ROLE OF HEPATOCYTE GROWTH FACTOR IN THE REPAIR PROCESS OF GASTRIC ULCER

胃潰瘍修復過程に於ける hepatocyte growth factor の役割

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Abstract

Various growth factors are suggested to be involved in gastric mucosal repair. In the present study, exogenous hepatocyte growth factor (HGF) has a proliferative effect on gastric epithelial cells, which is synergistic with EGF and insulin. Furthermore, comparison of the maximum proliferative effects and the optimum concentrations of several growth factors revealed that HGF was the most potent mitogen for gastric epithelial cells, as is the case for hepatocytes. In addition, restitution of gastric epithelial cell monolayers was assessed using a round wound restitution model. HGF was the most effective agent for facilitating gastric epithelial restitution among those tested. A binding assay revealed specific binding of HGF to its receptor on gastric epithelial cells. Northern blot analysis confirmed the expression of specific HGF receptor mRNA (c-met) by gastric epithelial cells but not by gastric fibroblasts. In order to investigate endogenous HGF production, we determined the effect of gastric fibroblast-conditioned medium on epithelial proliferation and restitution. The conditioned medium produced similar effects to HGF and its activity was neutralized by an anti-HGF antibody. In addition, expression of HGF mRNA was detected in gastric fibroblasts but not in gastric epithelial cells. Our immunohistochemical study confirmed these in vitro data by means of demonstrating the existence and localization of HGF at human native gastric mucosa. HGF was localized at fibroblasts under the epithelial cell layer. These results suggest that HGF may be a potent endogenous promoter of gastric epithelial cell proliferation and

migration, and may contribute to gastric mucosal repair through a paracrine mechanism.

INTRODUCTION

The epithelial lining of the stomach is rapidly renewed by the proliferation of immature gastric epithelial cells. These cells are located in the proliferating zone of the gastric glands (1) and are regulated by various autocrine, paracrine, and hormonal factors. The proliferative response of these cells to growth factors might be important in maintaining gastric mucosal integrity and in accelerating peptic ulcer healing. Several growth factors, including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), insulin, and insulin-like growth factor 1, have been reported to induce a mitogenic response of normal gastric epithelial cells in primary culture (2),(3),(4),(5). Among these factors, the expression of TGF-α has been reported to increase after gastric injury (6) and it is produced by gastric epithelial cells (7). In addition, Folkmann et al. have suggested that basic fibroblast growth factor (b-FGF), a potent growth promoter for vascular endothelial cells, might be important in duodenal ulcer repair (8),(9). They also reported that exogenously administered b-FGF accelerated duodenal ulcer healing to the same extent as a histamine H_2 receptor antagonist (10). These findings have suggested the importance of growth factors in the maintenance and repair of the gastric mucosa, although the role of each individual factor requires further investigation.

In addition to proliferation, previous reports have suggested that cell migration is an essential part of the early process of gastric mucosal repair (11). After various forms of gastric injury, mucosal integrity is re-

established by the rapid migration of epithelial cells across the wound margins in a process termed restitution. Stress ulcers of the gastric mucosa heal rapidly in rats and are almost completely repaired within 24 hr (12). The rapid process of mucosal restitution involves sloughing of the damaged epithelial cells, while viable cells migrate from adjacent to, or just beneath , the injured surface to cover the denuded area (13),(14). Early mucosal restitution appears to be an initial response which prevents deeper mucosal damage and occurs too rapidly to be accounted for by cell proliferation (15). The lost cells are subsequently replaced by proliferation, which is thought to begin 12-16 hr after injury and continues for 1-2 days (16). Therefore, both the migratory and the mitogenic responses of epithelial cells to various growth factors might be important in maintaining gastric mucosal integrity and accelerating peptic ulcer healing. It is of interest that growth factors such as EGF, TGF-α, and insulin have been shown to promote the migration of gastric epithelial cells.

Hepatocyte growth factor (HGF) is a hepatotrophic factor promoting liver regeneration that was initially purified from rat platelets (17),(18). HGF has been shown to stimulate the growth of various epithelial cells, such as renal tubular cells (19), epidermal melanocytes (20), and keratinocytes (21), suggesting that it might play an important role in the repair of these tissues. This factor is produced by mesenchymal cells, including fibroblasts, Kupffer cells, and endothelial cells, but not by epithelial cells (22),(23). However, the role of HGF in the gastric mucosa has not been well studied, despite the fact that gastric epithelial cells express c-met protein (24), the HGF receptor.

In the present study, we demonstrated that exogenous HGF induces the proliferation of rabbit gastric epithelial cells in primary culture (25) and that the conditioned medium of gastric fibroblasts also induces gastric epithelial cell proliferation. We also confirmed that the proliferative factor in the medium was HGF by demonstrating the expression of HGF mRNA and by a neutralizing experiment using anti-HGF antibody. We also characterized the effect of HGF on the migration of gastric epithelial cells using an in vitro restitution model. In order to confirm these in vitro data, we also performed immunohistochemical study, using human gastric biopsy samples. Our findings suggest the importance of HGF as a paracrine factor with a key role in the mesenchymal-epithelial interactions of the gastric mucosa. Although there have been numerous studies indicating that HGF functions in a paracrine fashion, it has never been clearly demonstrated that HGF derived from a particular tissue acts on epithelial cells of the same tissue. Therefore, this is the first actual demonstration that locally produced HGF stimulates the proliferation and migration of epithelial cells via a paracrine mechanism.

MATERIALS AND METHODS

Animals

Japanese white rabbits of both sexes (Doken Laboratory, Ibaraki) weighing 2.5-3.0 Kg were used.

Reagents

Human EGF was purchased from Wakunaga (Hiroshima, Japan) and human insulin was obtained from Shionogi (Osaka, Japan). Human recombinant HGF was purified from the conditioned medium of CHO cells transfected with an expression vector containing the complete human HGF cDNA (26). An anti-rabbit HGF antibody was kindly provided by Dr. Reza Zarnegar (University of Pittsburgh) (27). The reagents for gastric epithelial cell isolation and culture were as follows: Coon's modified Ham's F-12 medium (KC Biological Inc., Lenexa, KS), basal Eagle's medium (BME), minimal essential medium (MEM, Sigma, St. Louis, MO), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer (Sigma), bovine serum albumin (BSA) (fraction V, Sigma), Hanks' balanced salt solution (HBSS, Gibco, Grand Island, NY), crude type I collagenase (Sigma), and ethylenediaminetetraacetic acid (EDTA, Sigma). Genistein was purchased from Sigma chemical (St. Louis, Missouri). Crystal violet was purchased from Sigma. Indo-1 AM was from Wako (Tokyo, Japan). [^{3}H]-Thymidine was purchased from New England Nuclear (Boston, MA), a QuickPrep mRNA purification kit was purchased from Pharmacia Biotech (Uppsala, Sweden), a Megaprime DNA labelling kit was obtained from Amersham (Buckinghamshire, England), and Hybond-N+ was also obtained

from Amersham. A Quiaex DNA gel extraction kit was purchased from Quiagen (Chatsworth, CA)

Cell culture

Gastric fundic mucosal cells were isolated from adult rabbits and cultured as described previously (28). In brief, the fundic mucosa was quickly separated from rabbit stomachs, scraped bluntly, and minced into 2- to 3-mm^2 pieces, which were incubated in BME containing crude type I collagenase (0.35 mg/ml). This was followed by incubation in BME containing 1mM EDTA and further incubation in the former solution, which was performed twice serially at 37°C and pH 7.4 in an atmosphere of 5% CO_2 and 95% O_2. Cells from the final incubation were washed in HBSS and cultured at 37°C in a moist atmosphere containing 5% CO_2. The culture medium was F-12 medium supplemented with 10% heat inactivated (56°C for 30 min) fetal bovine serum (Gibco), 15 mM HEPES buffer, 100 units/ml penicillin, 100 units/ml streptomycin, and 5 μg/ml fungizone.

Gastric fundic fibroblasts were also obtained as described above by continuing the culture for more than one month, after which the mucosal cells died and fibroblasts became predominant. During culture, F-12 medium with 10% FBS was changed twice a week. Fibroblast-conditioned medium was obtained from these cultures after the fibroblasts became predominant. To produce the conditioned medium, fibroblasts were incubated for 24 hr at 37 °C in F-12 medium with 0.1% BSA.

Cell characterization

After culture for 48 hr, cells were examined morphologically,

histochemically, and electron microscopically as described elsewhere (28),(29). In brief, cultures were first examined with a phase-contrast microscope. To distinguish parietal cells, succinic dehydrogenase activity was determined by the method of Nachlas et al. (30). Bowie stain (31) was used to detect chief cells and periodic acid Schiff (PAS) staining was employed to identify mucous cells.

Assessment of cell proliferation

Crystal violet staining method; The Effects of the various growth factors on cell proliferation was assessed by staining with crystal violet. Isolated cells were inoculated on 96-well culture plates (Primaria, Falcon) at a density of 1.4 x 10^5 cells / cm^2, and then cultured for 24hr in F-12 medium supplemented with 10% FBS. After culture for 24 hr with serum-free F-12 medium, the culture medium was changed to serum-free F-12 medium supplemented with tested agents (EGF, insulin, HGF, conditioned medium of fibroblasts, or genistein) and 0.1% BSA. At 24 hr after the addition of agents, cultured cells were simultaneously fixed and stained by immersion for 10 minutes in 25% methanol containing 0.1% crystal violet . The stained cell layer was then washed and dried. The absorbance at 600 nm was measured using a multiwell spectrophotometer (Immunoreader 2000, InterMed, Japan).

[^{3}H]-thymidine incorporation method; The effects of various growth factors on DNA synthesis were determined by the [^{3}H]-thymidine incorporation method. Isolated cells were inoculated onto 24-well culture plates (Primaria, Falcon) at a density of 1.4 x 105 / well, and then cultured for 24 hr in F-12 medium supplemented with 10% FBS. After culture for a further 24 hr in

serum-free F-12 medium, culture was performed in serum-free F-12 medium supplemented with the test agents (EGF, insulin, HGF, or gastric fibroblast-conditioned medium), 0.1% BSA, and [3H]-thymidine (final concentration: 1.0 mCi/ml). Twenty-four hours later, the cells were washed and 5% trichloroacetic acid was added. Then the cells were let stand for 1 hr at 4°C, solubilized in 1 N NaOH, and neutralized with HCl. The solution was placed in a Readycap with XtalScint solvent-free scintillation medium (Beckman Instrument, Inc., CA) and air-dried overnight, after which the radioactivity was counted using a liquid scintillation counter. To allow accurate comparison of the effect of each agent on cell growth, all studies were done precisely at the same time using cells from a single rabbit stomach.

Restitution model

The effect of HGF and some other growth factors was studied using an in vitro model of gastric epithelial restitution. Confluent monolayers of primary cultured gastric epithelial cells in 24-well culture plates were wounded with a custom-made scraper that produced a round wound with a diameter of about 1.5 mm in each well. Then the monolayers were washed with fresh serum-free medium, and were further cultured in fresh serum-free medium in the presence or absence of growth factors including HGF, EGF, 10% FBS, and fibroblast-conditioned medium. Restitution of the epithelial cells was assessed in a blind fashion to avoid observer bias. Accordingly, determination of the uncovered area was performed by a person who was unaware of the details of the experiment. Photomicrographs

of the wounds were obtained at a 40-fold magnification using a Nikon microscope and camera. Then prints were made and wound area was cut out from each print and weighed. The weight was precisely related to the area, since the thickness of the prints was constant. Experiments were performed 6 times and the results were expressed as the mean ± SE. Morphological observation was also performed at a stronger magnification.

Radiolabeling of HGF

HGF was radioiodinated by the chloramine-T method as described previously (32). Briefly, 1.5 M sodium phosphate buffer, pH 7.0 (10 μl), 0.5μg of HGF (17μl), and 0.5mCi of $Na^{125}I$ (14 Ci/mg of iodine were placed in a siliconized tube, and the reaction was started by adding 5μl of chloramine-T (100 μg/ml), four times at 30-s intervals. The reaction was stopped by adding 20 μl of urea solution (1.2g/ml in 1M acetic acid). ^{125}I-HGF was separated by molecular sieve chromatography on a Sephadex G-25 column equilibrated with 4 mM HCl, 75 mM NaCl, and 1 mg/ml bovine serum albumin. ^{125}I-HGF prepared by this method had a specific activity of 70-160 mCi/mg.

Binding assay

The binding assay was carried out at 10°C by incubating ^{125}I-HGF with a monolayer of gastric epithelial cells as described elsewhere (32). Gastric epithelial cells in primary culture were incubated for 48 hr, after which the monolayer was washed with the binding buffer (20 mM Hepes and 0.2% BSA/Hanks, pH 7.0) and pre-incubated with the same buffer for 30 min at 10°C. After equilibration, fresh ice-cold binding buffer containing

various concentrations of ^{125}I-HGF with or without an excess of unlabeled HGF was added as indicated. Incubation was done for 1 hr at 10°C and halted by aspiration of the medium. The monolayer was washed 3-5 times with ice-cold buffer and the radioactivity bound to the cells was measured in a gamma-counter after solubilizing the monolayer with 1 N NaOH.

Neutralization experiment

Fibroblast-conditioned medium was incubated with chicken anti-rabbit HGF antibody or chicken IgG for 2 hr at 37°C and its effect on gastric epithelial cell proliferation was determined by the [^{3}H]-thymidine incorporation assay described above. F-12 medium with EGF was also incubated with the anti-rabbit HGF antibody and its effect on gastric epithelial cells was assessed to determine the specificity of the antibody.

Reverse transcription polymerase chain reaction for c-met

Total cellular RNA was isolated from cultured cells grown to confluence in 100-mm culture plates using RNAzol^{TM}B (Cinna/Biotecx Laboratories, Inc, Houston, Texas). Five micrograms of total RNA was reverse transcribed using M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD), after which the product was denatured at 95 °C for 5 min and cooled on ice. The polymerase chain reaction (PCR) was carried out in a final volume of 50 µl reaction buffer containing 50 mM KCl , 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 µM each of dATP, dGTP, dCTP, and dTTP, and 2.5 units of Taq polymerase (Promega, Madison, WI). Using 10 µl of the reverse transcription product, 30 cycles of amplification of the met first-strand cDNA were performed with 30 pmol of

each met primer (sense: 5' ^{3905}GGT TGC TGA TTT TGG TCA TGC^{3925} 3'; antisense: 5' ^{4126}TTC GGG TTG TAG GAG TCT TCT^{4146} 3'). Each amplification cycle consisted of denaturation at 93°C for 30 sec, annealing at 53.1°C for 45 sec, and polymerization at 72°C for 45 sec. PCR products (10 μl) were electrophoretically separated on 2% agarose gel in 1X TAE buffer, after which the gel was stained with ethidium bromide (0.5 μg/ml).

Northern blot hybridization

Total RNA was extracted from cultured gastric epithelial cells or gastric fibroblasts by the AGPC method (33) and purified to mRNA on an oligo(dT)-Cellulose Spun Column using a QuickPrep mRNA purification kit. Two micrograms of poly (A)+RNA was electrophoresed on 1% agarose gel containing 0.66 M formaldehyde and transferred to a HYbond-N+ nylon membrane filter. As a probe for HGF mRNA, a 1.4 kb HGF cDNA fragment including the 3'-portion of the α subunit, the β subunit, and part of the 3'-untranslated region was obtained from pRBC1 using the restriction enzyme EcoRI (34). As a probe for the detection of c-met, the PCR product obtained as described above was purified with a Quiaex DNA gel extraction kit. The cDNA was labelled with [α-^{32}P]dCTP by the random primer method using a Megaprime DNA labelling system, after which the membrane was hybridized with the radiolabelled cDNA probe at 65°C for 2 hr in rapid hybridization buffer. Then the membrane was washed in 2 X SSC with 0.1% SDS for 20 minutes at room temperature, followed by washing twice in 0.1 X SSC with 0.1% SDS for 15 min at 65°C. Finally, it was exposed to X-ray film for 48 hr at -70°C using an intensifying screen.

Measurement of intracellular free Ca^{2+}

Gastric mucosal cells were cultured on glass coverslips with F-12 medium. Cultured cells were incubated at 37°C for 15 min. in culture medium containing 5 mM indo-1 AM, the fluorescent Ca^{2+} probe. A coverslip was washed in indo-1 AM free solution for 30 min., and then placed in the flow-through cell chamber. The coverslip was continuously superfused with HEPES buffered solution containing 137 mM NaCl, 3.7 mM KCl, 0.5 mM MgCl2, 1.8mM CaCl2, 5.6mM glucose, and 4.0 mM HEPES. Intracellular free calcium concentration was measured as described by Peeters et al(15). The fluorescent light was collected by the objective lens and divided with a beam splitter to permit simultaneous measurement of both 410 nm and 480 nm wave lengths using two separate photomultiplier tubes. The $[Ca^{2+}]_i$ was calculated from the 410/480 fluorescence ratio of both fluorescence emission as: $[Ca^{2+}]_i$ = 250 nm (R-Rmin)/(Rmax-R)b. Rmax and Rmin are the values when indo-1 AM is Ca^{2+}-bound or Ca^{2+}-free respectively. To obtain Rmin, the gastric cultured cells were loaded with indo-1 AM, washed, and exposed to 140 mM KCl, 4 mM HEPES at the pH 7.05, and 50 mM digitonin in "0" Ca^{2+} (0.1 mM EGTA) for 2 min. at 37°C. After addition of Ca^{2+} sufficient to increase $[Ca^{2+}]o$ to 0.1mM ("High Ca^{2+}"), Rmax was obtained.

Reverse transcription polymerase chain reaction for HGF

16 pairs of biopsy specimens were obtained endoscopically from the edges of gastric ulcers and normal mucosa of 16 individual patients and were immediately frozen in liquid nitrogen. Later, total cellular RNA was

isolated, using RNAzol^{TM}B (Cinna/Biotecx Laboratories, Inc, Houston, Texas). Five micrograms of total RNA was reverse transcribed using M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD). The product was heated at 95 °C for 5 min and then cooled on ice. Then the PCR was carried out in the same buffer as that used for the c-met PCR. Using 10 μl of the reverse-transcribed product, 40 cycles of amplification were performed for the first-strand hHGF cDNA . Thirty picomoles of each of the hHGF primers was used (sense: 5'-CAG CGT TGG GAT TCT CAG TAT-3'; antisense: CCA ATG TTT GTT CGT GTT GGA-3').

These primers respectively represent the sense sequence in the K3 (exon 8) domain of the α chain (nucleotide 979-1000) and the antisense sequence in the 5' portion (exon 13) of the β chain (nucleotide 1497-1518) of hHGF mRNA (17). Each amplification cycle consisted of 90 s of denaturation at 94 °C, 1 min of annealing at 57 °C, and 2 min of polymerization at 72 °C. Ten microliters of each PCR product was electrophoretically separated on 2% agarose gel in 1 X TAE buffer. The gel was then stained with 0.5 μg/ml ethidium bromide and was visualized under ultraviolet light (35).

HGF immunohistochemistry

Anti-serum

A polyclonal antiserum was kindly donated by Dr. T. Nakamura which was raised in rabbits against recombinant human HGF purified from the culture fluid of transformed CHO cells.

Immunohistochemistry

Three pairs of biopsy specimens were obtained endoscopically from the

edges of gastric ulcers and from normal mucosa of three individual patients at the University of Tokyo Hospital (Tokyo, Japan). Written informed consent was obtained from all patients. Specimens were processed by the modified AMeX method (36). In brief, the tissues were first fixed in acetone at 4°C for 20 min and then at -20°C overnight, dehydrated in acetone at 4°C for 15 min and at room temperature for 15 min, cleared in methyl benzoate for 30 min and then in xylene for 30 min consecutively, and finally embedded in paraffin. Sections 2 μm thick were cut and deparaffinized with xylene, immersed in acetone, and incubated in methanol with 0.3% hydrogen peroxide for 30 min at room temperature to block endogenous peroxidase activity. The samples were incubated with heparinase-1 (0.5 units/ml; Sigma, St. Louis, MO) in phosphate-bufferred saline (PBS) at 37°C for 30 min. After rehydration with PBS, the sections were preincubated with 2% normal swine serum (NSS) in PBS, and then incubated for 2 hr at room temperature with either a polyclonal antibody against human recombinant HGF or with control non-immunized rabbit serum diluted to 1:2000 with 2% NSS. Next, the sections were washed three times in PBS and incubated for 30 min with biotinylated anti-rabbit IgG (Nichirei, Tokyo, Japan) diluted to 1:200 with 2% NSS. After washing three times in PBS, the sections were incubated for 30 min with avidin-biotin-peroxidase complex prepared using a Histofine ABC kit (Nichirei). Then the sections were washed in PBS, and incubated for 5 min in a solution containing 0.02% diaminobenzidine and 0.03% hydrogen peroxide in 0.1 M Tris-HCl buffer (pH 7.6). Nuclear counterstaining was performed with Mayer's hematoxylin.

Statistical Analysis

Data are presented as mean ± SE for n determinations. Comparisons between two groups were made by a Student's *t* test for grouped or paired data when appropriate; comparisons among several groups were made by analysis of variance, followed by Dunnett's test, when appropriate. In all analysises, statistical significance was attributed at a 95% or greater confidence level.

RESULTS

1. Cell culture and identification

Cultured cells formed subconfluent monolayers at 48 hr after inoculation and 93% of the cells in these monolayers had PAS-positive material in the cytoplasm (Fig. 1a). A previous report suggests that three percent of the cells showed a strong reaction for succinic dehydrogenase activity, indicating that they were parietal cells, and 2% of the cells had granules positive for Bowie staining, indicating that they were chief cells, and that transmission electron microscopy revealed that the majority of the cells contained electron-dense granules which are characteristic of mucous cells. These findings indicated that the cultures consisted mainly of mucous-producing cells (28). As mentioned earlier, these mucous-producing cells died after one month of culture and fibroblasts became predominant (Fig. 1b).

2. Effect of exogenous HGF on proliferation and restitution of gastric epithelial cells in primary culture

2-1. *Effect of exogenous HGF on gastric epithelial cell proliferation*

Cell proliferation was assessed by both crystal staining method and [^{3}H]-thymidine incorporation method. Crystal violet staining method reflect cell number and [^{3}H]-thymidine incorporation method reflects DNA synthesis. These methods altogether properly evaluate proliferation.

2-1-1. Cell proliferation assessed by the crystal violet staining method.

Human recombinant HGF stimulated cell proliferation significantly in a dose dependent manner (Fig.2-1-1a). EGF and insulin also stimulated the cell proliferation and acted synergistically with HGF(Fig. 2-1-1b, 2-1-1c).

2-1-2. DNA synthesis assessed by [^{3} H]-thymidine incorporation

HGF significantly stimulated DNA synthesis by gastric epithelial cells in a dose dependent manner (Fig.2-1-2a). EGF and insulin also stimulated DNA synthesis and acted synergistically with HGF (Fig.2-1-1b, 2-1-2c).

2-1-3. Comparison of maximum DNA synthesis among various growth factors

The dose-response profiles for EGF and insulin were also determined simultaneously. The maximal response was obtained with 15 ng/ml (180 pM) HGF, 10 ng/ml (1640 pM) EGF, and 20 mU/ml insulin (Fig. 2-1-3a). This optimum HGF concentration was extremely low, considering that the molecular weight of HGF is more than ten times greater than that of EGF. We simultaneously compared the maximal DNA synthesis of gastric epithelial cells stimulated by HGF, EGF, insulin, and 10% FBS, using cells from the same source to avoid variations due to differences in the background conditions. The maximal DNA synthesis induced by HGF was significantly higher than that induced by the other factors (HGF > insulin > 10% FBS > EGF) (Fig. 2-1-3b).

2-2. *Effect of HGF and other factors on restitution*

Confluent monolayers of gastric epithelial cells were wounded with a custom-made scraper that produced a round wound about 1.5 mm in diameter, and then were cultured with HGF and other factors. Cells from the edges of the wound gradually migrated to cover the defect. Figure 2-2a shows the time course of wound restitution in the presence of 10 ng/ml HGF. EGF (1-100 ng/ml), 10% FBS, and HGF (1-40 ng/ml) all significantly facilitated restitution when compared with the control. Figure 2-2b shows the dose-response of the effect of HGF on the restitution of gastric epithelial cells at 16 hours after wounding of the monolayers. It was found that 10 ng/ml (120 pM) was the optimum concentration. HGF showed by far the most potent promotion of restitution among the agents tested (Fig. 2-2c). Addition of cycloheximide at a concentration (10 nM) which completely blocked the induction of DNA synthesis by HGF (Fig. 2-2d) had no effect on the restitution process.

3. Signal transduction of HGF in gastric epithelial cells

3-1. The effect of HGF on Intracellular Ca^{2+} concentration

Neither HGF nor EGF increased intracellular Ca^{2+} concentration of gastric epithelial cell, while deoxycholic acid significantly increased intracellular Ca^{2+} concentration which is in accordance with previous study (37) (Fig. 3-1).

3-2. The effect of tyrosine kinase specific inhibitor, genistein, on HGF induced proliferation

Genistein, tyrosine kinase specific inhibitor inhibited significantly DNA synthesis caused by HGF, as well as EGF (Fig. 3-2a), as assessed by [^{3}H]-thymidine incorporation method. Genistein also inhibited the proliferation

induced by HGF and EGF (Fig. 3-2b), as assessed by crystal violet method.

4. HGF receptor on the gastric epithelial cells in primary culture

4-1. Binding assay

Figure 4-1a shows typical saturation curves for ^{125}I-HGF binding to its receptor on cultured gastric epithelial cells. The specific binding of HGF was saturated at about 50 pM. We performed Scatchard analysis, plotting bound ^{125}I-HGF / free ^{125}I-HGF as vertical axis and bound ^{125}I-HGF as horizontal axis (32). In order to obtain regression, we used the least squares method. Scatchard analysis yielded a rectilinear plot, suggesting the presence of a single class of high-affinity binding sites (Fig. 4-1b). The Kd value and the number of HGF receptors calculated from the Scatchard plot was 32 ± 19.7 pM and 488 ± 124 sites/gastric epithelial cell, respectively (95% confidence interval).

4-2. Expression of c-met mRNA

4-2-1. Expression of HGF mRNA, as demonstrated by RT-PCR

The reverse transcription polymerase chain reaction (RT-PCR) technique was initially used to detect the HGF specific receptor, c-met mRNA expression in primary cultures of gastric epithelial cells. The cells yielded a single amplified band with an estimated size of 242 bp (Fig. 4-2-1).

4-2-2. Expression of HGF mRNA by northern blot hybridyzation

We performed Northern hybridization with the RT-PCR product as a probe. The expression of HGF specific receptor, c-met mRNA was clearly seen in the gastric epithelial cells, while little expression, if any, was seen in

the gastric fibroblasts (Fig. 4-2-2).

5. Endogenous production of HGF by gastric fibroblasts

5-1. Effect of fibroblast-conditioned medium on gastric epithelial cell proliferation

Conditioned medium obtained from the cultured fibroblasts stimulated the DNA synthesis of gastric epithelial cells in a concentration (Fig. 5-1a) and time (Fig. 5-1b) dependent fashion (25), as assessed by [^{3}H]-thymidine incorporation method. Genistein, tyrosine kinase specific inhibitor, inhibited the DNA synthesis stimulated by the fibroblast conditioned medium.(Fig. 5-1c). The proliferative effect of the conditioned medium was neutralized by an anti-HGF antibody in a dose-dependent manner (Fig. 5-1d). In contrast, the proliferative effect of EGF was not influenced by this antibody (Fig. 5-1e).

5-2. Effect of fibroblast-conditioned medium on gastric epithelial cell restitution

Conditioned medium obtained from cultured fibroblasts also facilitated the restitution of gastric epithelial cell monolayers. The stimulatory effect of the conditioned medium on restitution was also neutralized by the anti-HGF antibody (Fig. 5-2).

5-3. Expression of HGF mRNA by gastric fibroblasts

Figure 5-3 shows the expression of HGF mRNA by gastric epithelial

cells and fibroblasts. Northern blot analysis clearly demonstrated the expression of HGF mRNA by gastric fibroblasts, but not by gastric epithelial cells.

6. Expression of HGF at the human gastric ulcer, in vivo.

6-1. HGF mRNA expression at the edges of gastric ulcers

We used the RT-PCR technique to detect HGF mRNA expression. We extracted total RNA from the biopsy samples of 16 patients. When the RT-PCR was performed, 14 out of 16 samples revealed a single band corresponding to a DNA fragment of the predicted size (539 bp), which suggested the production of HGF at the ulcer edges. Whereas, 9 out of 16 biopsies from normal gastric mucosa reveal a single band corresponding to HGF mRNA. The difference between ulcer edges and normal mucosa is significant ($p=0.02$). Figure 6-1 shows a representative demonstration of HGF mRNA expression in tissues from the edge of a gastric ulcer and from normal gastric mucosa. Since the PCR primers used corresponded to sequences in exon 8 and exon 13 between which there are introns, only the first strand cDNA of full length HGF would yield a PCR product of this size.

6-2. Distribution of HGF in human gastric ulcers and normal mucosa., using immunohistochemistry

We stained tissues obtained endoscopically from the edges of gastric ulcers or from normal gastric mucosa at a site sufficiently distant from any ulcers. The tissues were treated with heparinase in order to avoid the

binding of HGF to heparin or heparan sulfate in the extracellular matrix, since HGF has a strong affinity to heparin (38).

Spindle-shaped cells located beneath the epithelial cells, probably fibroblasts from the ulcer edges, were clearly and strongly stained by the anti-HGF antibody, suggesting the presence of HGF protein in these cells (Fig. 6-2a). These cells were not stained by non-immunized rabbit serum (Fig. 6-2b), indicating that the staining was specific for HGF. In contrast, there was no positive staining of the tissues from normal gastric mucosa (Fig. 6-2c). We stained three pairs of samples (ulcer edge and normal mucosa) from different patients and obtained the same results. These results indicates that HGF is localized at ulcer edges.

DISCUSSION

Various growth factors have been recognized as important in the maintenance and repair of organs throughout the body (38),(39). In the stomach, the gastric epithelial cells are continuously renewed and damage to the epithelium induces the common diseases of gastritis and peptic ulcer. Several growth factors, such as TGF-α and b-FGF, have been suggested to be involved in gastric mucosal repair. In the present study, we demonstrated that HGF had a strong proliferative effect on rabbit gastric epithelial cells in primary culture, which is synergistic with EGF and insulin, suggesting that the effect is mediated by distinct pathway. Furthermore, the maximum DNA synthesis induced was greater than that produced by insulin, EGF, or 10% FBS. The optimum HGF concentration producing this effect was also extremely low, considering the high molecular weight of this factor (more than ten times that of EGF). These findings indicate that HGF is one of the most potent mitogens for gastric epithelial cells, as was also the case for hepatocytes in a previous study (17) and thus may have an important influence on the gastric mucosa.

Although we demonstrated a proliferative effect of HGF on gastric epithelial cells, previous reports have suggested that cell migration is the principal force behind the early restitution of mucosal erosions in the gastrointestinal tract (40). However, there has been almost no investigation of the effect of various growth factors including HGF on either restitution or gastric epithelial cell migration. Nursat et al. reported that HGF facilitated

the separation, spreading, and migration of T84 intestinal epithelial cells and thus enhanced wound healing, using the same in vitro round wound restitution model (41). It is interesting that restitution is initiated by separation and spreading of the cells, according to their study. Their result is in accordance with ours in the sense that HGF facilitated the restitution, although the concentration of HGF that they used was 200 ng/ml, which seems too high to be physiological considering that the HGF concentration eliciting maximal activity ranges from 5 to 10 ng/ml for many kinds of cells (42). Besides, our study demonstrated that addition of cycloheximide at a concentration (10 nM) which completely blocked the induction of DNA synthesis by HGF had no effect on the restitution process, suggesting that HGF stimulated restitution by facilitating migration alone without any effect on mitogenesis. Furthermore, our experiments showed consistently that the effect of HGF on gastric epithelial restitution was greater than that of EGF or 10% FBS and that the optimum HGF concentration for promoting restitution was quite low, as was also the case for proliferation. All these results suggest the importance of HGF in gastric mucosal repair.

A binding assay using radiolabelled HGF revealed specific binding to gastric epithelial cells, and expression of the HGF receptor (c-met) by gastric epithelial cells was also confirmed. These results were compatible with the immunohistochemical study of Prat et al., which demonstrated the presence of c-met protein in gastric epithelial cells (43). Besides, the fact that genistein, specific tyrosine kinase inhibitor, inhibited the HGF-induced proliferation supported that the proliferative effect of HGF is mediated by c-

MET which is known to be a receptor of tyrosine kinase activity. Intracellular signal transduction system, by which the action of HGF is mediated, has not been clearly understood. Present study shows that intracellular Ca^{2+} was not increased by HGF, indicating that Ca^{2+} may not play a major role in the action of HGF to rabbit gastric epithellial cells. This is contradictory to the report by Kaneko et al (44), which suggested that intracellular Ca^{2+} of hepatocyte in primary culture was remarkably increased by HGF. These contradiction resulted from the difference of the cells employed in the experiments. This fact suggests that the effect of HGF on gastric epithelial cells is not mediated by intracellular Ca^{2+} concentration. Accordingly, the proliferative effect of HGF on gastric epithelial cells appears to be a specific HGF receptor-mediated response.

To establish the physiological role of HGF in the gastric mucosa, the source of this factor needs to be identified. EGF is reported to be present in the circulation and the gastric juice (45). TGF-α may be secreted by gastric epithelial cells and appears to act in an autocrine or paracrine fashion, so it may be an autocrine or paracrine growth factor for gastric epithelial cells (6), (46). The source and the secretory mechanism of b-FGF are not clearly understood, although the target of this factor has been suggested to be vascular endothelial cells (10). Since HGF is produced by various mesenchymal cells in other organs (22),(23),(47), we postulated that gastric fibroblasts might also produce this factor. The present study demonstrated that conditioned medium obtained from cultured gastric fibroblasts stimulates the growth of gastric epithelial cells and that this action is additive with that

of EGF or insulin, suggesting that it is not mediated by either of these factors and that the effect of fibroblast-conditioned medium was neutralized by an anti-HGF antibody, indicating that the proliferative effect of the conditioned medium was at least partly due to HGF produced by fibroblasts. In addition, gastric fibroblasts were shown to express HGF mRNA, while gastric epithelial cells did not. Furthermore, fibroblast-conditioned medium facilitated the restitution of gastric epithelial cells after the wounding of confluent monolayers, which was reversed by anti-HGF antibody, indicating that the stimulatory effect of the conditioned medium on restitution was at least partly due to HGF produced by fibroblasts. These findings confirm the production of HGF by gastric fibroblasts and support the role of HGF as a paracrine growth factor for gastric epithelial cells which is involved in "mesenchymal-epithelial interactions". In addition, we demonstrated the existence and production of HGF at the edges of human gastric ulcers by immunostaining with anti-HGF and the RT-PCR. In the immunohistochemical study, the biopsy samples were obtained endoscopically from gastric ulcer edges and normal mucosa. It may be assumed that deeper tissue are inclined to be obtained from the edges than normal mucosa owing to the positional differences, which might result in the difference of HGF expression. We evaluated the depth of the tisssue by observing whether or not they contain muscularis mucosae. There was no muscularis mucosae observed in either samples from the edges or normal mucosa. We also obtained gastric biopsy samples to assess HGF mRNA expression in which no evaluation could possibly be performed as for the depth of tissues. However, no difference could be assumed, since the samples were obtained in the same

way as was in the case of immunohistochemical study. It was found that HGF was mainly localized in the mesenchymal tissues, particularly in the fibroblasts. These findings support the role of HGF as a paracrine factor involved in gastric ulcer repair, as our in vitro studies also indicated. Although various authors have indicated that HGF functions in a paracrine manner, it has never been clearly demonstrated for a single organ that locally produced HGF can actually act on the neighboring epithelial cells. Therefore, this report provides the first actual demonstration of the paracrine role of HGF.

It is generally believed that gastric mucosal defects such as erosions or ulcers are first replaced by granulation tissue which is subsequently covered by epithelial cells (48). Since granulation tissue is mainly composed of fibroblasts, our results indicate that HGF may play an important role in the re-epithelialization process. Thus, it is possible that HGF might be a potential therapeutic agent for peptic ulcer disease.

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FIGURE LEGENDS

Figure 1. a: Phase-contrast micrograph of rabbit gastric epithelial cells in primary culture (x100). **b**: Phase-contrast micrograph of rabbit gastric fibroblasts in primary culture (x40).

Figure 2-1. a: Growth response of cells cultured for 24 hr with HGF, as assessed by crystal violet staining. HGF stimulated the proliferation of gastric epithelial cells significantly in a dose dependant fashion. The vertical axis shows a percentage of the control value converted from counts per minute. **b:** Proliferation of gastric epithelial cells cultured for 24 hr with HGF alone, EGF alone, or HGF plus EGF, as assessed by crystal violet staining method. EGF acted synergistically with HGF. **c:** Proliferation of gastric epithelial cells cultured for 24 hr with HGF alone, insulin alone, or HGF plus insulin, as assessed by crystal violet staining method. insulin acted synergistically with HGF. (mean+SE, *: $p<0.01$ compared to the control, **: $p<0.01$ compared to HGF alone, EGF alone, or insulin alone) INS: insulin.

Figure 2-1-2. a: DNA synthesis by cells cultured for 24 hr with HGF, as assessed by [^{3}H]-thymidine uptake. HGF stimulated DNA synthesis of gastric epithelial cells significantly in a dose dependant manner. **b:** DNA synthesis by cells cultured for 24 hr with HGF alone, EGF alone, or HGF plus EGF, as assessed by [^{3}H]-thymidine incorporation. EGF acted synergistically with HGF. **c:** DNA synthesis by cells cultured for 24 hr with HGF alone, insulin

alone, or HGF plus insulin, as assessed by [^{3}H]-thymidine uptake. Insulin acted synergistically with HGF. (mean+SE, *: $p<0.01$ compared to the control, **: $p<0.01$ compared to HGF alone, EGF alone, or insulin alone) INS: insulin.

Figure 2-1-3. DNA synthesis by gastric epithelial cells cultured for 24 hr with various agents, as assessed by [^{3}H]-thymidine incorporation. Every agent significantly stimulated DNA synthesis in a dose-dependent manner. These experiments were performed simultaneously using epithelial cells from a single source. The vertical axis shows a percentage of the control value converted from counts per minute. **a:** Hepatocyte growth factor (HGF), epidermal growth factor (EGF), and insulin all stimulated DNA synthesis in a dose-dependent manner. The maximal response was obtained with 180 pM HGF, 1640 pM EGF, and 20 mU/ml insulin. The optimum HGF concentration was extremely low, being less than a ninth of that for EGF. **b:** Comparison of the maximum DNA synthesis induced by HGF, EGF, insulin, and 10%FBS indicated that the relative mitogenic potency was in the order of HGF > insulin > 10% FBS > EGF. (mean+SE, *: $p<0.01$ vs. control, **: $p<0.01$ vs. HGF.)

Figure 2-2. Effect of HGF and other factors on epithelial restitution.
a: The time course of wound restitution in the presence of 120 pM HGF. Confluent monolayers of gastric epithelial cells were wounded with a custom-made scraper to produce round wounds about 1.5 mm in diameter.

Then the monolayer was cultured with HGF and other factors. Cells from the wound edges gradually migrated to cover the defect. **b:** Restitution was assessed by determining the residual uncovered area 16 hours after wounding. HGF facilitated restitution of gastric epithelial cells in a dose-dependent manner, with 10 ng/ml (120 pM) being the optimum concentration. (mean+SE, *: $p<0.01$ vs. control) **c:** Restitution was also assessed by determining the residual uncovered area over time. The vertical axis indicates the residual uncovered area expressed as a percentage of the original wound area and the horizontal axis shows time. EGF, 10% FBS, and HGF significantly facilitated restitution when compared with the control. The potency of the effect on restitution was in the order of 120 pM HGF > 10% FBS > 3280 pM EGF. HGF with 10 nM cycloheximide, which does not have any proliferative effect, facilitated restitution to the same extent as in its absence. Each plot represents the mean ± SE. (HGF10: 10 ng/ml (120 pM) HGF, HGF+CH: 10 ng/ml (120 ng/ml) HGF with 10 nM cycloheximide, EGF20: 20 ng/ml (3280 pM) EGF) **d:** Cycloheximide suppressed DNA synthesis induced by 120 pM HGF in a dose-dependent manner and at the concentration of 10 nM, it completely blocked the DNA synthesis. (HGF: 120 pM HGF, CH: cycloheximide (nM), mean+SE, *: $p<0.01$ vs. control, **: $p<0.01$ vs. HGF.)

Figure 3-1. Intracellular Ca^{2+} concentration of cultured cells with stimulation by HGF and EGF. Deoxycholic acid was used as a positive control. Neither HGF nor EGF increased the intracellular Ca2+

concentration, while deoxycholic acid did.

Figure 3-2. a: Suppression of HGF induced DNA synthesis by genistein as assessed by [^{3}H]-thymidine incorporation. **b:** Genistein suppressed proliferation of gastric epithelial cells induced by HGF, as assessed by crystal violet staining method (mean+SE, *: $p<0.01$ compared to HGF plus DMSO). DMSO: dimethyl sulfoxide. GS: genistein.

Figure 4-1. a: Saturation curve for the binding of ^{125}I-HGF to its receptor on cultured gastric epithelial cells. Sparse (1.4×10^{5} cells/well) monolayers of rabbit gastric epithelial cells were incubated for 1 hr at 10 °C with ^{125}I-labeled HGF alone or with 10 nM unlabeled HGF. **b**: Scatchard plot. We performed Scatchard analysis, plotting bound ^{125}I-HGF / free ^{125}I-HGF as vertical axis and bound ^{125}I-HGF as horizontal axis. In order to obtain a regression line, we used the least squares method. The Kd value was 32 ± 19.7 pM. The number of HGF receptors was 488 ± 124 sites/cell (95% confidence interval).

Figure 4-2-1. Expression of HGF specific receptor, c-met mRNA. The RT-PCR technique was initially used to detect c-met mRNA expression. RT-PCR was performed using a set of primers (sense: 5' ^{3905}GGT TGC TGA TTT TGG TCA TGC^{3925} 3'; antisense: 5' ^{4126}TTC GGG TTG TAG GAG TCT TCT^{4146} 3') and RNA from cultured gastric epithelial cells, which yielded a single amplified band with an estimated size of 242 bp.

Figure legend 4

Figure 4-2-2. Then, northern blot hybridization was done using the RT-PCR product as a probe to confirm that c-met mRNA was expressed by gastric epithelial cells, while little expression, if any, was seen in gastric fibroblasts.

Figure 5-1. The conditioned medium (CM) was F-12 medium containing 0.1% BSA incubated with gastric fibroblasts at 37°C for 24 hr, which contained factors produced and secreted by the fibroblasts. **a:** DNA synthesis by cells cultured for 24 hr with stimulation by the fibroblast conditioned medium, as assessed by [^{3}H]-thymidine incorporation. The conditioned medium stimulated DNA synthesis significantly in a concentration dependent manner. **b:** DNA synthesis by cells cultured for 24 hr with stimulation by the conditioned medium which was left with cultured fibroblasts for indicated periods of time, as assessed by [^{3}H]-thymidine incorporation. The conditioned medium stimulated DNA synthesis in a time dependent manner. (mean+SE, *: $p<0.01$ compared to the control) **c:** Suppression of conditioned medium induced DNA synthesis by genistein, a tyrosine kinase specific inhibitor, as assessed by [^{3}H]-thymidine incorporation. Genistein suppressed the DNA synthesis of gastric epithelial cells induced by fibroblast conditioned medium. (mean+SE, *: $p<0.01$ compared to the conditioned medium of fibroblasts plus DMSO). DMSO: dimethyl sulfoxide. GS: genistein. FIB: fibroblast condioned medium. **d:** Suppression of DNA synthesis induced by fibroblast-conditioned medium in the presence of an anti-rabbit HGF antibody as assessed by [^{3}H]-thymidine incorporation. The

conditioned medium had a proliferative effect on gastric epithelial cells, which was neutralized by an anti-HGF antibody, suggesting that factor in the medium was HGF. The control was F-12 medium containing 0.1% BSA without being incubated with the fibroblasts. (mean+SE, *: $p<0.01$ vs. conditioned medium alone. CM: conditioned medium. Ab4000X: antibody at a dilution of 1:4000, etc.) **e:** EGF-induced DNA synthesis was not affected by the anti-rabbit HGF antibody, demonstrating that its neutralizing effect was specific to HGF. (mean+SE)

Figure 5-2. Effect of fibroblast-conditioned medium on gastric epithelial cell restitution. Conditioned medium also facilitated the restitution of gastric epithelial cell monolayers and this effect was suppressed by an anti-HGF antibody (dilution: 1 : 500), suggesting that HGF in the medium was involved in promoting restitution. Anti-HGF antibody alone at this dilution did not have any effect on restitution. Each plot represents the mean ± SE.

Figure 5-3. Expression of HGF mRNA by gastric cells, as assessed by Northern blot hybridization. Northern blot hybridization was performed after extraction of poly A RNA from either gastric epithelial cells or gastric fibroblasts, using 1.4 kb HGF cDNA. Expression of HGF mRNA was seen in gastric fibroblasts, but not in gastric epithelial cells, indicating the production of HGF in the gastric fibroblasts, which was confirmed immunohistochemically (Fig. 9). (Ep: gastric epithelial cells. Fib: gastric fibroblasts.)

Figure 6. Representative demonstration of HGF mRNA expression in human tissue from the edge of a gastric ulcer. The 539 bp DNA fragment was produced by RT-PCR of total RNA the tissue using a pair of primers designed to amplify an HGF cDNA sequence. It was visualized with UV light following separation in 2% agarose gel and treatment with ethydium bromide solution. Fourteen out of 16 biopsy samples from gastric ulcer edges revealed HGF mRNA expression, while 9 out of 16 biopsies from normal gastric mucosa reveal HGF mRNA expressions. The difference between ulcer edges and normal mucosa is significant ($p=0.02$). 1-3 represent the biopsies from ulcer edges and 3-4 represent the biopsies from normal gastric mucosa.

Figure 6-2. Immunohistochemical study of human gastric mucosa using anti-HGF antiserum. **a**: Tissue obtained endoscopically from the edge of a gastric ulcer. The spindle-shaped cells, probably fibroblasts, under the epithelial cell layer are clearly and strongly stained by anti-HGF antiserum (arrow). **b**: The same tissue as that shown in a was also stained using non-immunized rabbit serum as a control. No detectable staining was seen, indicating that the staining with anti-HGF antiserum was specific for HGF. **c:** Normal gastric mucosa obtained from the same patient at a site distant from the ulcer. No strong staining is seen with the anti-HGF antiserum.

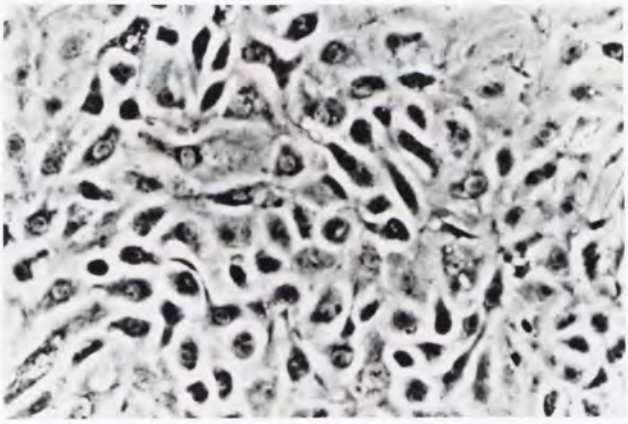


Figure 1a. Phase-contrast micrograph of rabbit gastric epithelial cells in primary culture (x100)

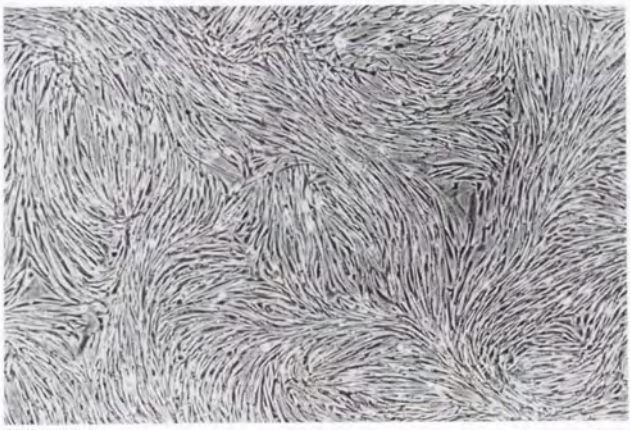


Figure 1b. Phase-contrast micrograph of rabbit gastric fibroblasts in primary culture (x40)

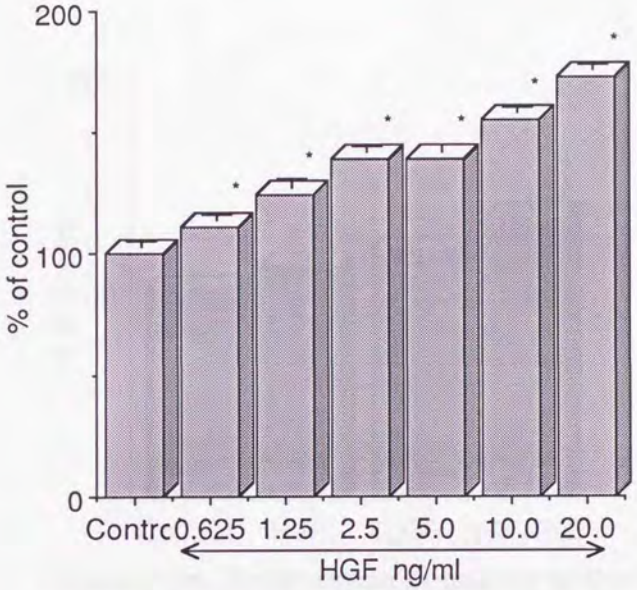


Figure 2-1a. Growth response of cells cultured for 24 hr with HGF, as assessed by crystal violet sataining

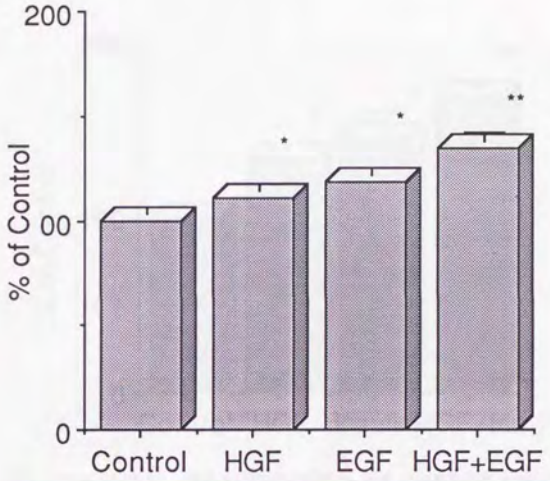


Figure 2-1b. Proliferation of gastric epithelial cells cultured for 24 hr with HGF alone, EGF alone, or HGF plus EGF, as assessed by crystal violet staining method

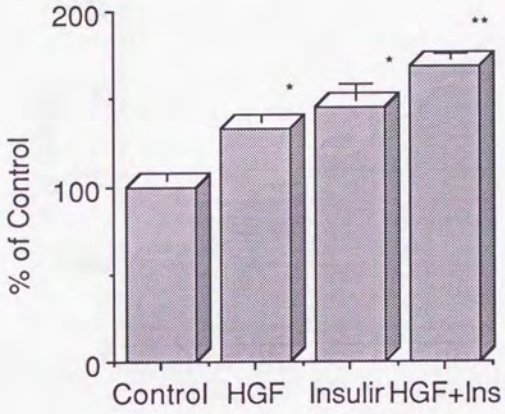


Figure 2-1c. Proliferation of gastric epithelial cells cultured for 24 hr with HGF alone, insulin alone, or HGF plus insulin, as assessed by crystal violet staining method

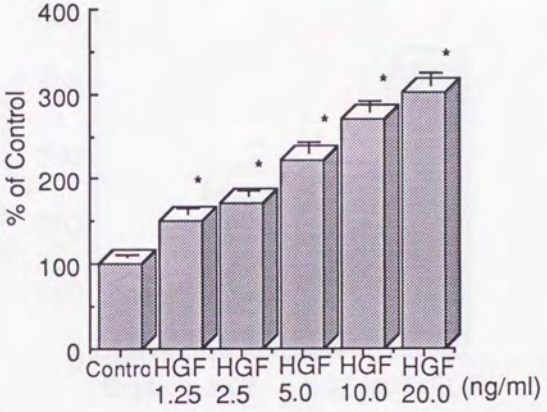


Figure 2-1-2a. DNA synthesis by cells cultured for 24 hr with HGF, as assessed by [3H]-thymidine uptake

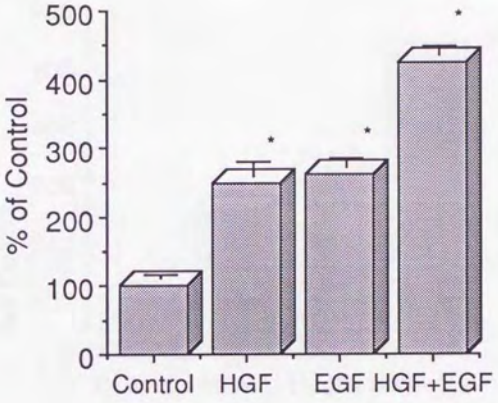


Figure 2-1-2b. DNA synthesis by cells cultured for 24 hr with HGF alone, EGF alone, or HGF plus EGF, as assessed by [3H]-thymidine incorporation

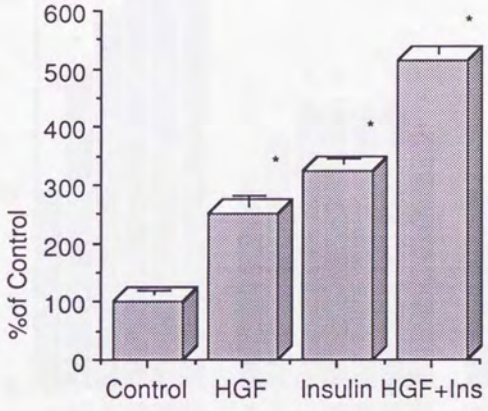


Figure 2-1-2c. DNA synthesis by cells cultured for 24 hr with HGF alone, insulin alone, or HGF plus insulin, as assessed by [3H]-thymidine incorporation

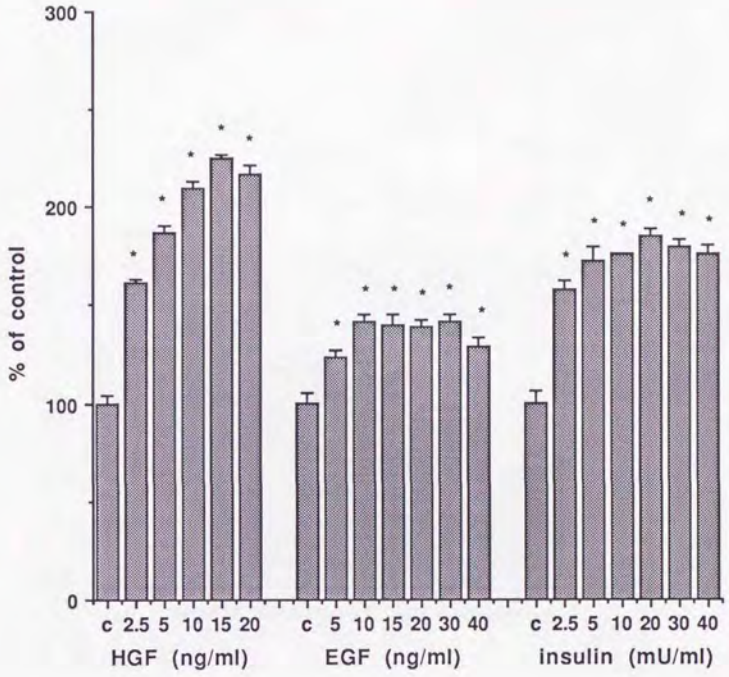


Figure 2-1-3a. DNA synthesis of gastric epithelial cells induced by various agents

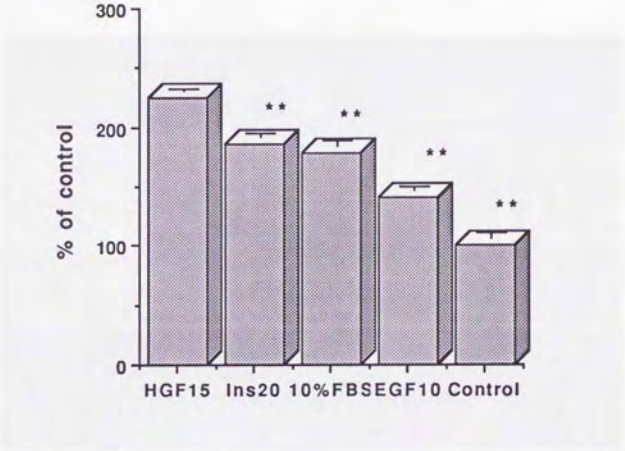


Figure 2-1-3b. Comparison of the maximum DNA synthesis induced by HGF, EGF, insulin, and 10%FBS

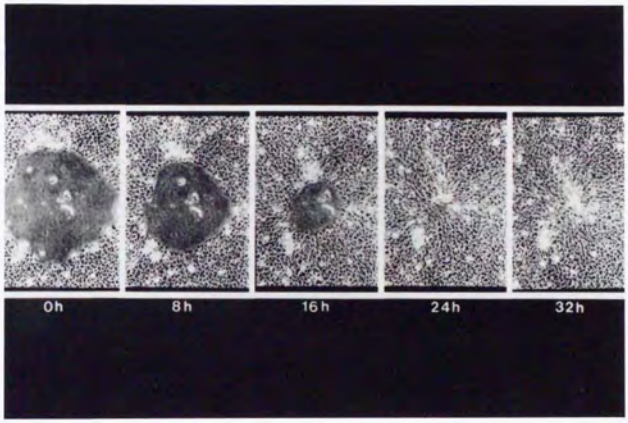


Figure 2-2a. The time course of wound restitution in the presence of 120 pM HGF

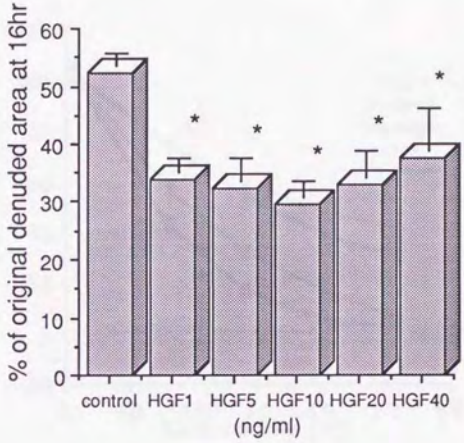


Figure 2-2b. Dose dependency of HGF effect on gastric restitution

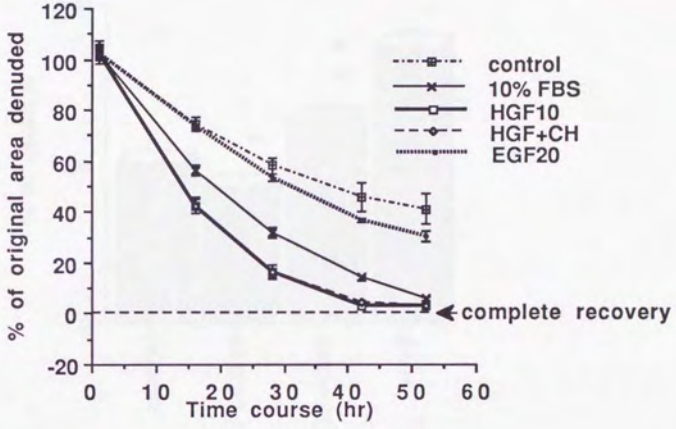


Figure 2-2c. Restitution induced by various agents

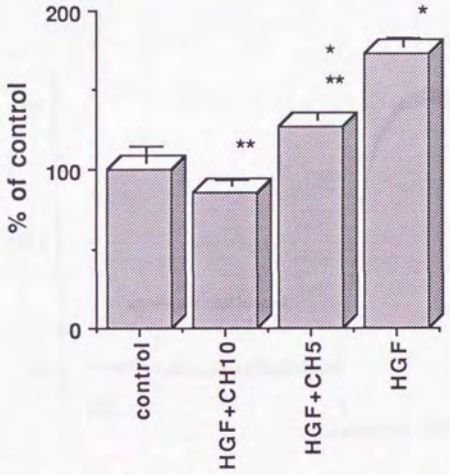


Figure 2-2d. DNA synthesis indeced by HGF is inhibited by cycloheximide

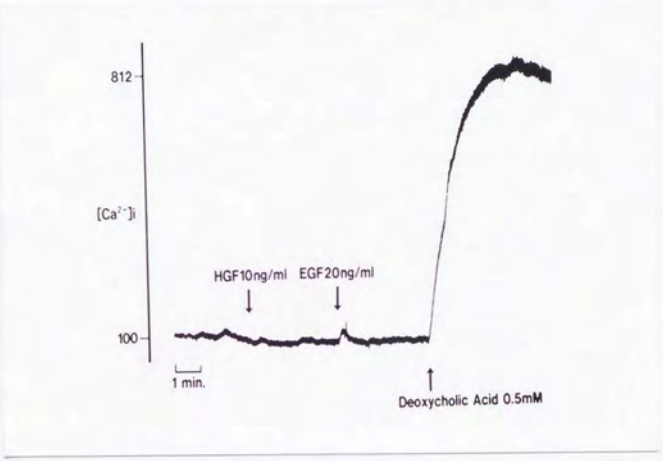


Figure 3-1. Intracellular Ca2+ concentration of cultured cells with stimulation by HGF and EGF

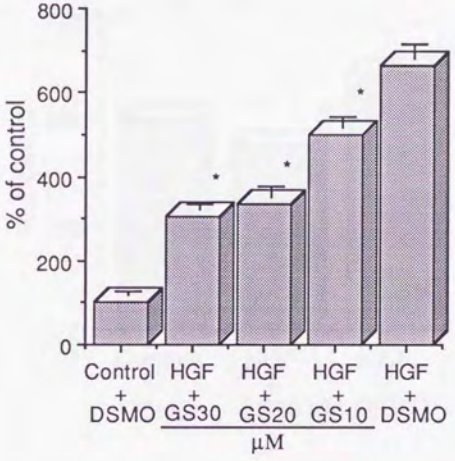


Figure 3-2a. Suppression of HGF-induced DNA synthesis by genistein, as assessed by [3H]-thymidine incorporation

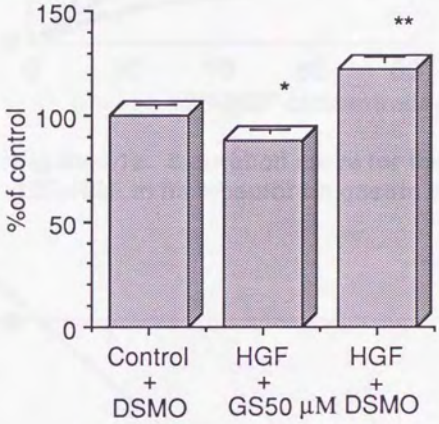


Figure 3-2b. Suppression of HGF-induced DNA synthesis by genistein, as assessed by crystal violet staining

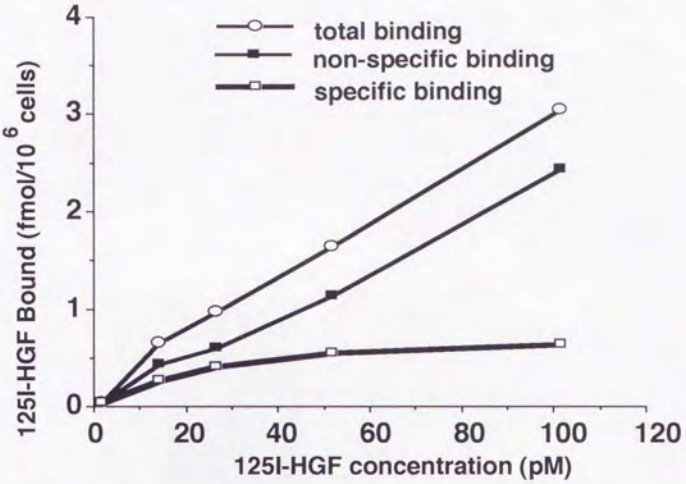


Figure 4-1a. Saturation curve for the binding of 125I-HGF to its receptor on gastric epithelial cells

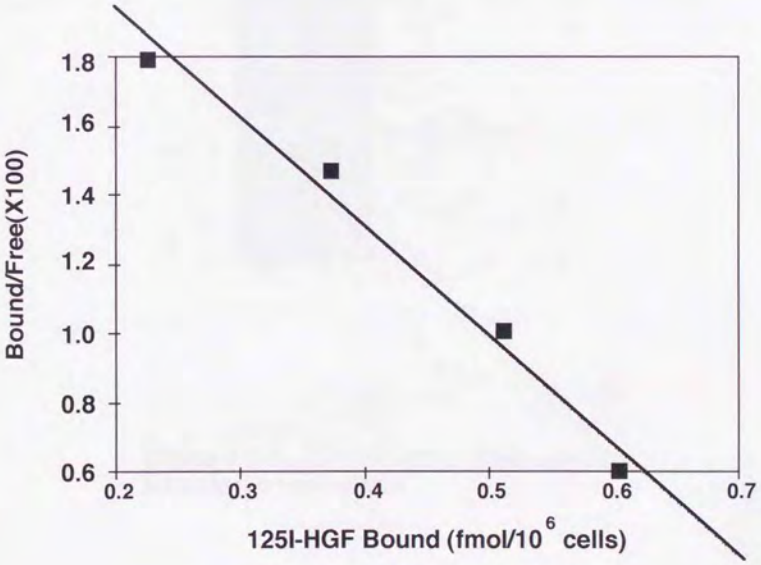


Figure 4-1b. Scatchard plot

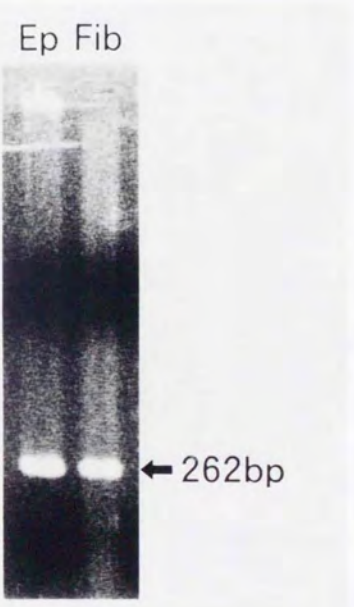


Figure 4-2-1. Expression of HGF specific receptor, c-met mRNA

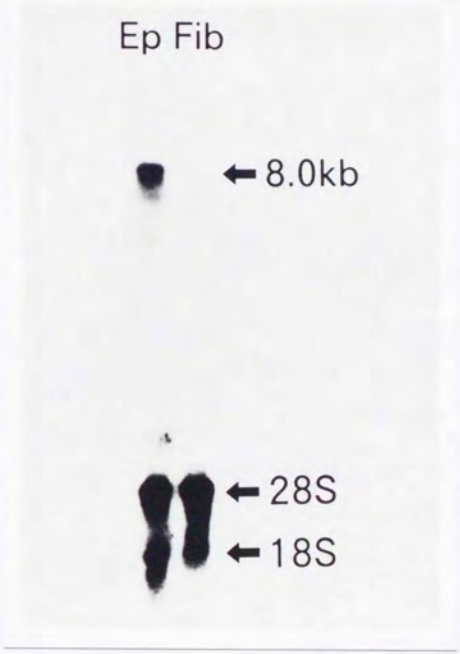


Figure 4-2-2. C-met expression by gastric cells, as assessed by northern blot hybridization

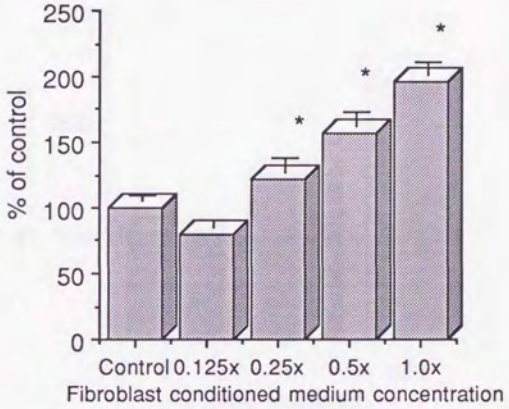


Figure 5-1a. DNA synthesis induced by fibroblast-conditioned medium, as assessed by [3H]-thymidine incorporation

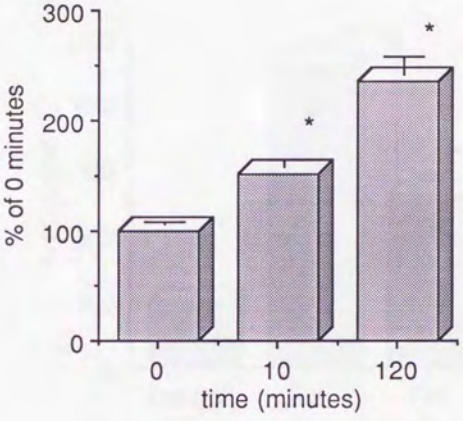


Figure 5-1b. DNA synthesis induced by conditioned medium which was left with gastric fibroblasts for indicated periods of time, as assessed by [3H]-thymidine incorporation

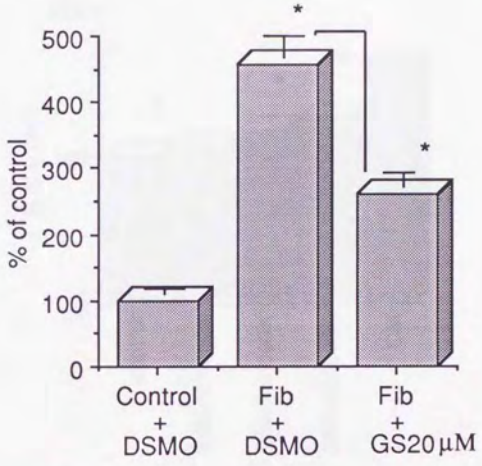


Figure 5-1c. Suppression of conditioned medium-induced DNA synthesis by genistein, as assessed by [3H]-thymidine incorporation

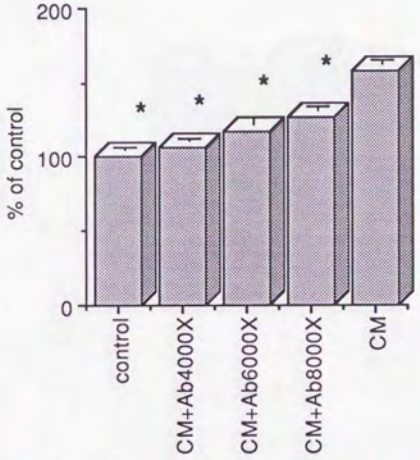


Figure 5-1d. Suppression of conditioned medium-induced DNA synthesis by anti-HGF antibody, as assessed by [3H]-thymidine incorporation

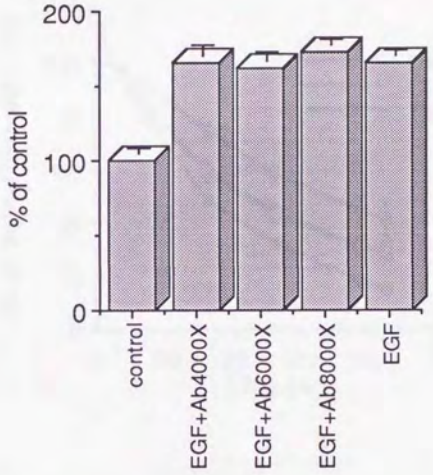


Figure 5-1e. Suppression of EGF-induced DNA synthesis by anti-HGF antibody, as assessed by [3H]-thymidine incorporation

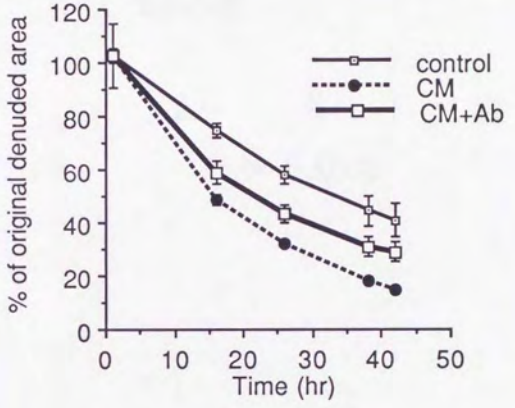


Figure 5-2. Effect of fibroblast-conditioned medium on gastric epithelial restitution

Figure 5-3. Expression of HGF mRNA by gastric cells, as assessed by northern blot hybridization

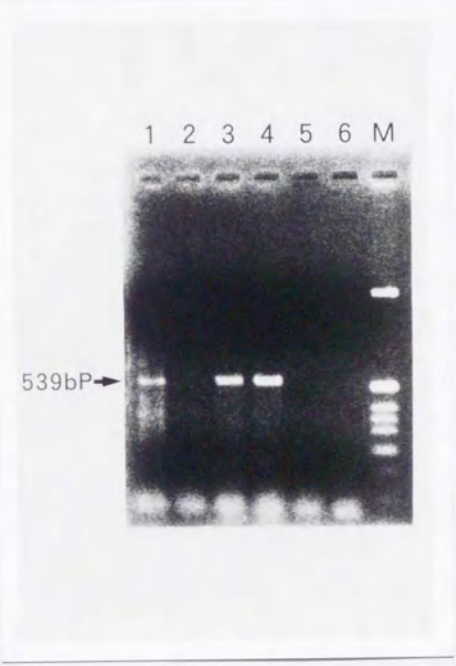


Figure 6-1. Representative demonstration of HGF mRNA expression in human tissue from the edge of a gastric ulcer

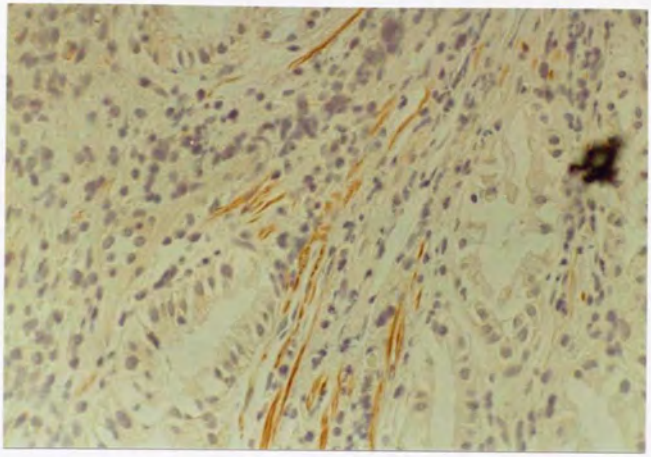


Fig. 6-2a

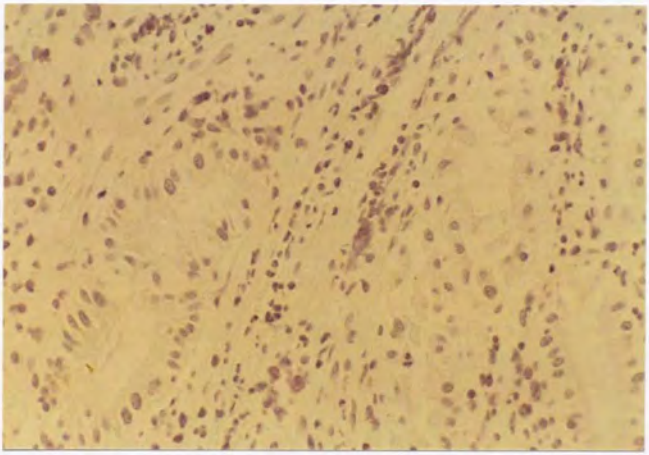


Fig. 6-2b

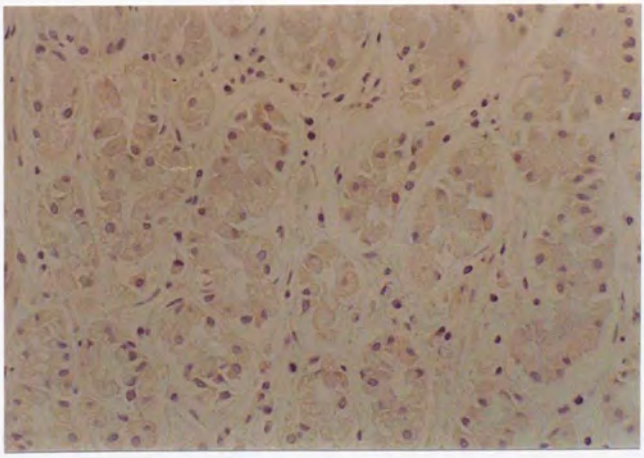
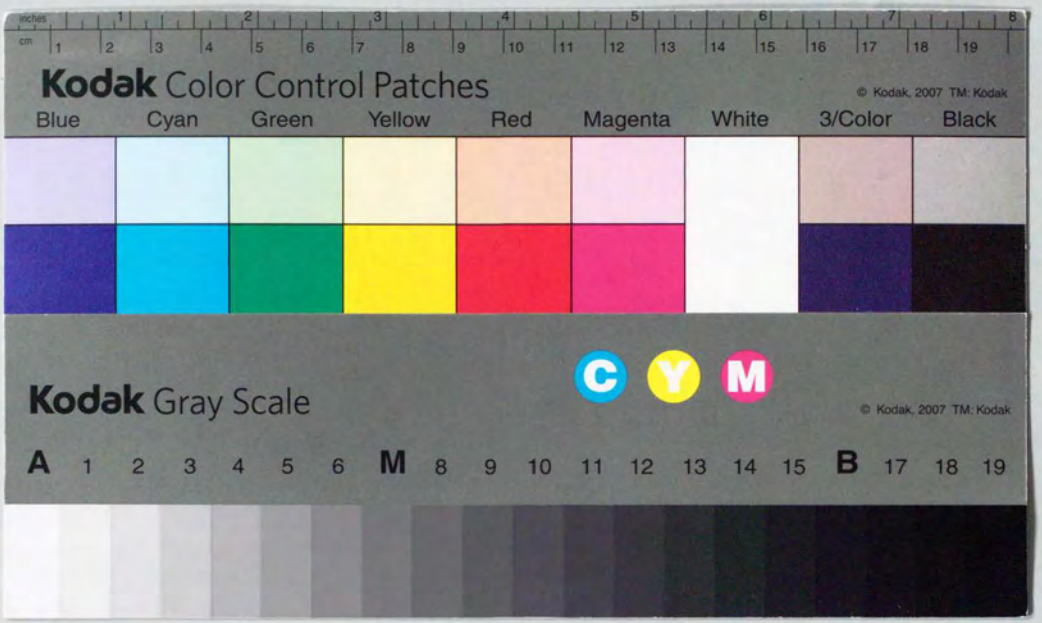


Fig. 6-2c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