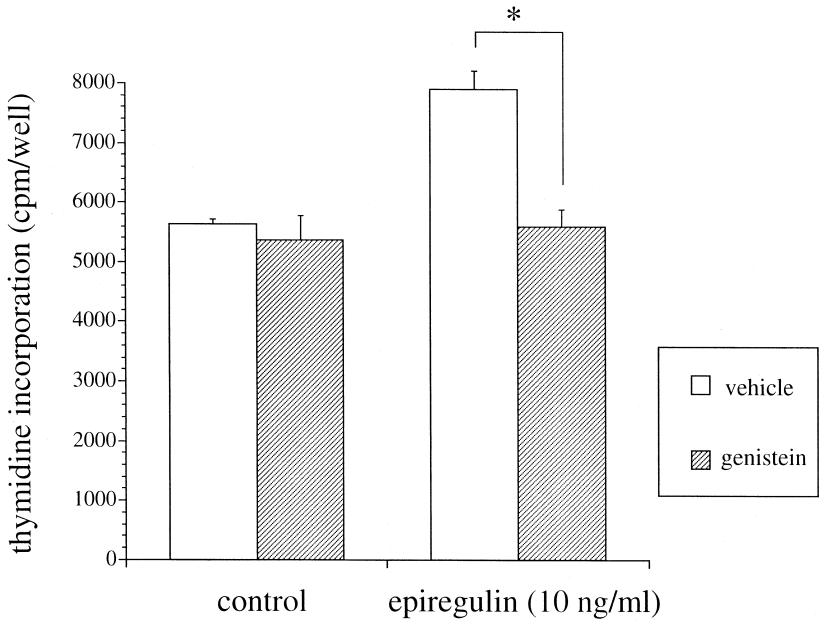

Fig. 4. Effect of genistein on stimulation of gastric cell proliferation by epiregulin. Genistein (20 μg/ml) completely blocked the gastric cell proliferation induced by epiregulin (10 ng/ml). Mean ± SEM of six experiments are shown. * $p < 0.01$ versus controls.

2.3.1. Thymidine incorporation study

Cells were incubated with 0.5 μCi/ml of [6-^{3}H]-thymidine (specific activity, 0.96 Bq/mmol; Amersham, Poole, UK) for 24 h. Cells were then washed three times with ice-cold PBS and incubated in 5% trichloroacetic acid (TCA) for 20 min at 4°C. The material precipitated in TCA was lysed in 1 M NaOH and radioactivity was measured with a liquid scintillation counter (LS-5801, Beckman Instruments, Fullerton, CA, USA). To examine the role of tyrosine kinase in cell proliferation, 20 μg/ml genistein (a tyrosine kinase inhibitor) was added to the cells 30 min before epiregulin treatment (Akiyama et al., 1987).

2.3.2. Mitotic index and bromodeoxyuridine labelling index

5-Bromodeoxyuridine (BrdU; Sigma), 10 μg/ml, was added and incubated for 24 h. After fixation, cells were stained with a monoclonal antibromodeoxyuridine antibody (Dakopatts, Glostrup, Denmark), and the labelling index of BrdU and the mitotic index were assessed (Fujiwara et al., 1993).

2.4. Electrophoresis and Western blot analysis

Subconfluent monolayers of gastric cells were used in these experiments. Cells were incubated with epiregulin or

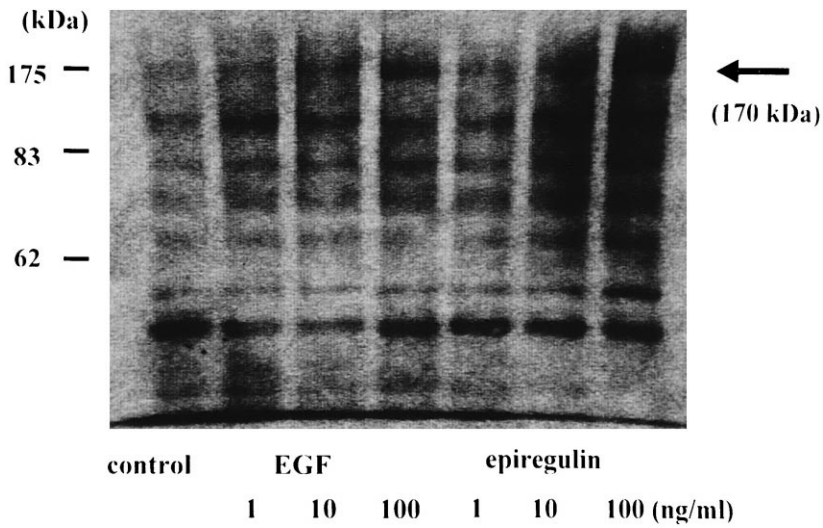

Fig. 5. Effect of epiregulin and EGF on autophosphorylation of cultured gastric cells. Epiregulin and EGF stimulated tyrosine phosphorylation of some proteins. Tyrosine phosphorylation of a 170 kDa protein, which corresponded to the EGF receptor, was stimulated the most.

EGF for 15 min at 37°C and then washed with sterile HBSS, and 0.5 ml/well of Laemmli SDS sample buffer (Wako) was added. Samples were heated at 95°C for 5 min and 15 μg protein aliquots were loaded onto 7.5% SDS–polyacrylamide gels. After separation, proteins were electrophoretically blotted onto Immobilon-P (Millipore, Bedford, MA, USA), using a transfer buffer containing 48 mM Trizma Base, 39 mM glycine and 20% methanol (Towbin et al., 1979). The Immobilon-P was soaked in Tris-buffered saline–Tween (TBST) containing 2% bovine serum albumin and incubated overnight at 4°C. It was then incubated with mouse monoclonal $IgG2b_k$ anti-phosphotyrosine antibody (clone 4G10, 0.5 μg/ml in blocking solution; Upstate Biotechnology, Lake Placid, NY, USA) for 1 h at room temperature, and washed with TBST buffer. The bound antigen–antibody complexes were detected with horseradish peroxidase-conjugated affini pure F(ab′)2 fragment goat anti-mouse IgG(H + L) (Immunotech, France), using enhanced chemiluminescence as described by the manufacturer's instructions (Amersham), and exposed to Amersham ECL detection film. The EGF-receptor autophosphorylation band at 170 kDa was quantified by 2-dimensional laser scanning densitometry.

2.5. Statistical analysis

Differences between experimental groups were evaluated statistically by the Mann–Whitney *U*-test. Values are expressed as means and standard errors of the mean (SEM). *P*-values less than 0.05 were considered significant.

3. Results

3.1. Cell population

PAS-positive cells were counted in 10 microscope fields ($n = 4$). The majority of cells ($90.6 \pm 1.1\%$) were PAS-positive (Fig. 1). Therefore they had the characteristics of gastric mucus-producing cells.

3.2. Effect of epiregulin and EGF on proliferation of cultured gastric cells

3.2.1. Thymidine incorporation

Epiregulin significantly stimulated thymidine incorporation into rabbit gastric cells at concentrations of 1–100 ng/ml (Fig. 2). The maximal effect was observed at a dose of 10 ng/ml epiregulin, which stimulated thymidine incorporation from 4382.5 ± 118.5 cpm/well in controls to 6873.0 ± 329.2 cpm/well ($p < 0.01$). This effect was similar to that of EGF at 10 ng/ml (Fig. 3).

3.2.2. Mitotic index and BrdU labelling index

Epiregulin significantly increased both the mitotic index and the Bromodeoxyuridine labelling index of rabbit gastric cells at concentrations of 10–100 ng/ml ($p < 0.01$, Table 1). This effect was similar to that of EGF.

Table 1
Effect of epiregulin and EGF on proliferation of cultured rabbit gastric cells.

	BrdU LI (%)[a]	Mitotic index (%)[b]
Control	0.2 ± 0.1	0.2 ± 0.1
Epiregulin (ng/ml)		
1	0.3 ± 0.1	0.2 ± 0.1
10	2.0 ± 0.8[c]	0.5 ± 0.0[c]
100	3.6 ± 2.7[c]	1.5 ± 0.1[c]
EGF (ng/ml)		
1	0.2 ± 0.1	0.2 ± 0.1
10	0.8 ± 0.3[c]	0.7 ± 0.1[c]
100	2.6 ± 1.1[c]	1.4 ± 0.3[c]

Epiregulin and EGF stimulated proliferation of gastric cells to a significant extent (10–100 ng/ml). Mean ± SD of four separate experiments are shown.
[a] BrdU labelling index (LI) (%): BrdU-positive cells/total cells in field.
[b] Mitotic index (%): dividing cells/total cells in field.
[c] $p < 0.01$ versus controls.

3.3. Effect of genistein on proliferation of cultured gastric cells induced by epiregulin

Genistein (20 μg/ml), a tyrosine kinase inhibitor, did not affect cell proliferation when administered alone, but completely blocked the epiregulin-induced cell proliferation, from 7889.0 ± 314.2 cpm/well to 5590.0 ± 299.9 cpm/well ($p < 0.01$) (Fig. 4).

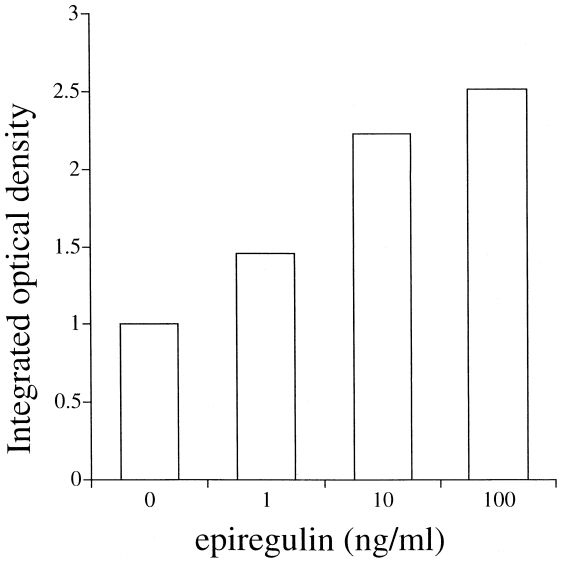

Fig. 6. Two-dimensional laser scanning densitometry analysis of autophosphorylation of the EGF receptor. Epiregulin stimulated autophosphorylation of the EGF receptor in a dose-dependent fashion.

3.4. Effect of epiregulin and EGF on tyrosine phosphorylation

Treatment with both epiregulin and EGF stimulated the tyrosine phosphorylation of some proteins. The phosphorylation of a 170 kDa protein, which corresponded to the EGF receptor, was stimulated the most (Fig. 5). Epiregulin promoted tyrosine phosphorylation of the 170 kDa protein in a dose-dependent manner, and the strongest stimulation was observed at 100 ng/ml, which evoked a 2.5-fold increase compared to control by 2-dimensional laser scanning densitometry analysis (Fig. 6).

4. Discussion

The present study demonstrated that epiregulin stimulates the proliferation of rabbit gastric cultured cells to a significant extent. This effect was similar to that of EGF. The potency of transforming growth factor-α (TGF-α) on the proliferation of rabbit gastric cells has been shown to be similar to that of EGF (Nakajima et al., 1993). Thus it appears that epiregulin, EGF, and TGF-α have similar potency in stimulating rabbit gastric epithelial proliferation.

Since epiregulin-induced proliferation was completely blocked by genistein, and both epiregulin and EGF stimulated phosphorylation of a 170 kDa protein, which corresponded to the EGF receptor, epiregulin-induced proliferation of gastric cells was mediated mainly via a tyrosine kinase pathway through EGF receptors.

Continuous cell renewal and proliferation is one of the major defensive mechanisms of the gastric mucosa. This mechanism is responsible for maintaining mucosal integrity, and enables healing of injuries and ulcers (Fujiwara et al., 1995). Gastric epithelial proliferation is controlled by various growth factors. In vitro, EGF, TGF-α, insulin, and hepatocyte growth factor have potent mitogenic effects on cultured gastric cells (Chen et al., 1991; Rutten et al., 1991, 1993; Takahashi et al., 1993, 1995; Yoshiura et al., 1994).

EGF is produced by the salivary glands and is present in the normal gastric lumen. However, during ulceration of the gastrointestinal mucosa, EGF is also locally produced by a novel cell type, which grows from the base of the glands lining the ulcer margin (Wright et al., 1990). In addition to the increased production of EGF during ulceration, EGF receptors, which are the major target of EGF, are highly expressed in mucosal cells at the ulcer margin (Tarnawski et al., 1992). These findings indicate that EGF plays an important role in gastric ulcer healing.

EGF initially binds to the EGF receptor, which is a 170 kDa glycoprotein possessing an extracellular binding domain and an intracellular tyrosine kinase domain linked by a transmembrane region. Ligand binding to the extracellular domain stimulates tyrosine kinase activity (Carpenter, 1987).

Although tyrosine kinase induces phosphorylation of various intracellular substrates, a major substrate for this kinase is the receptor itself (Yang et al., 1994). A group of cellular proteins, containing a src homology 2 (SH2) domain, bind to sites of tyrosine autophosphorylation on receptor tyrosine kinases and mediate information transfer to the nucleus (Chen et al., 1987). The intrinsic tyrosine kinase is crucial for EGF-induced biological responses such as transformation, normal receptor trafficking, ligand-induced mitogenesis, and signal transduction (Chen et al., 1987; Filhol et al., 1993).

It has been reported that epiregulin mediates signals which stimulate or inhibit the proliferation of target cells of various types, but that its biological characteristics differ from those of other members of the EGF family. Since epiregulin bound to the EGF receptors of epidermoid carcinoma A431 cells much more weakly than did EGF, epiregulin may bind to the EGF receptor with low affinity. Alternatively, other receptors which bind EGF-related ligands, such as HER2/erbB2, HER3/erbB3, and HER4/erbB4, may serve as receptors for epiregulin (Toyoda et al., 1995). However, in the present study, epiregulin stimulated autophosphorylation of only the 170 kDa protein, which corresponds to the EGF receptor, and did not affect the phosphorylation of other receptors such as erbB2, erbB3, and erbB4, which are 185 kDa (Akiyama et al., 1986), 148 kDa (Kraus et al., 1989) and 180 kDa (Plowman et al., 1993) proteins, respectively. Therefore, epiregulin may bind only to the EGF receptor and not to other EGF- related ligands.

In conclusion, epiregulin is a potent stimulator of proliferation of rabbit cultured gastric cells, and its effect is mediated through autophosphorylation of EGF receptors.

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