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Constitutive Expression of Hepatocyte Growth Factor May Maintain the Sheet Construction of Gastric Epithelial Cells through Facilitating Actin-Myosin Contractile System

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Previously, we have demonstrated that hepatocyte growth factor (HGF) plays an important role in the repair process of gastric ulcer, showing that HGF expression is specifically increased at gastric ulcer edge. In the present study, we demonstrated the constitutive expression of HGF mRNA in normal mucosa, which is as much as 0.1-1.0 attomole/μg total RNA. In order to evaluate the hypothesis that HGF might have some role in maintenance of gastric mucosa or prevention of injury initiation, we developed an in vitro model using rabbit gastric epithelial cell in primary culture. 1% ethanol destroys the cell to cell contact to disrupt the monolayer sheet of the cells without causing any damage to cell viability, indicating that irritants may initiate the mucosal injury. HGF remarkably prevented the disruption induced by the ethanol without eliciting proliferation or migration. This action of HGF was suppressed by actin selective inhibitor, cytochalasin B, indicating that it was mediated by an actin-myosin contractile system. In conclusion, constitutively expressed HGF may prevent the initiation of gastric epithelial disruption, which is dependent on some sort of mobile action of the cells.

Gastro-intestinal tract is constantly exposed to foreign substances. Mucosal epithelial cell layer is an important first line of physical defense. Epithelial cells make up a sheet in which each cell sticks together with cell to cell junction including tight junction so as to protect the internal tissues. Previous reports suggested that mucosal damage is repaired by various growth factors through their potential ability to stimulate proliferation and migration of gastric epithelial cell (1), (2), (3), (4), (5). We have also previously shown that endogenously produced HGF has a stimulative effect on proliferation and restitution of gastric epithelial cells and HGF is specifically expressed at gastric ulcer edges (6). These findings directly suggests that the growth factor is specifically induced at damaged site and facilitate the gastric ulcer repair. However, the maintenance of mucosal integrity or initiation to the mucosal injury, as compared with the repair, has never been focused on previously. In the present study, we demonstrate that HGF is constitutively expressed at normal gastric mucosa, which suggests that it might have some role in maintenance of gastric mucosa or prevention of injury initiation. In order to evaluate the role of integration of cultured gastric epithelial cell monolayer, which may represents gastric mucosal epithelial layer.

MATERIALS AND METHODS

Reverse Transcription Polymerase Chain Reaction (RT-PCR) for HGF and Competitive RT-PCR

Total cellular RNA was isolated from biopsy tissue obtained from human normal gastric mucosa endoscopically, using RNAzol B (Cinna/Biotec Laboratories, Inc, Houston, Texas). One microgram 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

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of amplification of the HGF first-strand cDNA were performed with 20 pmol of each met primer (sense: 5″-^{979}CAGCGTTGGGATTCTCAGTAT^{1000}-3″ from exon 8; antisense: 5″-^{1497}CCTATGTTTGTTCGTGTTGGA^{1518}-3″ from exon 13, which should yield a single amplified band with a size of 539 bp. Each amplification cycle consisted of denaturation at 94°C for 30 sec, annealing at 52.2°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).

Competitive RT-PCR was performed to quantify the HGF mRNA expressed in normal gastric mucosa. Competitive PCR is a established method of quantification of mRNA level, during which one set of primers is used to amplify both a target gene cDNA and another DNA fragment; in essence the second DNA fragment competes with the target DNA for the same primers and thus act as an internal standards. The competitor was constructed to be used as competitive internal standards in PCR amplification for quantitation of mRNA level of target genes, using MIMIC construction kit (Clontech laboratories, Inc., Palo Alto, CA) according to the manufacturer's instruction. The competitor sequence is '5′-CAGCGTTGGGATTCTCAGTAT TGTTATACAG GGAGATGAAA GGATGCACTT GCCTAGCCCT ACAGATTCCA AGTTTTATCG CACCCTGATG GAGGAGGAGG ACATGGAAGA CATTGTGGAT GCAGATGAGT ATCTTGTCCC ACACCAGGGC TTTTTCAACA TGCCCTCTAC ATCTCGGACT CCTCTTCTGA GTTCATTGAG CGCTACTAGC AACAATTCTG CTACAAACTG CATTGACAGA AATGGGCAGG GGCACCCTGT GAGGGAAGAG GCTTCCTGCC TGCTCCAGAG TATGAAACC AGCTGATGCC CAAGAAACCA TCTACTGCCA TGGTCCAGAA TCAAAT TC-CAACACGAACAAACATAGG-3′′. (Underlined are the target gene primer sequences.) Serial dilutions of the competitor are added to PCR amplification reactions containing constant amounts of the target cDNA samples. The competitor and target templates compete for the same primers in the reaction. By knowing the amount of the competitor added to the reaction, one can determine the mRNA level.

Animals

Japanese white rabbits of either sex (Doken Laboratory, Ibaraki, Japan) weighing 2.5–3.0 kg were used.

Reagents

Human recombinant Hepatocyte Growth Factor (HGF) was purified from the conditioned medium of CHO cells transfected with expression vector containing full size human HGF cDNA (7). The reagents for gastric epithelial cell isolation and culture were as follows: Coon's modified Ham's F-12 medium (KC biological Inc., Lenexa, Kanzas), Basal medium Eagle (BME), minimal essential medium (MEM) (Sigma), amino acid, N-2-hydroxyethylpiperadine-N-2-ethanesulonic acid (HEPES) buffer (Sigma), bovine serum albumin (BSA) (fraction V, Sigma), Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, New York), crude type I collagenase (Sigma), ethylenediamine-tetraacetic acid (EDTA) (Sigma). Cytochalasin B was purchased from Sigma chemical (St. Louis, Missouri).

Cell Culture

Gastric fundic mucosal cells were isolated from adult rabbits and cultured as described previously (8). In brief, the fundic mucosa was quickly separated from rabbit stomach, scraped bluntly, and minced into 2- to 3-mm^2 pieces. The minced tissues 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 with an atmosphere of 5% CO_2 and 95% O_2. Cells from the final incubation were washed with HBSS, and cultured at 37°C in a moist atmosphere containing 5% CO_2. The culture was F-12 medium supplemented with 10% heat inactivated (56°C for 30 min) fetal bovine serum (Gibco), 15mM HEPES buffer, 100 units/ml penicillin, 100 units/ml streptomycin, and 5 μg/ml fungizone.

Cell Characterization

Cultured cells were examined at 48 hr by periodic acid-Schiff (PAS) staining as described elsewhere (9).

MTT Assay

MTT assay is a convenient way to estimate the number of viable cells and has been mainly used to assess cell growth (10). Cells were incubated with test agents for an experimental period under culture conditions. Culture plates were centrifuged for 5 min at 4°C. Media were carefully aspirated and cells were incubated with MTT (0.5 mg/ml) in EBSS for 1 hr. Culture plates were centrifuged for 5 min at 4°C. Media were aspirated and 50 μl of absolute ethanol was added. Culture plates were vigorously shaken (Plate Mixer MPM-2N, Katagaki) to insure solubilization of the blue formazan. The optical density of each well was measured using and automatic plate reader (model 2550 EIA Reader, BIO-RAD) with a 570 nm test wave length.

Assessment of Cell Sheet Integration

Gastric fundic mucosal cells were isolated as explained above and inoculated on 24-well culture plate. Cells were incubated in F-12 medium with 1% ethanol supplemented with test agents. The cell sheet gradually forms mesh like denuded area, of which Photomicrographs were obtained at a 40-fold magnification using a Nikon microscope and camera. The images were taken into the Macintosh computer by a scanner and the denuded areas were measured, using an image editing software, AdobePhotoshop™ (Adobe, Mountain View, CA).

RESULTS

The Expression of HGF mRNA in Human Normal Gastric Mucosa

Normal gastric mucosa was obtained endoscopically from the body of human stomach. HGF mRNA was clearly observed in the normal mucosa, which was as much as 0.1-1.0 attomole/μg total RNA (Fig. 1). HGF is constitutively expressed by normal human mucosa and HGF mRNA level is quite much, as compared with the 1.0–10.0 attomole/μg total RNA of GAPD, house keeping gene (data not shown), indicating that HGF might have some role in maintenance of gastric mucosa.

Cell Culture and Identification

Cultured cells formed confluent monolayers at 72 hr after inoculation. Figure 2 shows the phase contrast microphotograph of the monolayer culture at 72 hr after inoculation. The cells have PAS positive material in the cytoplasm, which was not affected by 15 min amylase treatment (data not shown).

Effect of Ethanol on Cell Viability

After 20 hours incubation, low concentration of ethanol, 2% and less, did not have any effect on viability of gastric epithelial cells, while ethanol of 3% and more decreased the cell viability in a dose dependent fashion, as assessed by MTT assay (Fig. 3).

Effect of Ethanol on Disruption of Gastric Cell Monolayer

The observation by ultrarapid motion pictures reveal that the 1% of ethanol, which dose not have any effect on viability as shown above, disrupted the monolayer construction of gastric epithelial cells; the cell to cell contact is disrupted to create the mesh-like denuded areas. The figure 4a shows the serial pictures obtained from the motion pictures. The disruption of the monolayer was assesses by measuring the denuded area, using computer system. The figure 4b show how the computer recognized the denuded area, which seems quite equivalent to human recognition.

Effect of HGF on Maintenance of Monolayer Construction against the Ethanol

HGF remarkably maintained the integrity of gastric cell monolayer in a dose dependent fashion against ethanol induced injury, without facilitating proliferation or migration, which was observed by ultra rapid motion pictures (Fig. 5a). The effect was suppressed by the actin inhibitor, cytochalasin B, indicating that cytoskeleton plays a important role in maintaining the monolayer

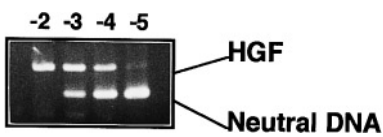

FIG. 1. Expression of HGF mRNA in normal gastric mucosa. Total RNA was extracted from biopsy samples obtained endoscopically from human normal gastric mucosa. Competitive RT-PCR was performed to quantify the level of HGF mRNA expression, using the competitor which was constructed from neutral DNA fragment. HGF mRNA expression level of gastric mucosa is estimated as 0.1–1.0 attomole per 1 μg total RNA. (-2: 10 attomole/μg, -3: 1 attomole/μg, -4: 0.1 attomole/μg, -5: 0.01 attomole/μg).

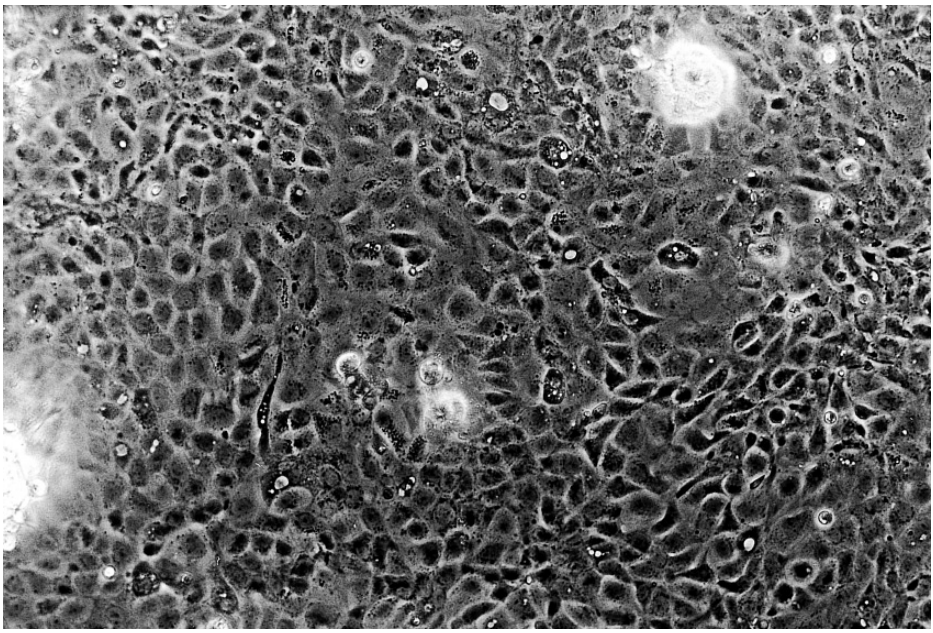

FIG. 2. Phase-contrast micrograph of rabbit gastric epithelial cells in primary culture (×40).

construction of gastric epithelial cells; HGF maintained the integrity by facilitating actin-myosin contractile system (Fig. 5b).

DISCUSSION

Numerous reports have been published to demonstrate the roles of growth factors, including epidermal growth factor, basic fibroblast growth factor, transforming growth factor α, β, platelet derived growth factor, and hepatocyte growth factor, in the repair process of gastric mucosal injuries (1), (2), (3), (4), (5), in which their stimulative action of proliferation, migration, angiogenesis, and mucous secretion were discussed. However, their direct roles in gastric mucosal maintenance has never been discussed before. The effect of HGF on viability of cultured hepatocytes was lately mentioned, although the effect was very modest (11). Our unpublished data

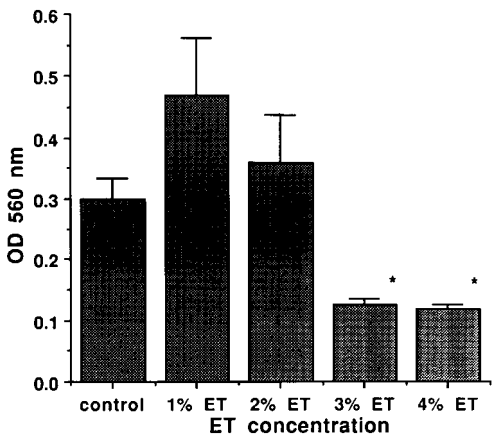

FIG. 3. Effect of ethanol on viability of gastric epithelial cells, as assessed by MTT assay (mean + SE, *: $p < 0.01$ vs. control).

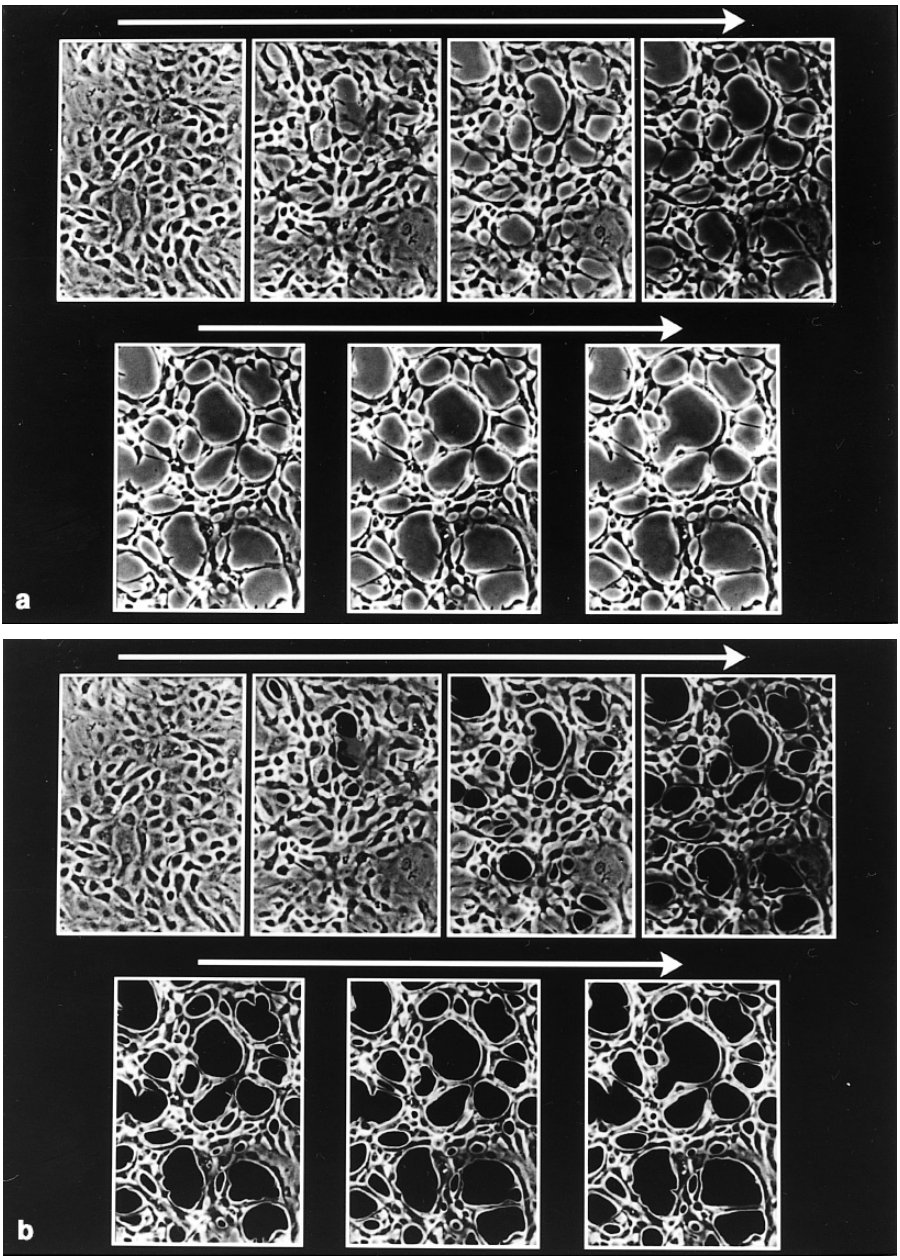

FIG. 4. (a) The time course of gastric epithelial monolayer sheet destruction induced by ethanol. (b) The destruction was assessed by determining the denuded area among the cells using a computer system. The picture shows how the computer recognizes the denuded areas. Black stained areas are recognized as denuded.

suggest that HGF dose not have protective action for gastric epithelial cell viability. Previously, we demonstrated that HGF plays an important role in the repair process of gastric ulcer repair, showing that the expression of HGF is specifically increased at the gastric ulcer edge (6). In the present study, we further performed competitive RT-PCR to show the constitutive expression of HGF mRNA in the normal gastric mucosa, and the amount of HGF mRNA is as much as 0.1-1.0 attomole/μg total RNA. What is the role of HGF constitutively expressed in the mucosa? We

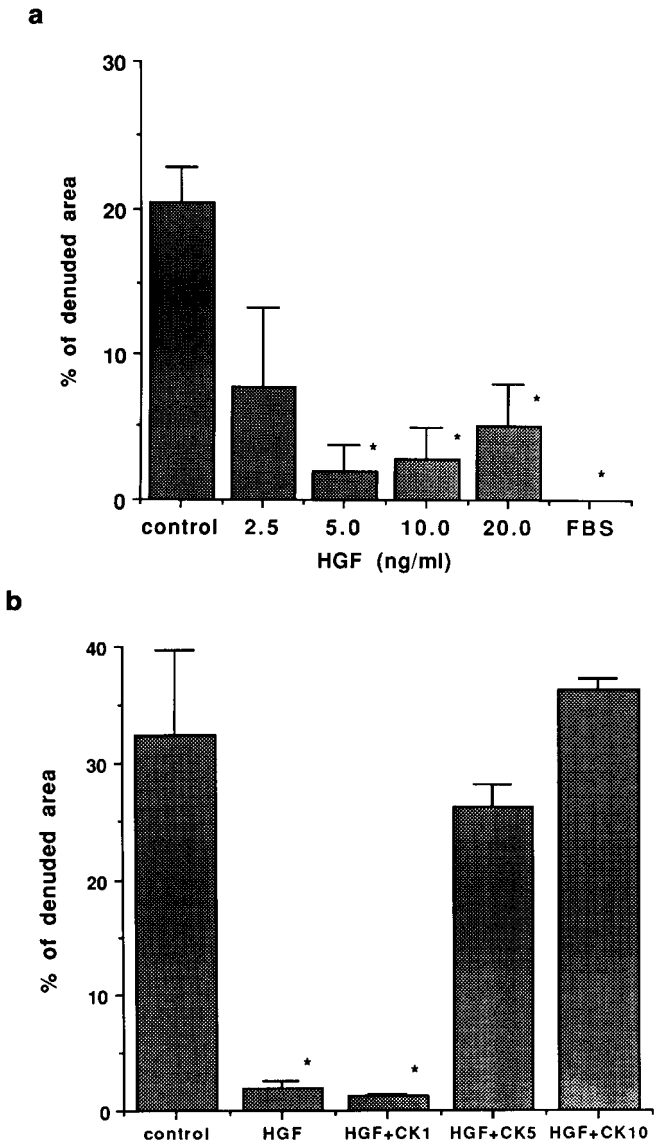

FIG. 5. (a) HGF prevented the destruction significantly in a dose dependent fashion (mean + SE, : $p < 0.01$ vs. control). (b) The preventive action of HGF was inhibited by actin selective inhibitor, cytochalasin B, indicating that cytoskeleton plays an important role in maintaining the monolayer construction of gastric epithelial cells; HGF maintained the integrity by facilitating actin-myosin contractile system (mean + SE, *: $p < 0.01$ vs. control).

speculated that not only dose HGF play a role in the process of gastric repair, it has the role in maintenance of gastric mucosa or prevention of injury initiation. Speaking of the maintenance of gastric mucosa, we have to evaluate the integrity of gastric epithelial layer which is an important first line of defense of internal tissue from harmful substances such as acid. Of course, cytotoxicity is another important event. However, as it turned out in the course of our present report, the disruption occurs easier than loss of cell viability. Therefore, so far as initiation of gastric injuries or maintenance of gastric mucosa is concerned, the evaluation of epithelial disruption is important, and so we established an in vitro system to evaluate the effect of hepatocyte growth factor on the maintenance of gastric mucosa. Monolayer construction of our primary culture system resembles

gastric mucosal epithelial layer in many ways. For example, epithelial cells are attached to each other to form a sheet construction by tight junction (9) which is the essential device for the tissue construction. In this sense, the destruction of monolayer culture of gastric epithelial cells may represent the actual initiation of gastric mucosal injuries, and it enables us to investigate the mechanism in a simplified system. In addition, considering the gastric juice and mucous layer of the stomach, ethanol concentration which reach to the epithelial layer maybe quite low in the normal condition. And low concentration ethanol of 1% which is used and did not have any effect on cell viability in the present study, may be practical for investigating the actual initiation of gastric mucosal disruption.

It is previously demonstrated that HGF is a pleiotropic factor, which has mitogenic, motogenic, and sometimes morphogenic effects (12). Our data indicate that HGF maintained the monolayer construction without eliciting its potential ability for migration or proliferation. Therefore, the effect of HGF here appears something new and different from previous acknowledged effects of HGF at a glance. The concept, 'protection' seems quite static. However, our data indicated that the effect was mediated by restoring actin-myosin contractile system. The previous reports indicated that the effect of HGF on migration was mediated by induction of actin polymerization (13). Actually, the observation of ultrarapid motion pictures shows that even in confluence, the gastric epithelial cells are changing their shapes dynamically and incessantly, so as to cover the opening among them, though their relative locations are not changed. Therefore, we speculate that integrity of gastric epithelial cell sheet is very much dependent on cell movement which is quite dependent on the endogenously and constitutively expressed HGF.

In conclusion, constitutively expressed HGF may prevent the initiation of gastric epithelial disruption, which is dependent on some sort of mobile action of the cells.

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