J Mater Sci: Mater Med (2014) 25:573–582
DOI 10.1007/s10856-013-5088-x

Endoscopic application of EGF-chitosan hydrogel for precipitated healing of GI peptic ulcers and mucosectomy-induced ulcers

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Received: 10 August 2013 / Accepted: 27 October 2013 / Published online: 14 December 2013

Abstract The gastrointestinal (GI) endoscopy has become a standard diagnostic tool for GI ulcers and cancer. In this study we studied endoscopic application of epidermal growth factor-containing chitosan hydrogel (EGF-CS gel) for treatment of GI ulcer. We hypothesized that directional ulcer-coating using EGF-CS gel via endoscope would precipitate ulcer-healing. EGF-CS gel was directly introduced to the ulcer-region after ulceration in acetic acid-induced gastric ulcer (AAU) and mucosal resection-induced gastric ulcer (MRU) rabbit and pig models. The ulcer dimensions and mucosal thicknesses were estimated and compared with those in the control group. Healing efficacy was more closely evaluated by microscopic observation of the ulcer after histological assays. In the AAU model, the normalized ulcer size of the gel-treated group was 2.3 times smaller than that in the non-treated control group on day 3 after ulceration ($P < 0.01$). In the MRU model, the normalized ulcer size of the gel-treated group was 5.4 times smaller compared to that in the non-treated control group on day 1 after ulceration ($P < 0.05$). Histological analysis supported the ability of EGF-CS gel to heal ulcers. The present study suggests that EGF-CS gel is a promising candidate for treating gastric bleeding and ulcers.

1 Introduction

Peptic ulcers (PU) are one of the most common diseases around the world. More than 10 % of people worldwide suffer from PU in their lifetimes. In the United States, 14.5 million cases were reported to have peptic ulcer disease in 2007 [1, 2]. Complications of PU, typically known as bleeding or perforation, cause an estimated 6,500 deaths each year. Recently, age-related gastrointestinal (GI) ulcers, more likely in those aged 60 or older, are increasing. Over 50 % of older than 65 year-olds take NSAIDs on a daily basis which attributes to a greater incidence of old-age GI ulcers [3, 4]. Most GI ulcers are diagnosed based on the symptoms, particularly abdominal pain, dyspepsia and chest pain. However, diagnosis based on symptoms is not always appropriate because symptoms often overlap with other disease like upper GI cancers and other diseases. Endoscopies are the most powerful tools for the diagnosis of GI lesions; they allow one to visualize the ulcers, to identify the bleeding focus, and to detect early malignant deformations of mucous and discriminate margins between benign and malignant tissues [5]. Current developing technologies enable physicians to beneficially perform simultaneous endoscopic treatments for defined GI lesions [1, 2]. Endoscopic mucosal resection (EMR), endoscopic

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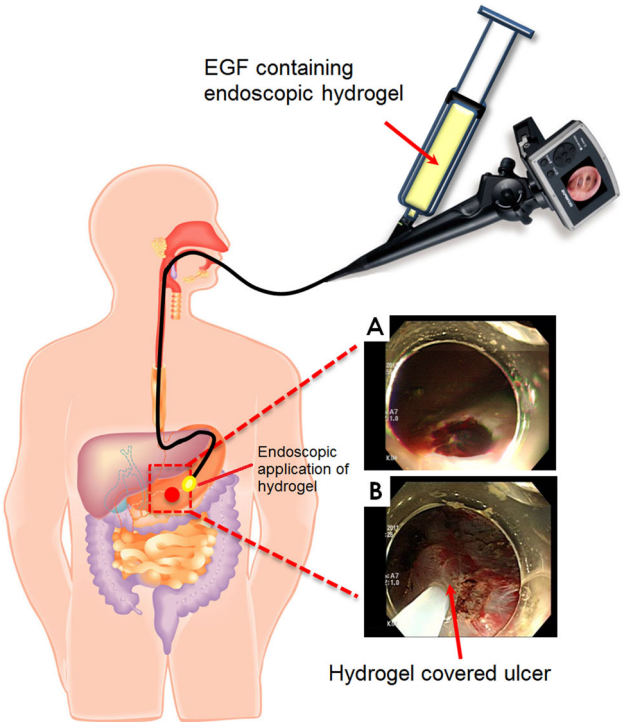

Scheme 1 Endoscopic application of ulcer-covering EGF-Chitosan hydrogel (EGF-CS gel). **a** Experimental model of mucosectomy-induced gastric ulcer with bleeding in micro-pigs. **b** EGF-CS gel was applied via an endoscopic catheter to cover the bleeding ulcer and precipitate ulcer-healing

submucosal dissection of early gastric cancer and colon polypectomy are readily performed directly during the diagnosis [6–8]. These endoscopic resections (ER) have distinct advantages over conventional surgery, i.e., they are less invasive and more economical and have now become standard practice all over the world. Paradoxically, ERs can provoke post-operative acute ulcers. ER-induced ulcers always accompany bleeding which can evolve into life-threatening complications [9, 10]. Saito et al. [11] reported that around 7–8 % of patients undergo bleeding after the mucosal resection and they may require additional haemostatic treatments. But temporal homeostatic treatment possesses re-bleeding issues. Re-bleeding can always happen if there is no proper treatment of the exposed ulcers from corrosive gastric environments (i.e., bile acid, digestive enzyme, gastric acid and food).

Until now we don't have any recommendable endoscopic method for therapeutic treatment of PU and ER-induced acute ulcers without hemostatic agents for bleeding control. Herein we describe an endoscopic application of ulcer-coating hydrogel (epidermal growth factor-containing chitosan hydrogel; EGF-CS gel) for GI ulcer treatment. Our EGF-CS gel can be directly applied to ulcers via an endoscopic catheter after en-bloc resection (Scheme 1). We selected chitosan for a gelling agent. Chitosan is biocompatible, biodegradable, hemostatic, anti-infective and, more importantly, it accelerates wound healing [12–16]. Chitosan has been widely studied as a biomaterial and now chitosan films are commercially available for wound healing and haemostatic application [17–20].

Our endoscopic gel contains epidermal growth factor (EGF). EGF acts by binding to the EGF receptor (tyrosine kinase), thereby initiating a series of events which accelerates regeneration of ulcer mucous [21–24]. EGF exerts not only cell growth but also protective healing actions on the wound area. Recently, several formulations of EGF have been studied regarding their ability to accelerate wound healing. The most commonly used form is solution; there are only a few studies reported dealing with bioadhesive gels, microemulsions and liposomes [25]. Alemdaroglu et al. [26] reported EGF-containing chitosan hydrogel for an application of skin wound healing. But endoscopic application of EGF-CS gel is new area of gastroenterology.

In this study, we have examined the therapeutic efficacy and feasibility of the EGF-CS gel for endoscopic treatment of GI ulcers. The significance of the EGF-CS gel in ulcer healing is identified by a marked increase of the healing process of in vitro (wounded cell monolayer) and in vivo ulcer models (rabbits and micro-pig GI ulcers). Of special note, the EGF-CS gel demonstrated accelerated ulcer healing on an endoscopic mucoresection-induced acute ulcer model of micro-pigs. The healing process was also observed via histology studies [Masson's trichrome (MT) and proliferating cell nuclear antigen (PCNA) staining] for every ulcer tissue in this study.

2 Materials and methods

2.1 Animals

Two female micro-pigs (Micro-pig®; Medi Kinetics, Pyeongtack, South Korea) weighing 35–40 kg, 12 male rabbits (New Zealand White; Orient Bio, Seongnam, South Korea) weighing 2–2.5 kg, and 20 male CrljOri:CD1 (ICR) mice (CD-1®; Orient Bio, Seongnam, South Korea) weighing 20–25 g were used for our experiments. All animal care and experimental procedures were conducted in accordance with the guidelines of the Experimental Animal Research Committee of Inha University (protocol number; Inha 120622-145, Incheon, South Korea).

2.2 Preparation of EGF-CS gel

The EGF-CS gel was fabricated as follows: 6 g of chitosan powder (M.W. 130 K, 93 % of degree of deacetylation,

Biopolytech Co. Korea) were dissolved in distilled water with thickening agents (10 g of sorbitol, 2 g of HPMC and 5 g of PVP K-30) under magnetic stirring. Methylparaben (0.36 g) and propyl paraben (0.04 g) were dissolved in 20 ml of distilled hot water, added to the chitosan solution. The final volume was adjusted to 200 ml. EGF (Daewoong Pharm. Co., Korea) was dissolved at a final concentration of 1 μg/ml.

2.3 Cellular wound healing assay

The effect of the EGF-CS gel on cellular restitution was studied on a defected monolayer of SNU-484 human gastric cancer cells (Korean Cell Line Bank, Seoul, South Korea). The assay was performed based on the method described by Takahashi et al. [27] with modifications. SNU 484 cells were cultured in 60-mm culture tissue dishes and injured with a pipette tip to produce a round wound (~1 mm of tip end diameter) as an acute ulcer in each well. Then the monolayers were washed with phosphate-buffered saline (PBS, pH 7.4) and further cultured in fresh serum-free medium with PBS, 1 % (v/v) of 1 μg/ml of EGF solution (final 50 ng EGF), 1 % (v/v) of EGF-CS gel (final 50 ng EGF), or 1 % (v/v) of CS gel for up to 72 h. Time-dependent infiltration of cells at 0, 6, 24, 48, and 72 h after the wounding was estimated with a digital image processor connected to a microscope (Axio Observer D1; Carl Zeiss, Oberkochen and Jena, Germany).

2.4 Acetic acid-induced chronic gastric ulcer models in rabbits

The chronic ulcer model was developed using acetic acid [acetic acid-induced ulcer; (AAU)]. Rabbits were deprived of food for 24 h prior to the operation. Anesthesia was induced with intramuscular injection of a mixture of ketamine and xylazine (40 and 11 mg/kg body weight, respectively). A median laparotomy was performed to expose the stomach. The stomach was surgically opened along the greater curvature. Gastric mucosa of the upper corpus was exposed to 60 % acetic acid solution in a glass bottle for 15 s. The contact area of the bottle orifice was determined as the initial size of the ulcer. The exposed area was rinsed with isotonic saline to minimize adhesion. For the test, ulcers were treated with 1 ml of EGF-CS gel. Rabbits were sacrificed 3 days after the treatments to assess the ulcer healing process.

2.5 Gastric-mucosa resection models in rabbits

A gastric-mucosa resection model (MRU), which can substitute for an EMR, was developed as follows. After the laparotomic opening of the stomach, two hundred micro liters of isotonic saline was injected into the submucosal layer of the gastric walls. Then, the gastric mucosa with a diameter of 7–10 mm was resected using a pair of operating scissors. Ulcers were randomly divided into a control (non-treated) and a treated group (1 ml of EGF-CS gel treated). Rabbits were sacrificed 1 day after the ulcer treatment, and the gastric tissues were recovered for observation.

2.6 Micro-pig endoscopic muco-resection gastric ulcer models

Micro-pigs were starved for 24 h before the operation being allowed tap water only. After 24 h of no food, anesthesia was induced with an intramuscular injection of tiletamine/zolazepam (Zoletil®, Virbac Korea, Seoul, South Korea) and xylazine (Narcoxyl-2®, Intervet Korea Ltd., Seoul, South Korea), and maintained by inhalation of isoflurane (Ifran®; Hana Pharm., Kyonggi-do, South Korea) during the operation. The endoscope (GIF-Q260; Olympus Medical Systems, Tokyo, Japan) was inserted with the animals in a lateral decubitus position. The target areas were marked with an argon plasma coagulator, and isotonic saline was injected into the submucosal layer. The ER was performed to produce acute ulcers with diameters of 2.0–3.0 cm. Saline (control) or 10 ml of the EGF-CS gel was applied to each ulcer. Micro-pigs were then starved for another 24 h after ER. After the procedure, the micro-pigs were introduced to individual cages for recovery. Any other treatments were not allowed during the experiment such as the administration of proton pump inhibitor or antibiotics. Healing progress of the resected-mucosa was observed via endoscopy at 1, 3, and 6 weeks after ER. Then, the micro-pigs were killed and the gastric ulcer tissues were harvested for histological assessment.

2.7 Histological studies

Gastric glandular structures and the thickness of the regenerated gastric mucosa were observed after hematoxylin-eosin staining of the recovered gastric tissues. The mucosal thickness was measured in different fields of each slide and averaged. Recovery of mucosal glycoproteins and fibrosis of the muscularis mucosae were observed via periodic acid schiff (PAS) staining and MT staining, respectively. Immunofluorescence staining specific for PCNA was performed to verify regeneration of the mucosa in the AAU model.

2.8 Apparent gastric retention of EGF-CS gel

Ulcer covering may be one of the major mechanisms underlying the therapeutic activities of the EGF-CS gel, along with the direct effects of EGF itself. Our EGF-CS gel

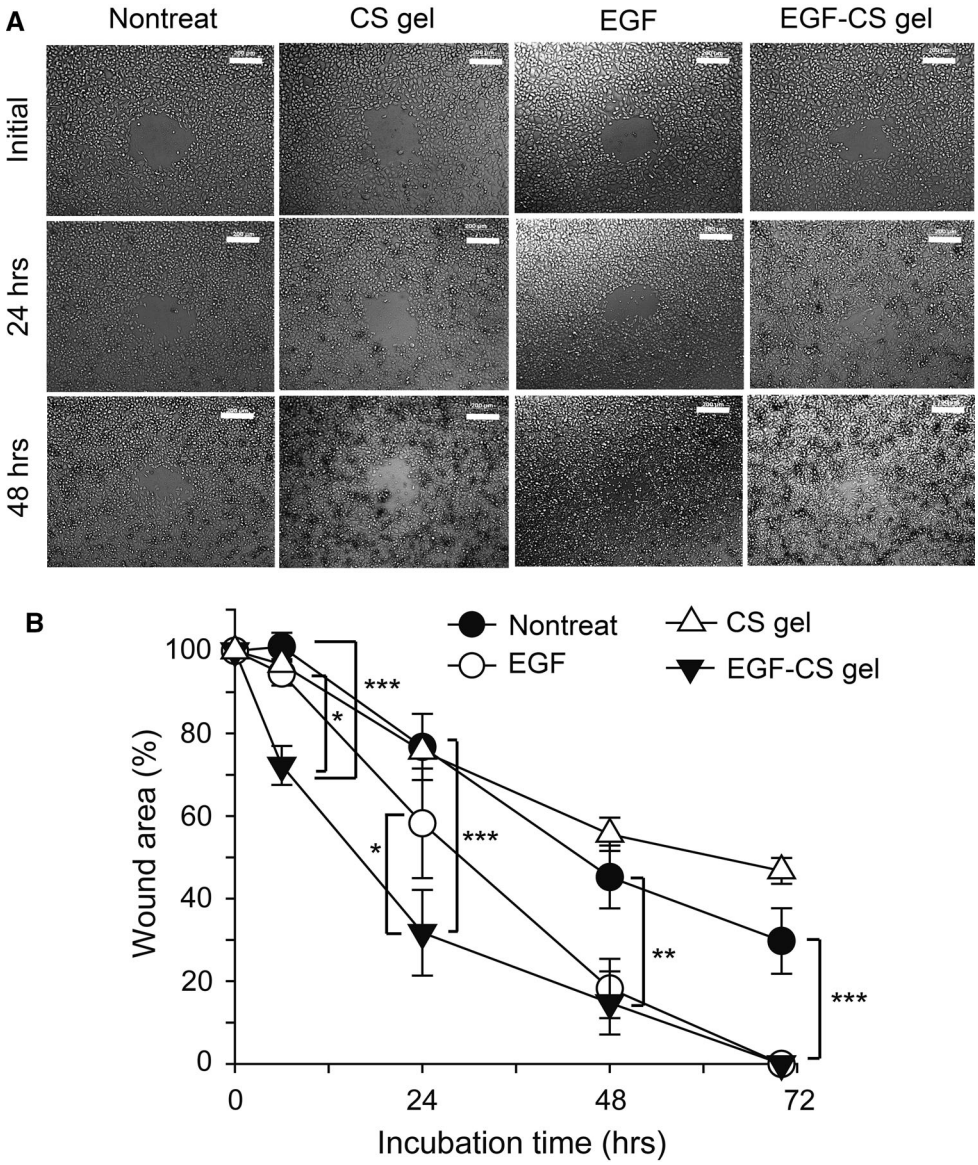

Fig. 1 Restitution of the wounded SNU-484 cell lines under the treatments of control (non-treated), CS gel, EGF solution, and EGF-CS gel. **a** Microscopic images of each treatment by times (scale bar represents 200 μm). **b** % decrease of the wound area. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

is supposed to cover the ulcers for an extended time. In this study, we estimated apparent resident time of the EGF-CS gel in the stomach using a fluorescence imaging system. Twenty mice were starved but given water for 24 h prior to treatment. The EGF-CS gel (experimental group, $N = 8$) or water (control group, $N = 8$) containing 1 % (v/v) of 10 mg/ml methylene blue was administered orally to the mice. Four mice served as the negative control (pre-treated) and were not treated with methylene blue as a fluorescent dye. Each mouse was killed after 0, 30, 60, and 120 min and the stomachs were harvested. Fluorescence was then detected against the negative control at 616–661 nm using an in vivo fluorescence imaging system (Maestro^{TM}; CRi, Inc., Waltham, MA). The signal intensity in the stomach region was measured with Maestro^{TM} software. The relative signal intensity was calculated as the ratio of the signal intensity at 0 min divided by the signal intensity at 30, 60, or 120 min, respectively.

2.9 Statistical analysis

All data are reported as the mean ± SEM. A statistical analysis was performed via an unpaired student's *t* test. A *P* value of <0.05 or <0.001 was considered statistically significant.

3 Results

3.1 Effect of EGF-CS gel on cellular restitution

Monolayers of human gastric epithelial cancer cells (SNU-484) with ~1 mm of a wounding defect were treated with

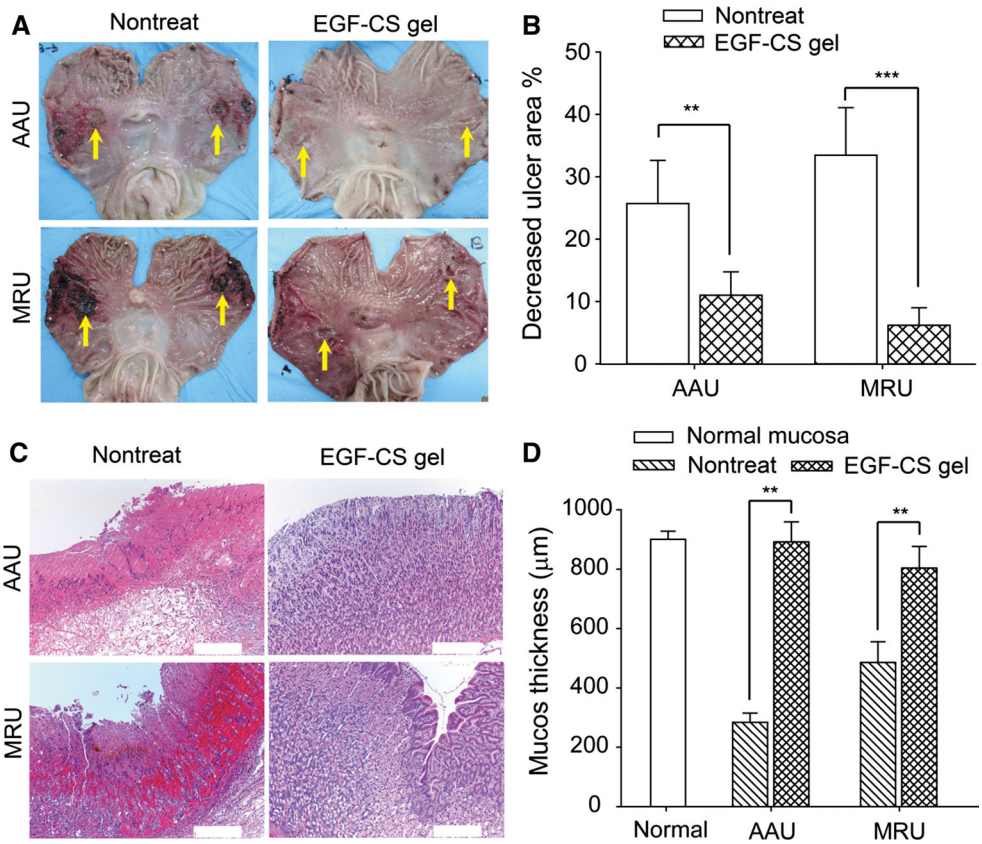

Fig. 2 Ulcer healing efficacy of EGF-CS gel on rabbit ulcer models. **a** Macroscopic images of rabbit stomach after gel treatment. Non-treated and EGF-CS gel-treated ulcer (*arrow*) in AAU (day 3) and MRU (day 1). **b** Relative decrease (%) of ulcer area. Data are present as the mean ± SEM for 6 and 4 ulcers in AAU and MRU, respectively. *** $P < 0.001$, ** $P < 0.01$. **c** Non-treated and EGF-CS gel-treated mucosa of AAU and MRU in rabbit (*scale bar* represents 200 μm). **d** Mucus layer thickness. Data are presented as the mean ± SEM for 6 and 4 ulcers in AAU and MRU, respectively. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

EGF, CS gel and EGF-CS gel. The concentration of EGF in each treatment was 10 ng/ml. Cells gradually migrated and covered the wounded area. Figure 1 shows the time course of the wound restitution of the SNU-484 cell line. The infiltration rate was varied as per the treatments. The EGF solution and EGF-CS gel significantly facilitated restitution compared with the non-treated and CS gel. The EGF-CS gel dose and time dependently decreased the wound area (Data is not shown).

3.2 Healing effect of EGF-CS gel on rabbit acetic acid-induced gastric ulcer model

Ulcer healing efficacy of EGF-CS gel was evaluated against the acetic acid- and mucosal resection-induced gastric ulcer rabbit models. In the AAU groups, the initial ulcer area was 221 mm^2. The relative ulcer areas at 3 days after ulcer treatments decreased to 25.7 ± 2.8 and 14.4 ± 2.8 % for the non-treated group and the gel-treated group, respectively (Fig. 2).

3.3 Healing effect of EGF-CS gel on rabbit resection-induced gastric ulcer model

In the MRU groups, the initial wound area was 199 ± 26.7 mm^2, and the relative ulcer areas on 1 day were 33.5 ± 3.8 and 6.2 ± 1.4 % for the non-treated group and the gel-treated group, respectively (Fig. 2). The EGF-CS gel treated animals displayed significantly precipitated ulcer healing compared with the non-treated group in both the AAU and MRU groups (*P*-values: 0.002 and 0.029, respectively).

3.4 Histological observations of gastric mucosa in a rabbit ulcer models

Tissue histology clearly showed precipitated ulcer healing by applying the EGF-CS gel. The untreated ulcer in both AAU and MRU showed extensive deep damage in the gastric mucosa of the untreated rabbits. On day 1 and 3 after ulcer induction, we found deep open ulcers in the untreated MRU and AAU. These severe lesions were characterized by coagulative necrosis of the glands with diffuse hemorrhage in the mucosa, accompanied by atrophic gastric mucosa and loss of glandular tissues. In contrast, ulcers in the gel-treated rabbits showed progressive healing with regenerating mucosa. Nearly normal gastric mucosa with a small area of atrophied surface epithelium was also observed (Fig. 2c). EGF-CS gel accelerated the ulcer-healing process, resulting in almost full recovery of the mucosa (Fig. 2d). Among the AAU, the mucosal thickness on day 3 after ulcer treatment was 284 ± 16 μm for untreated rabbits and 892 ± 34 μm for gel-treated animals. In the MRU groups, mucosal thickness

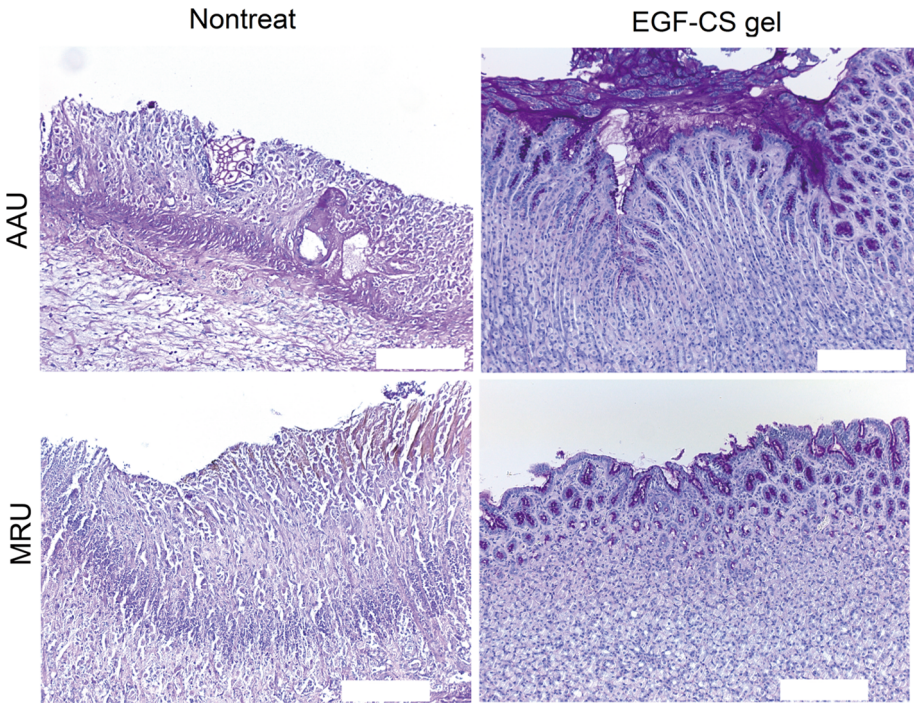

Fig. 3 PAS staining of non-treated and EGF-CS gel treated mucosa in AAU and MRU. Mucosal glycoprotein specific staining (*violet blue*) is observed at surface and neck mucus cells in the EGF-CS gel-treated ulcer (*scale bar* represents 200 μm) (Color figure online)

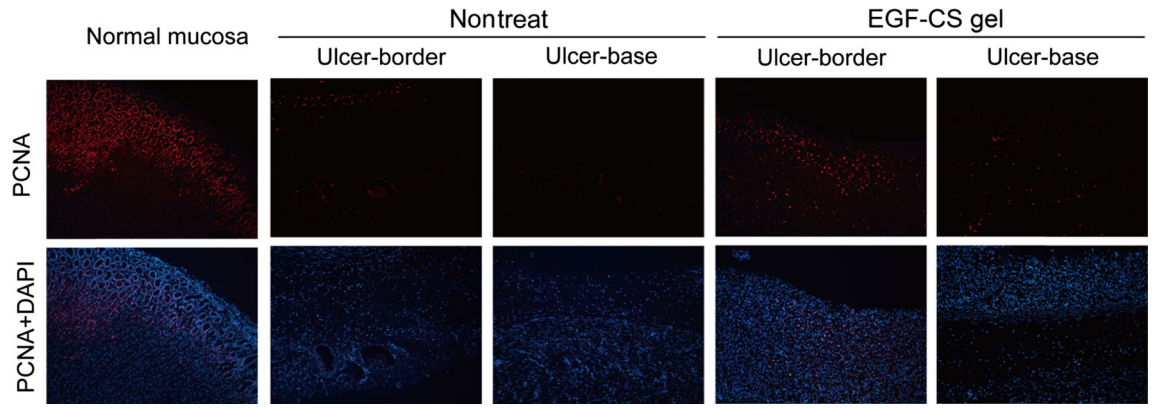

Fig. 4 Antibody to proliferating cell nuclear antigen (Anti-PCNA) staining of control (non-treated) and EGF-CS gel-treated mucosa in AAU. PCNA specific staining (*pink*) is observed in muscularis mucosae and submucosa of the ulcer base in the EGF-CS gel group unlike that in the non-treated control group (Color figure online)

on day 1 after ulcer induction and treatment was 486 ± 35 μm for untreated rabbits and 804 ± 36 μm for gel-treated animals. These results demonstrated that EGF-CS gel promoted significant healing of ulcers relative to the control ($P < 0.01$).

PAS and PCNA staining also supported the facilitated ulcer healing by EGF-CS gel (Figs. 3, 4). PAS typically binds to neutral muco-glycoproteins and deep purple staining indicates higher muco-glycoprotein density and recovery of the mucous layer. The untreated group showed weakly positive PAS staining in the mucous neck cells. Conversely, the gel-treated group presented strong PAS-positive reactions in surface mucous cells and mucous neck cells. PCNA is a factor for processing DNA polymerase δ, and an abundance of PCNA is associated with higher levels of cell proliferation during ulcer repair [28, 29]. In the control group, expression of PCNA was observed in only some of the muscularis mucosae in the healing zone (ulcer border). In contrast, PCNA expression in the EGF-CS gel group was found in the muscularis mucosae and submucosa of the ulcer base and in most of the mucosa in the healing zone (Fig. 4).

3.5 Apparent resident time of EGF-CS gel in the stomach

The time duration of the EGF-CS gel covering the ulcer after application was indirectly estimated by estimation of

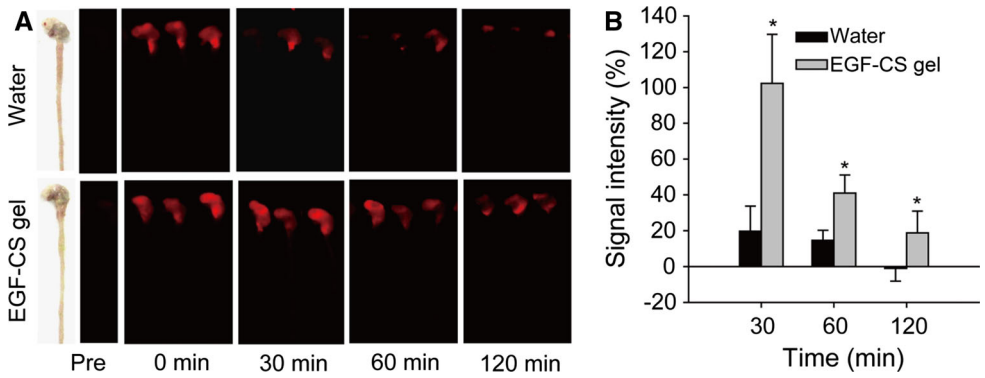

Fig. 5 Retention of EGF-CS gel on gastric mucosa. **a** Macroscopic and fluorescence images of residual EGF-CS gel in mouse stomach. Methylene blue as a fluorescence marker was pre-mixed with water or EGF-CS gel and orally administered. Mice were killed at 0, 0.5, 1 and 2 h after oral administration. **b** The fluorescent signal intensity from stomach EGF-CS gel is retained at least 120 min after administration in the stomach. Data are presented as the mean ± SEM. * $P < 0.05$

the gastric retention time of the EGF-CS gel. Gastric retention of the EGF-CS gel was assessed by fluorescent imaging of the GI tract. The mean relative signal intensity for the water-treated group decreased to 20 ± 14, 15 ± 6, and −5 ± 7 % at 30, 60, and 120 min after administration, respectively. However, the mean relative signal intensity for the gel-treated group was 102 ± 27, 41 ± 10, and 26 ± 11 % at 30, 60, and 120 min after administration, respectively (Fig. 5). Signal intensity of the gel-treated group was five times higher than that of the water-treated group ($P < 0.05$). These data suggest that the EGF-CS gel stays over time on the ulcer, releasing EGF and synergistically facilitating the healing process.

3.6 Healing effect of EGF-CS gel on micro-pig resection-induced gastric ulcer

Ulcer-healing efficacy of the EGF-CS gel against the ER-induced gastric ulcer on micro-pigs was observed using an endoscope. One week after the treatment, the control group showed two round clear gastric ulcers with diameters of 2.0 and 2.5 cm. Gel-treated micro-pigs showed the diameters of the ulcers were less than 2.0 cm. The depth of the untreated ulcers was greater than that of the gel-treated lesions and the underlying muscle layer remained exposed. Six weeks after ER, scar formation was observed and the sizes of the scars in the gel-treated lesions were smaller than those of the untreated lesions (Fig. 6). The surrounding mucosa of the untreated animals had radial folds, indicating deep scar formation. Conversely, the micro-pigs treated with the EGF-CS gel exhibited moderate gastric wall formation without severe scarring. Histological examination (Fig. 7) revealed that all ulcers displayed irregular foveolar and fibrotic submucosa. There were also broken muscularis mucosa and foveolar hyperplasia. However, the submucosa and muscularis propria of the gel-treated ulcers were much thicker than those of the untreated lesions. The muscularis propria of the untreated ulcer was also very thin and severed.

4 Discussion

In the present study, we evaluated the therapeutic efficacy and feasibility of an EGF-CS gel for an endoscopic treatment of GI ulcers. The EGF-CS gel is applied to the ulcer via an endoscopic catheter and acts as an ulcer-coating patch that protects the lesion from the corrosive gastric environment while releasing EGF into the mucosal defects.

The endoscopic EGF-CS gel demonstrated facilitated ulcer healing in rabbit and micro-pig ulcer models. The recovery of the ulcers in the gel-treated group was accelerated by approximately 1.8- and 5.4-times in the AAU and MRU of rabbits, respectively, in comparison with the non-treated group. Histology studies supported a significant increase in the thickness of the mucosa. Definitely well-developed glandular structures of regenerated gastric mucosa at the ulcer margin were observed in the EGF-CS gel groups (Figs. 3, 4).

The PAS stain especially confirmed the regeneration of mucosa based on glycoprotein contents. PAS typically stains glycoproteins and indicates an increase or decrease in glycoprotein content [30]. PAS stains of the gastric ulcer of non-treated rabbit displayed positive PAS stains mainly in the apical portion of the mucosal cells which was decreased by acetic acid. The results in the micro-pig model more confidently proved the therapeutic potentials and the endoscopic feasibility of the EGF-CS gel (Figs. 6, 7).

We demonstrated that the retention time of the EGF-CS gel is more than 120 min using an in vivo imaging system in mice. Gastric emptying scintigraphy against laboratory

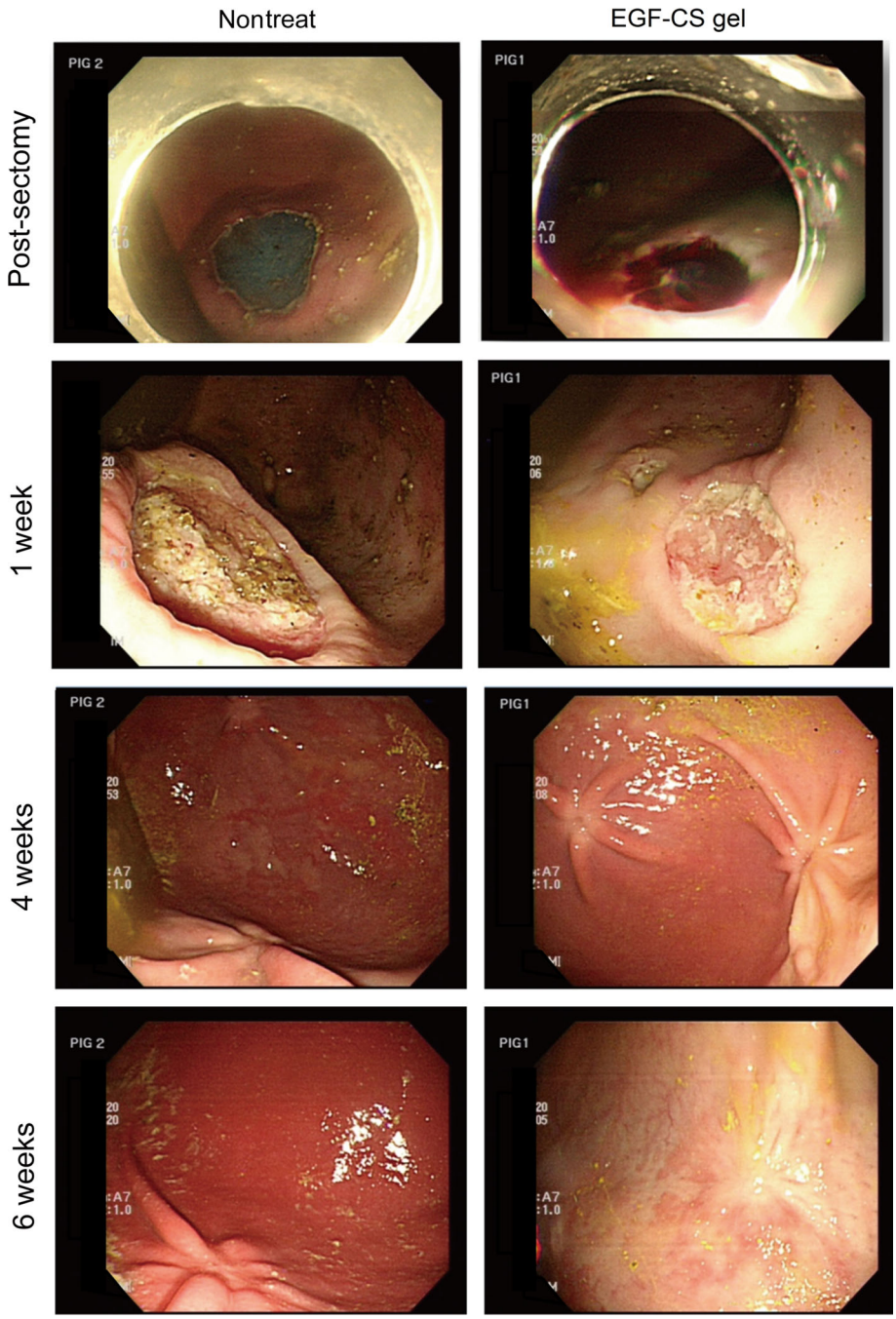

Fig. 6 Gastric endoscopic images of MRU in micro-pig. Mucoresection was performed to create ulcer models on the gastric mucosa and the EGF-CS gel was directly applied via an endoscopic catheter. Ulcers were endoscopically observed after 0, 1, 3, 6 weeks of the treatments. Endoscopic images proved the EGF-CS gel accelerates ulcer healing and reduced scar formation. In this study, the feasibility of the EGF-CS gel for endoscopic application was also confirmed

animals proved around 30 % of liquid meal remained at 15 min after oral administration. The EGF-CS gel maintained almost 100 % of intensity at least for 30 min after the administration.

The results of our retention study revealed that the EGF-CS gel resided on the gastric mucosa for a prolonged time. Relan et al. reported that the expression level of EGFR increased at the ulcer margin at 30 min after ulceration [31, 32]. EGF binds to its receptor (EGFR) on the surface of target cells and is rapidly internalized and degraded with the receptor. The enhanced ulcer healing by the EGF-CS gel might be due to the prolonged release of EGF from the chitosan hydrogel and the concomitant sustained activation of EGFR for a longer duration of time.

Until now, upper endoscopies have been performed to detect bleeding foci and achieve primary hemostasis but endoscopic therapy for ulcer healing has not been reported as of yet. As mentioned before, ulcer bleeding is another major issue of GI ulcer treatments. Based on the reported data, we expect muco-adhesive EGF-CS gel possibly functions as a hemostatic agent for treatment of ulcer-related bleeding, even though it was not proved in this study.

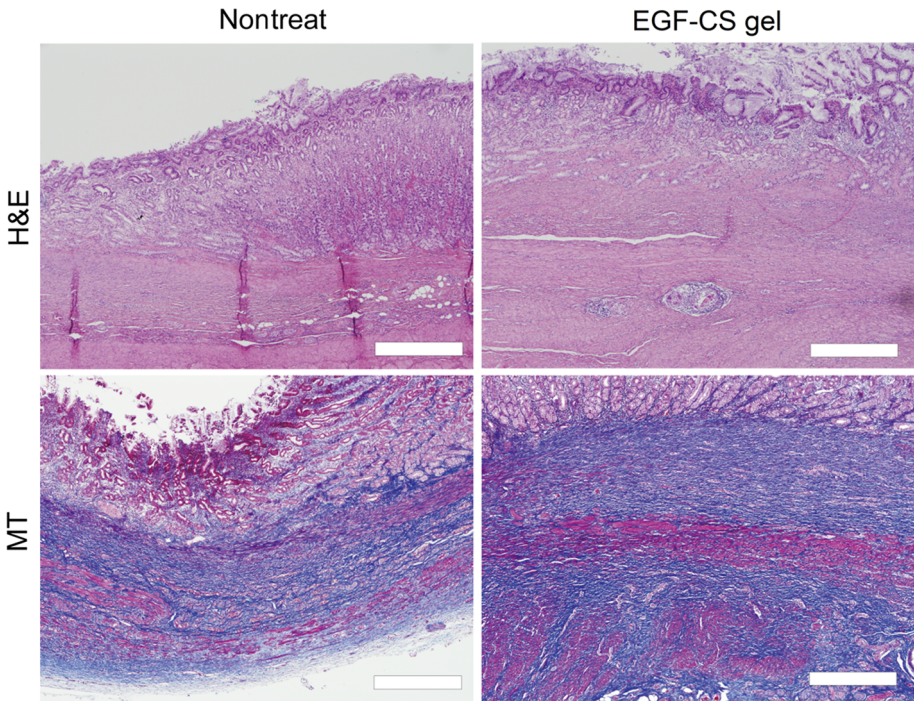

Fig. 7 Histological images of the ulcer region in micro-pigs treated with or without the EGF-CS gel. The ulcer area in non-treated and EGF-CS gel treated group displays irregular foveolar and fibrotic submucosa. In the control group, broken muscularis externa were observed as well as atrophy of gastric pits, unlike that found in the EGF-CS gel treated group (*scale bar* represents 200 μm)

In conclusion, we observed the therapeutic efficacy and feasibility of endoscopic application of EGF-CS gel for the treatment of GI ulcers. Our EGF-CS gel can be applied for hemostatic and ulcer-healing purposes after EMR or colon polypectomy for accelerating ulcer healing and preventing rebleeding.

Acknowledgments This study was supported by a grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A111060). The large animal study was supported by the National Center of Efficacy Evaluation for the Development of Health Products Targeting Digestive Disorders (NCEED).

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