Promoters of epithelialization induce expression of vascular endothelial growth factor in human gastric epithelial cells in primary culture

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Abstract Both epithelialization and angiogenesis are indispensable processes in gastric ulcer healing. Coordination between these processes has not been well studied. In the present study, we have established a new primary culture system of human gastric epithelial cells and investigated the effect of epithelialization stimulants on a specific angiogenic factor, vascular endothelial growth characterized as epithelial cells. Both epithelialization stimulants, hepatocyte growth factor (HGF) and epidermal growth factor (EGF), significantly stimulated vascular EGF expression in gastric epithelial cells. HGF and EGF receptors were expressed by the cells, suggesting that regulation may be mediated through specific receptors.

Key words: Vascular endothelial growth factor; Hepatocyte growth factor; Epidermal growth factor; Gastric epithelial cells; Primary culture

1. Introduction

Gastric ulcer healing requires a blood supply for cellular nutrient inflow as well as waste outflow. Therefore, angiogenesis is one of the most important processes in gastric ulcer healing [1]. Several growth factors are responsible for angiogenesis. We recently reported that vascular endothelial growth factor (VEGF), which is a strong angiogenic factor, plays an important role in gastric ulcer healing [2]. Epithelialization is another important process in gastric ulcer healing, on which the quality of ulcer healing is dependent. Epithelialization is also dependent on growth factors, such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF) [3–5].

Angiogenesis and epithelialization are both essential processes in gastric ulcer healing. In addition, these processes should occur in a coordinated way: epithelialization should accompany angiogenesis to provide sufficient blood supply to newly formed epithelium. Therefore, coordination between these growth factors is important. However, the coordination between angiogenesis and epithelialization, accordingly between these factors, has not previously been addressed. Therefore, the role of VEGF and the regulation of its expression in gastric cells must be investigated in order to gain a complete understanding of the whole story of gastric ulcer healing.

An immunohistochemical study showed that the sources of VEGF are gastric epithelial cells and fibroblasts [2]. Therefore, the regulation of VEGF expression in gastric epithelial cells is important, which should be inevitably investigated using an in vitro culture system. As Zhang et al. demonstrated, cell lines express various genes differently from normal tissues. Therefore, primary culture systems may be the best alternative. Presently, primary culture systems of gastric epithelial cells derived from non-humans, such as rabbits and rats, are available [6] and these culture systems have brought about many fruitful achievements. Human systems still have great advantages over these non-human systems, such as the availability of various commercial ELISA kits and gene information. In spite of the advantages and the efforts we and other groups have made to establish a primary culture system of human gastric epithelial cells suitable for investigating protein synthesis and its regulation by certain factors, it is not yet available. Terano et al. previously developed a system but it was derived from an endoscopically obtained biopsy sample and formed only a few small colonies [7]. Smoot et al. also developed a system which was similar to Terano's and produced only a few small colonies [8]. These systems are not suitable for investigating protein synthesis. Therefore, for this study, we further improved the system of Terano et al. and established a new primary culture system of human gastric epithelial cells derived from human stomach tissue obtained at surgery.

In the present study, we investigated the effects of HGF and EGF on VEGF expression using a primary culture system of gastric epithelial cells. We also performed RT-PCR to demonstrate the expression of HGF and EGF receptors in gastric epithelial cells.

2. Materials and methods

2.1. Cell culture

Human gastric mucosal epithelial cells were isolated from the adult human stomach. Gastric mucosa was obtained at surgery and incubated in PBS containing pronase (400 units/ml) (Kaken, Tokyo, Japan). The submucosal layer was carefully removed by scissors. The epithelial layer was then minced into 0.5–1 mm^2 pieces and incubated in BME containing collagenase B (0.35 mg/ml) (Boehringer Mannheim GmbH, Germany), for 10 min. This was followed by incubation in BME containing 1 mM EDTA for 5 min and again in BME containing collagenase B for 20–40 min. Cells from the final incubation were washed and cultured at 37°C in a moist 5% CO_2 atmos-

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Abbreviations: VEGF, vascular endothelial growth factor; VPF, vascular permeability factor; HGF, hepatocyte growth factor; EGF, epidermal growth factor; BME, basal Eagle's medium; MEM, minimal essential medium; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; RT-PCR, reverse transcription polymerase chain reaction

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phere. Culture medium was F-12 medium supplemented with 10% heat inactivated (56°C for 30 min) fetal bovine serum (Gibco BRL, Gaithersburg, MD), 15 mM HEPES buffer, 100 units/ml penicillin, 100 units/ml streptomycin, and 5 mg/ml fungizone. Subconfluent gastric epithelial cells were obtained after 48 h. When expression studies were performed, F-12 medium was supplemented with 0.1% bovine serum albumin (BSA) instead of FBS, besides test agents.

Gastric fibroblasts were also obtained from the procedure described above after cultured for more than a month, when mucosal cells died and fibroblasts were dominant. During the period, F-12 medium with 10% FBS was changed twice every week.

2.2. Reverse transcription polymerase chain reaction for c-met and EGF receptor mRNA

Total cellular RNA was isolated from endoscopically obtained gastric mucosal biopsy samples using RNAzol B (Cinna/Biotecx Laboratories, Inc., Houston, TX). 0.1 μg total RNA was reverse transcribed using M-MLV reverse transcriptase (Gibco BRL), after which the product was subjected to PCR with the 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′) or EGF receptor primer (sense: 5′-^{1290}AGG ACG GGG ACC AGA CAA CT^{1309}-3′; antisense: 5′-CAC CAC CAG CAG CAA GAG GA^{1535}-3′) [9]. Each amplification cycle consisted of denaturation at 94°C for 45 s, annealing at 53.1°C (c-met), or at 59.1°C (EGF receptor) for 45 s, and polymerization at 72°C for 90 s.

2.3. Determination of VEGF and IL-8 concentration

VEGF concentrations were determined by enzyme-linked immunosorbent assay (ELISA) with a human VEGF EIA kit (Immuno-Biological Laboratories Co. Ltd., Fujioka, Gunma, Japan). The IL-8 concentration was also determined with a human IL-8 EIA kit (PerSeptive Biosystems, Framingham, MA).

2.4. Reverse transcription polymerase chain reaction for VEGF

Total cellular RNA was isolated from cultured human gastric epithelial cells using RNAzol B. 0.1 μg total RNA was reverse transcribed using M-MLV reverse transcriptase (Gibco BRL), after which the product was subjected to PCR analysis with VEGF primers (sense: 5′-GAG TGT GTG CCC ACT GAG GAG TCC AAC-3′; antisense: 5′-CTC CTG CCC GGC TCA CCG CCT CGG CTT-3′) [10]. Each amplification cycle consisted of denaturation at 93°C for 30 s, annealing at 55°C for 60 s, and polymerization at 72°C for 60 s.

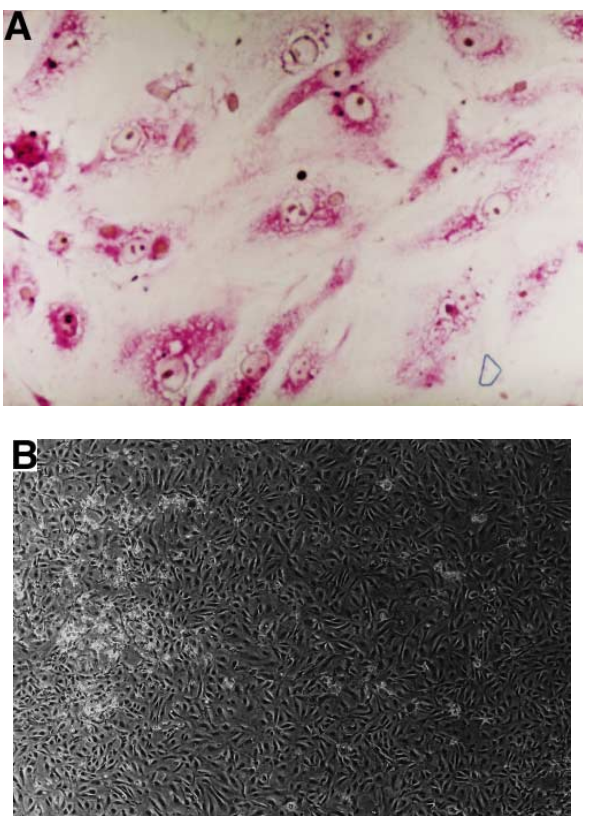

Fig. 1. A: PAS staining of human gastric epithelial cells cultured for 48 h (×100). The pink material in the cytoplasm represents PAS-positive material. B: Phase-contrast micrograph of human gastric epithelial cells in primary culture (×40).

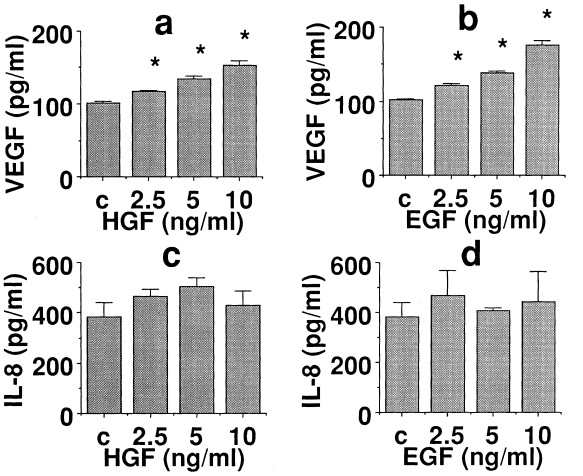

Fig. 2. a: The effect of EGF on the production of VEGF protein by human gastric epithelial cells, as assessed by ELISA. HGF induced VEGF production by gastric epithelial cells in a dose-dependent manner (mean+S.E., $*P < 0.01$ vs. control). b: The effect of EGF on the production of VEGF protein by human gastric epithelial cells, as assessed by ELISA. EGF induced VEGF production by gastric epithelial cells in a dose-dependent manner (mean+S.E., $*P < 0.01$ vs. control). c: The effect of HGF on IL-8 release by gastric epithelial cells (mean ± S.E.). d: The effect of EGF on IL-8 release by gastric epithelial cells (mean+S.E.).

3. Results

3.1. Cell culture and identification

Cultured cells had formed subconfluent monolayers by 48 h after inoculation. Fig. 1A shows a phase contrast micrograph of the monolayer. 93% of the cells in the monolayers had PAS-positive material in the cytoplasm, indicating that the cultures consisted mainly of mucus-producing cells (Fig. 1B).

3.2. Effect of HGF and EGF on VEGF and IL-8 expression in gastric epithelial cells as assessed by ELISA

FBS-free medium supplemented with or without agents, conditioned with human gastric epithelial cells in a 24-well culture dish for 18 h, was obtained and the VEGF concentration of the medium was determined by ELISA. Both HGF and EGF significantly increased VEGF expression in gastric epithelial cells in a concentration-dependent manner (Fig. 2a,b). The VEGF concentrations (100–200 pg/ml) seemed too low, considering that the optimal concentration for gastric endothelial proliferation is around 1–100 ng/ml [11]. However, the local concentration, when it acts as a paracrine factor, should be higher and physiologically effective, as it is determined after dilution with 0.5 ml medium in this study. To rule out the possibility that HGF and EGF stimulated general protein synthesis in the target cells prior to cell proliferation, or synthesis was due to an increase in the number of producing cells, we evaluated the IL-8 concentration in the same medium in which we determined the VEGF concentration, since VEGF and IL-8 are both specifically expressed at gastric

ulcer edges [12]. Neither HGF nor EGF stimulated the production of IL-8 (Fig. 2c,d), indicating that the effects of EGF and HGF on VEGF production are specific. All experiments were done in triplicate.

3.3. Expression of HGF and EGF receptors in primary cultures of gastric epithelial cells

RT-PCR demonstrated that cultured gastric epithelial cells express both HGF and EGF receptor mRNA. EGF receptor mRNA is also expressed in gastric fibroblasts, but to a lesser extent (Fig. 3).

3.4. Splicing variants of VEGF mRNA of human gastric epithelial cells by HGF

RT-PCR demonstrated that cultured gastric epithelial cells revealed three bands, corresponding to $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ (Fig. 4). These findings indicated that gastric epithelial cells express these three isoforms of VEGF, as in the case of other normal tissues [13].

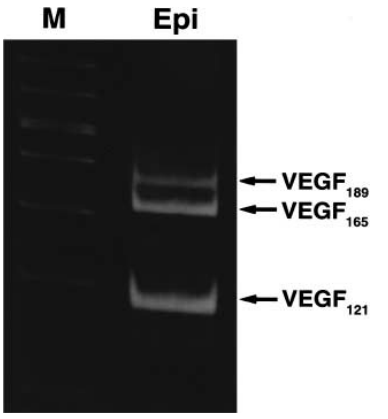

Fig. 4. VEGF mRNA expression by gastric epithelial cells, as assessed by RT-PCR. Three bands corresponding to three splicing variants can be seen in the gastric epithelial cells. (M: marker; Epi: gastric epithelial cells in primary culture).

4. Discussion

Gastric ulcer healing consists of various processes, including angiogenesis and re-epithelialization. Various reports provide a good body of evidence that growth factors play important roles in these processes. Several growth factors, including EGF [14] and HGF [2–4], play important roles in epithelialization in gastric ulcer healing, which includes epithelial proliferation and migration. There are also several growth factors, including bFGF [15], which are involved in angiogenesis and gastrointestinal ulcer healing. Among them, VEGF is the only factor that specifically acts on vascular endothelial cells and promotes angiogenesis. Szabo et al. demonstrated that exogenous VEGF accelerated the healing of cysteamine-induced chronic duodenal ulcers in an in vivo rat experimental model [16]. Moreover, to obtain a good quality of healing, coordination and communication between these processes is important and concomitantly so among these factors. However, this aspect of gastric ulcer healing has hardly been touched upon. In the present study, we have shown that HGF and EGF induce the synthesis of VEGF in gastric epithelial cells in primary culture. This demonstrates communication between two important processes: epithelialization and angiogenesis. Since angiogenesis induced by VEGF provides a blood supply to growing target cells, the effect of growth stimulants on VEGF expression by target cells is very reasonable. Otherwise, the ulcer scar may be exposed to the risk of ischemia and thus, recurrence of the ulcer. Also, exogenous as well as endogenous HGF and EGF may promote overall ulcer healing, not just epithelialization.

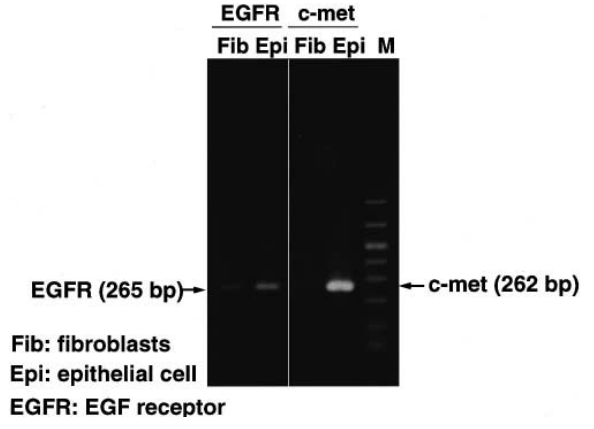

Fig. 3. The expression of the HGF receptor, c-met and EGF receptor in human gastric epithelial cells in primary culture, as assessed by RT-PCR. Both receptors are seen in gastric epithelial cells.

A previous study demonstrated that VEGF expression is seen in gastric epithelial cells in ulcer healing [5]. Therefore, the investigation of the regulation of VEGF expression in gastric epithelial cells is indispensable for understanding the angiogenesis in ulcer healing. Zhang et al. demonstrated that only 47 out of 228 genes which were expressed at higher levels in colorectal cancer cell lines were also expressed at high levels in the primary colorectal cancers, using the serial analysis of gene expression (SAGE) method [17]. This comprehensive study confirmed our impression that cancer cell lines are sometimes not suitable for gene expression studies, especially for the investigation of physiological function. In this sense, a primary culture system of gastric epithelial cells is preferable for gene expression studies. There are primary culture systems of gastric epithelial cells of non-humans, such as rats and rabbits [6,18]. However, in spite of the great advantages of the human primary culture system of gastric epithelial cells, including a wide variety of accumulated information, such as gene sequences and the established ELISA system, not to mention that it best simulates the human in vivo mechanism, there have been virtually no human primary culture systems suitable for gene expression studies. Many attempts, including ours, have been made to establish an appropriate primary culture system, in vain. Previous methods of primary culture systems adapted collagenase for the digestion of the gastric epithelium, which is the main procedure of the method. However, human epithelium could not be successfully digested by collagenase alone. In the present study, we adapted pronase (Kaken, Tokyo, Japan), a very strong proteinase, in combination with collagenase and succeeded in the digestion of the gastric epithelium without damaging a majority of the cells.

In conclusion, this study demonstrated the effect of HGF and EGF, growth promoters for epithelial cells on the up-regulation of VEGF, an angiogenic factor, using a newly established primary culture system of human gastric epithelial

cells, indicating the coordination between epithelialization and angiogenesis in gastric ulcer healing.

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