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HEPATOCYTE GROWTH FACTOR STIMULATES WOUND REPAIR OF THE RABBIT ESOPHAGEAL EPITHELIAL CELLS IN PRIMARY CULTURE

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We have recently established an in vitro primary culture system for esophageal epithelial cells, which enabled us to investigate the effect of hepatocyte growth factor (HGF) and other factors on esophageal restitution. HGF remarkably stimulated restitution of these cells. So did epidermal growth factor (EGF), though moderately. Restitution velocity of esophageal cells was remarkably higher than that of gastric epithelial cells. The expression of c-met, specific HGF receptor was demonstrated by the esophageal cells, suggesting that the effect of HGF was mediated by its specific receptor. The expression level of c-met mRNA was the same as that of gastric epithelial cells, as assessed by competitive RT-PCR technique. These results suggest that HGF might be involved in the repair process of esophageal mucosal damage.

The lining of esophagus is a stratified squamous epithelium, which is as strong as dermal epithelium. However, it is constantly exposed to foreign substances such as food and hydrogen chloride from stomach, which sometimes causes mucosal damage (1). As in the case of gastric mucosa, mitogenic response of the cells to various growth factors might be important to maintain the integrity of the esophageal mucosa and to accelerate the healing process of esophageal mucosal damage (2), (3). Some growth factors, including epidermal growth factor (EGF), transforming growth factor (TGF), insulin, and insulinlike growth factor 1 have been reported to induce mitogenic response to normal gastric epithelial cells using primary culture models (4), (5). However, the mechanism of esophageal repair has not been very much discussed before. One of the reasons is that in vitro model of esophagus, especially primary culture system for esophageal epithelial cell has not been established. Although some attempts have been performed to establish primary systems of esophageal epithelial cells, each served only as a particular and limited usage (6).

Hepatocyte growth factor (HGF) is a hepatotrophic factor for liver regeneration, purified from rat platelet (7). Not only does it have the proliferative effect on hepatocyte, but also on other various epithelial cells, such as renal tubular cells (8), epidermal melanocytes (9), and keratinocytes (10). In addition, we have previously demonstrated the effect of HGF on proliferation and restitution of gastric epithelial cells through paracrine mechanism, which is stronger than those of formerly established growth factors (11), (12). The source of HGF have been demonstrated to be mesenchymal cells such as fibroblasts, Kupffer cells, and endothelial cells but not epithelial cells (13),(14). In the case of gastric mucosa, fibroblasts were demonstrated to be one of the source (12).

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At present study, we newly developed a primary culture system for esophageal epithelial cells and demonstrated the effect of HGF and EGF on restitution and compare the effect between esophageal and gastric epithelial cells. We also compare the HGF specific receptor, c-met mRNA expression level between them.

Materials and methods

Animals

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

Reagents

Human EGF was purchased from Wakunaga, Hiroshima, Japan. Human recombinant Hepatocyte Growth Factor (HGF) was purified from culture fluids from transformed CHO cells. The reagents for esophageal and gastric epithelial cell isolation and culture were as follows: Coon's modified Ham's F-12 medium (KC biological Inc., Lenexa, Kansas), 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).

Cell culture

Gastric mucosal cells were isolated from adult rabbits and cultured as described previously (15).

Esophageal mucosal cells were isolated from adult rabbits as follows. Esophagus was quickly resected from rabbit after sacrifice. The epithelium was separated from the underlying muscular layer by sharp scalpel, producing a sheet of epithelium. It was then minced into less than 1 mm 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 Cells from the final incubation were washed with 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), 15mM HEPES buffer, 100 units/ml penicillin, and 100 units/ml streptomycin.

Cell characterization

Cultured cells were examined at 48 hr by periodic acid-Schiff (PAS) staining as described elsewhere (16), as well as phase contrast microscopy.

Restitution model

The effects of HGF and EGF were studied using an in vitro model of gastric epithelial restitution (12). Confluent monolayers of primary cultured esophageal epithelial cells in 24-well culture plates were wounded with a custom-made scraper that produced a round wound approximately 1.5 mm in diameter in each well after 24 hr of serum free period. 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 and medium with 10% FBS. Restitution of the epithelial cells was assessed in a blind fashion to avoid observer bias; 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 regular. Experiments were performed 6 times and the results were expressed as the mean ± SE.

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 B (Cinna/Biotecx 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 of amplification of the c-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'), which should yield a single amplified band with a size of 242 bp. 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).

Competitive reverse transcription polymerase chain reaction

Competitive RT-PCR was performed to quantify the c-met mRNA expressed in esophageal and gastric epithelial cells for comparison. 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 used as competitive internal standards in PCR amplification for quantitation of mRNA level of target genes, using MIMIC^{TM} construction kit (Clontech laboratories, Inc., Palo Alto, CA) according to the manufacturer's instruction. The competitor sequence is "5'CCAACGACTAAAACCAGTACG TGTTATACAG GGAGATGAAA GGATGCACTT GCCTAGCCCT CATTGTGGAT GCAGATGAGT ATCTTGTCCC ACACCAGGGC TTTTTCAACA TGCCCTCTAC ATCTCGGACT CCTCTTCTGA GTTCATTGAG CGCTACTAGC AACAATTCTG CTACAAACTG CATTGACAGA AATGGGCAGG GGCACCCTGT GAGGGAAGAG GCTTCCTGCC TGCTCCAGAG TATGTAAACC AGCTGATGCC CAAGAAACCA TCTACTGCCA TGGTCCAGAA TCAAA TCTTCTGAGGATGTTGGGCTT3'". (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.

Results

Cell culture and identification

Cultured cells formed sub-confluent monolayers at 72 hr after inoculation. Figure 1a shows the phase contrast micrograph of the monolayer culture at 72 hr after inoculation. The cells have PAS positive material in the cytoplasm, which disappear after 15 min amylase treatment (Fig.1b and Fig.1c), indicating that the material is glycogen. It is well known that mature esophageal epithelial cells contains glycogen in the cytoplasm. Therefore, these cells may be considered as esophageal epithelial cells.

Effect of HGF and EGF on restitution

Confluent monolayers of esophageal and 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 2a shows the time course of esophageal wound restitution in the presence of 10 ng/ml HGF. Figure 2b shows the dose-response of the effect of HGF on the restitution of esophageal epithelial cells at 10.5 hours after wounding of the monolayers. It was found that 10 ng/ml (120 pM) was the optimum concentration. Figure 2c shows the dose-response of the effect of EGF on the restitution of esophageal epithelial cells at 10.5 hours after wounding of the monolayers. It was found that 10 ng/ml (120 pM) was the optimum

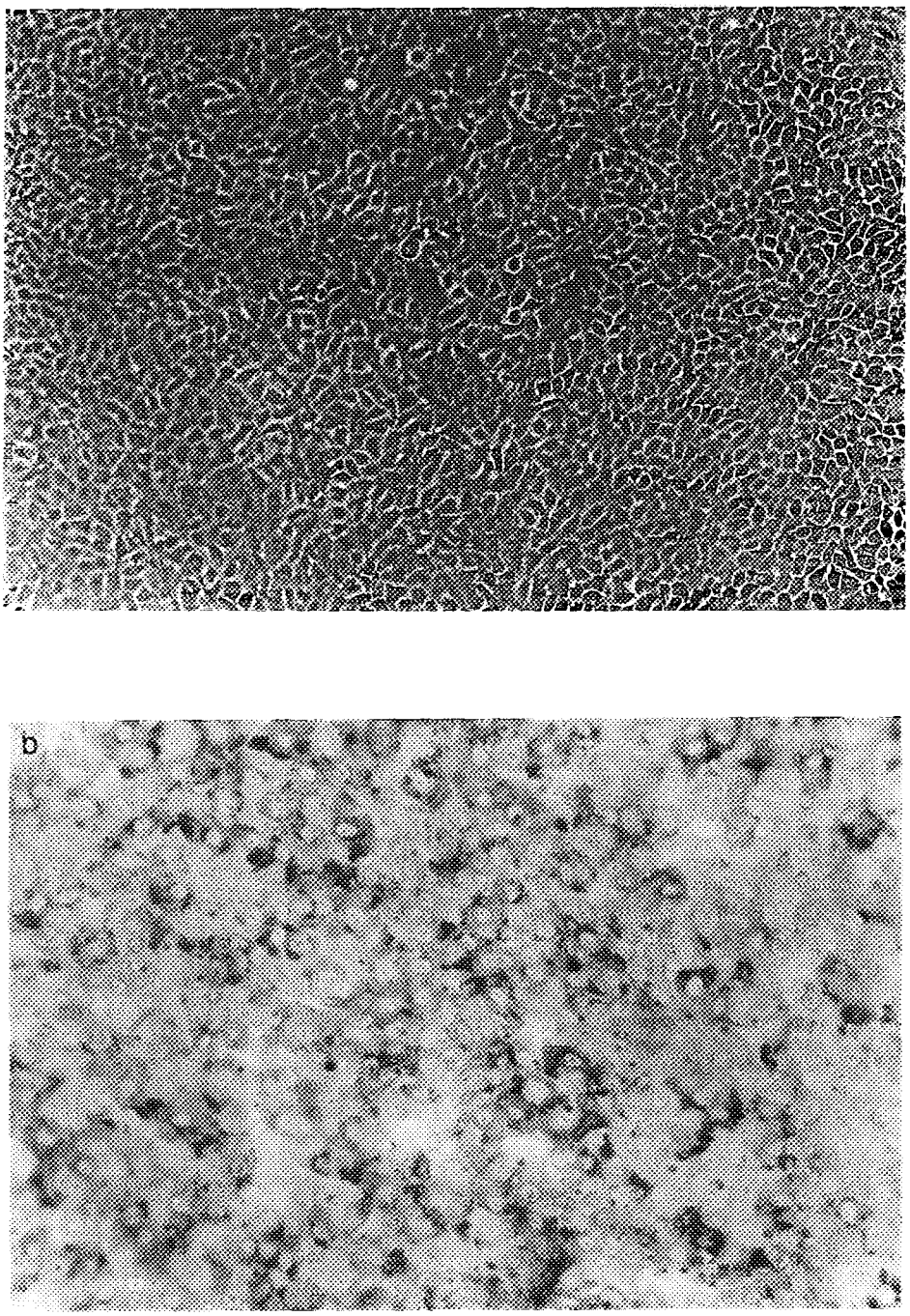


Figure 1. a: Phase-contrast micrograph of rabbit esophageal cells in primary culture (x40). **b**: PAS staining of cells cultured for 72 hr (x100). Dark materials in the cytoplasm are PAS-positive materials. **c:** PAS staining of the cultured cells after 15 min amylase treatment. The PAS-positive materials disappeared, suggesting that they are glycogen. Therefore, the cells are identified as matured esophageal epithelial cells.

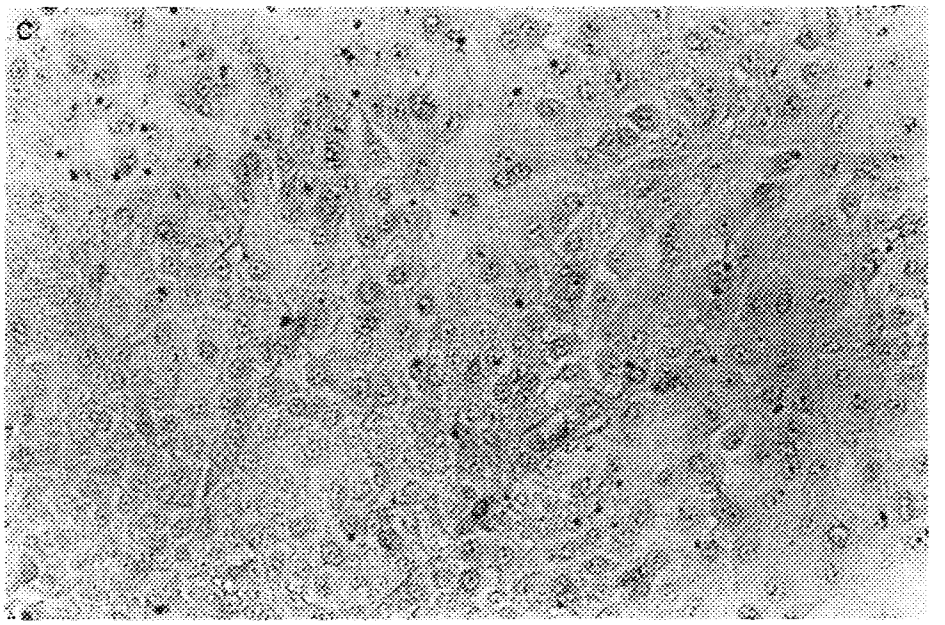


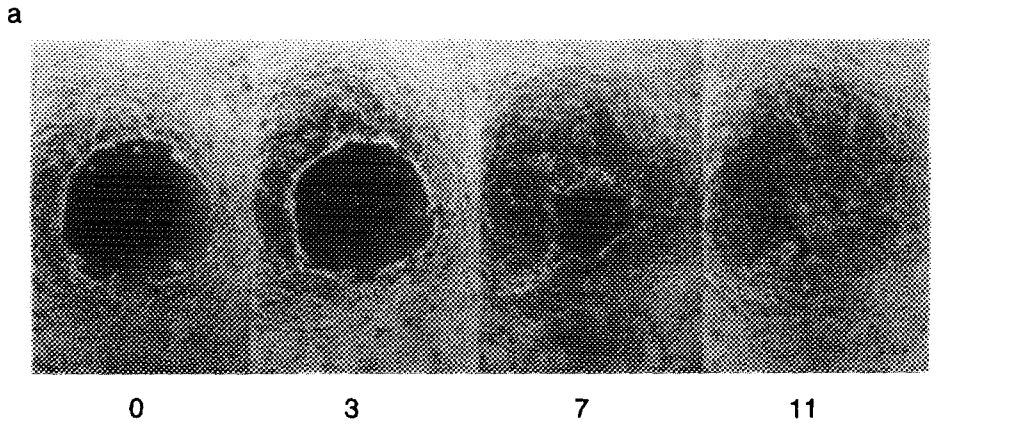
Figure 1 – *Continued*

concentration. HGF showed the promotion of restitution equivalent to 10% FBS. However, the effect of EGF was not so remarkable as HGF though significant compared with control ($p<0.01$) (Fig. 2d). The restitution of esophageal cells was much more remarkable than that of gastric epithelial cells (Fig. 2e).

Expression of HGF specific receptor, c-met mRNA

RT-PCR revealed that Esophageal cells yielded a single amplified band with an estimated size of 242 bp. Competitive RT-PCR further revealed that the c-met mRNA

Figure 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 esophageal 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 10.5 hours after wounding. HGF facilitated restitution of esophageal 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:** EGF was also tested at concentrations from 2.5-20 ng/ml (410-3280 pM), with 10 ng/ml (1640 pM) being found to stimulate restitution maximally (mean+SE, *: $p<0.01$ vs. control). **d:** 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 > 1640 pM EGF. Each plot represents the mean ± SE. (HGF10: 10 ng/ml (120 pM) HGF, EGF10: 10 ng/ml (1640 pM) EGF) **e:** Comparison of restitution between gastric and esophageal epithelial cells induced by 10 ng/ml HGF. The restitution assay on both gastric and esophageal cells was performed without serum free period. Esophageal restitution was far more remarkable than gastric restitution.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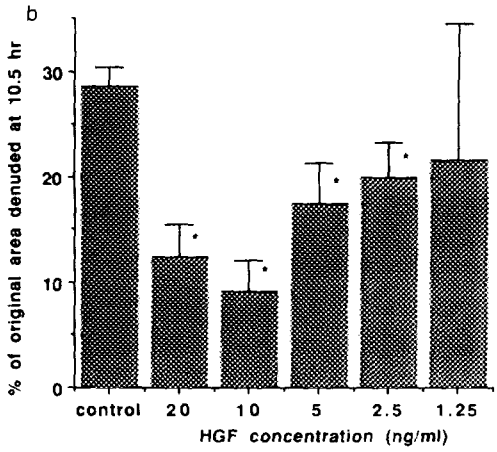
b
% of original area denuded at 10.5 hr
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control
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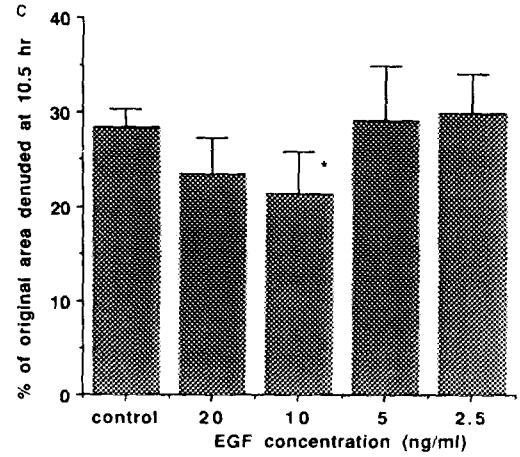
HGF concentration (ng/ml)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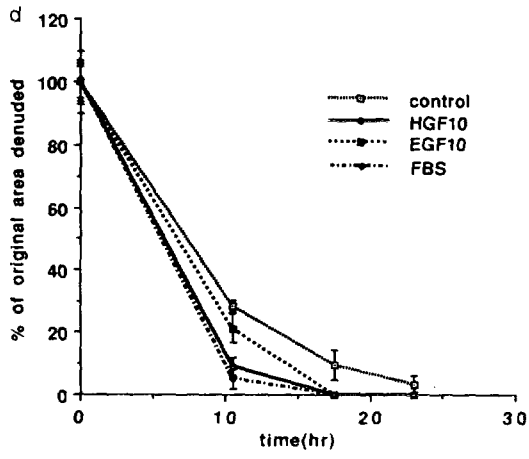
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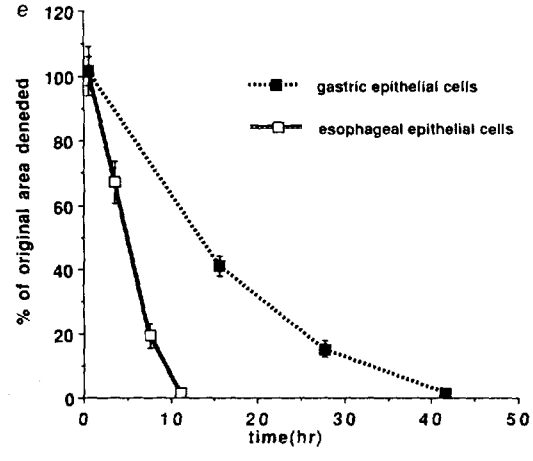
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gastric epithelial cells
esophageal epithelial cells

expression by esophageal cells was as much as 10 attomoles/ 1μg total RNA, which was the same as by gastric epithelial cells (Fig. 3).

Discussion

Esophageal mucosal impairment is clinically quite frequent. Although several reports indicates that growth factors, especially EGF are important in the repair (1), (2), (3), (17), (18), (19), the mechanism of repair process was neglected for a long time. In the present study, we developed new primary culture system for esophageal epithelial cells, which enabled us to perform in vitro restitution assay. The esophageal epithelial cells are composed of three layers called stratum germinativum, stratum spinosum and stratum corneum. The cells move from basal location (stratum germinativum) towards luminal surface (stratum spinosum and stratum corneum) as they evolve, being differentiated and matured. Matured cells were characterized as glycogen containing cells. Since our cells are demonstrated to have glycogen, these cells may be regarded as those actually exist on the luminal surface and at the edge of the wound of esophageal mucosa.

As we have stated in the introduction, HGF has been found to induce mitogenic reaction to various kinds of epithelial cells, including gastric epithelial cells. However, its effects on esophageal mucosal cells has not been studied before. Using these cells, we performed in vitro wound restitution assay. Early repair of digestive tract is mainly dependent on movement of the adjacent cells called restitution rather than proliferation (21), (22). Growth factors are considered to play important roles in this process (22). In the present study, we investigated the effects of HGF on the restitution of rabbit esophageal epithelial cells in primary culture. We have found that there was a clear dose-dependent response in restitution of these cells to HGF. The level of specific HGF receptor, c-met mRNA expressed by esophageal epithelial cells was the same as that of gastric epithelial cells. Therefore, the action of HGF may be mediated by the HGF receptor, as in the case of gastric epithelial cells. The restitution of esophageal epithelial cells was much more remarkable than that of gastric epithelial cells. Further studies are

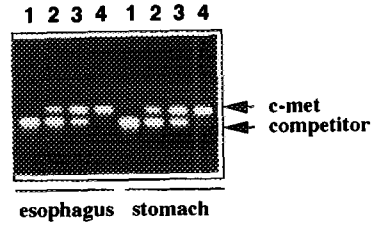

Figure 3. Expression of HGF specific receptor, c-met mRNA. Total RNA was extracted from esophageal epithelial cells and gastric epithelial cells. Competitive PCR was performed to quantify the level of c-met expression, using the competitor which was constructed from neutral DNA fragment. C-met expression levels of both esophageal and gastric epithelial cells are estimated as $6x10^{7.5}$ copies per 0.1μg total RNA. (1: $6x10^{9}$, 2: $6x10^{8}$, 3: $6x10^{7}$, 4: $6x10^{6}$).

necessary to clarify the mechanism of the difference in restitution between esophageal and gastric epithelial cells.

In conclusion, HGF may be an important factor for the repair process of esophageal mucosa.

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