Article

JOURNAL OF
biomaterials
applications

Journal of Biomaterials Applications
2014, Vol. 28(7) 1113–1121

DOI: 10.1177/0885328213499948
jba.sagepub.com
SAGE

rhEGF-containing thermosensitive and mucoadhesive polymeric sol–gel for endoscopic treatment of gastric ulcer and bleeding

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Abstract

Gastrointestinal endoscopy is a standard diagnostic tool for gastrointestinal ulcers and cancer. In this study, we have developed recombinant human epidermal growth factor-containing ulcer-coating polymeric sol–gel for endoscopic application. Chitosan and pluronic F127 were employed for their thermoresponsive and bioadhesive properties. At temperatures below 21°C, polymeric sol–gel remains liquid during endoscopic application and transforms to gel at body temperature after application on ulcers. In an in vitro cellular wounding assay, recombinant human epidermal growth factor sol–gel significantly enhanced the cell migration and decreased the wounding area (68%) compared to nontreated, recombinant human epidermal growth factor solution, and sol–gel without recombinant human epidermal growth factor (42, 49, and 32 % decreased at day 1). The in vivo ulcer-healing study was performed in an acetic acid-induced gastric ulcer rat model and proved that our recombinant human epidermal growth factor endoscopic sol–gel facilitated the ulcer-healing process more efficiently than the other treatments. Ulcer sizes in the recombinant human epidermal growth factor sol–gel group were decreased 2.9- and 2.1-fold compared with those in the nontreated group on days 1 and 3 after ulceration, respectively. The mucosal thickness in the recombinant human epidermal growth factor sol–gel group was significantly increased compared to that in the nontreated group (3.2- and 6.9-fold on days 1 and 3 after ulceration, respectively). In a gastric retention study, recombinant human epidermal growth factor sol–gel stayed on the gastric mucosa more than 2 h after application. The present study suggests that recombinant human epidermal growth factor sol–gel is a prospective candidate for treating gastric ulcers via endoscopic application.

Keywords

Gastric ulcer and bleeding, rhEGF, pluronic F127, chitosan, thermosensitive sol–gel, endoscopic treatment

Introduction

According to US National Institute of Diabetes and Digestive and Kidney Diseases, more than 14.5 million Americans suffer from peptic ulcers, and every year, 350,000 people are newly diagnosed.[1,2] Mortality related to peptic ulcers is approximately 3000 per year. Ulcer-related bleeding and perforation are the major causes of death.[3] Recently, antibiotic treatment of *Helicobacter pylori* has greatly reduced the incidence of GI ulcers in western countries.[1,4] But gastric ulcer remains a serious disease in underdeveloped countries, accounting for 4% of all deaths caused by digestive diseases including GI cancers. Incidence of GI ulcer increases linearly with age, putting a heavy burden on

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the healthcare system all over the country as the proportion of people aged 65 and older is steadily increasing. Long-term and multiple use of nonsteroidal anti-inflammatory drugs (NSAIDs) by the elderly is mainly implicated in the higher incidence rate. Approximately 50% of people 65 years of age or older take NSAIDs on a daily basis.[5,6] Recent clinical investigation proved that cyclo-oxygenase-2 selective inhibitors, which should theoretically be safer than nonselective NSAIDs, also raise the risk of adverse gastric events like nonselective NSAIDs.[7]

Ulcers induced by long-term use of NSAIDs are always accompanied by gastric bleeding.[6] As mentioned before, ulcer-related bleeding can be life-threatening and must be considered seriously. However, GI ulcer-bleeding is very latent and can be diagnosed mostly under an endoscopy.

Endoscopic technology is rapidly developing due to emerging medical engineering. Traditional endoscopy was previously confined to diagnosis of ulcers and ulcer-bleeding and identification of malignant lesions.[8] Now, endoscopic surgery for the treatment of malignant lesions and colon polyps has become prevalent. Endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) were developed for the removal of abnormal of malignant flat lesion confined to the superficial layers (mucosa and submucosa) of the GI tract.[9–11] The clinical benefits of EMR have been widely proved, and operations are increasing dramatically all over the world. Although EMR is regarded as a relatively noninvasive procedure, endoscopic resection (ER) of malignant lesions inevitably induces ulcers and bleeding. However, ER-induced ulcers and bleeding have not been treated very effectively. Bleeding is mostly observed during the procedure or within the first 24 h.[12] Delayed bleeding occurs in up to 13.9% of patients.[13,14] Exposure of the ulcer exposed to corrosive gastric juice may induce the delayed bleeding, which can threaten the life of the patient.

In this study, we designed an ulcer-coating sol–gel containing epidermal growth factor (rhEGF). Ulcer-coating sol–gel can be administered through an endoscopic catheter during the operation. In particular, we functionalized the delivery system with thermosensitive polymer. Hydrogel remains liquid below body temperature, which allows it to pass easily through the endoscopic catheter; at body temperature, it transforms into a gel that can attach and cover the ulcers. Additionally, rhEGF facilitates wound repair and accelerates the regeneration of the mucous layer.[15–17]

Thermosensitive polymers transform their physical states (i.e. swell, shrink, and sol to gel) in response to environmental temperature. The transition temperature can be tailored by modifying hydrophobic side groups. This interesting characteristic (e.g. transformation at body temperature) has attracted scientist to extensively study thermosensitive polymers for the development of biomedical applications.[18,19]

N-substituted polyacrylamides, polyphosphazenes, and poly(ethylene glycol) (PEG)–polyesters block polymers are typical synthetic thermosensitive polymers.[20] They show excellent thermosensitivity and versatile potency for biomedical applications. However, clinical application of these polymers is very limited because of their undissolved toxicity, especially in the case of polyacrylamides.[21] One of the PEG–polyesters (PEG-*b*-PLGA-*b*-PEG) containing paclitaxel has been used for clinical tests in the treatment of breast cancer.[22] Pluronics, block copolymer of poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) (PEO–PPO–PEO), is the most commonly used thermoreversible polymer that has been approved by the FDA and EPA for food additives and pharmaceutical ingredients.[23,24] However, the low mechanical strength and nondegradability of pluronic hydrogel are drawbacks for the design of long-term drug-delivery systems.

Unlike synthetic polymers, naturally derived polymers have excellent biocompatibility. Methyl cellulose, gelatin, chitosan, and agarose have been studied for thermosensitive hydrogel. However, their less versatile thermosensitivity requires additional chemical modifications or blending with synthetic thermosensitive polymers.[19,25]

Our thermosensitive hydrogel is composed of pluronic F127 and chitosan to take advantage of the properties of both naturally derived and synthetic polymers and can be introduced to clinical trials without any toxicity concerns. Chitosan, a natural polysaccharide composed of acetylated *b*-(1,4)-D-glucosamine, has been widely used for biomaterials due to its superior biocompatibility, bioadhesive properties, and wound-healing efficacy. With constitutional blending with pluronic F127, our hydrogel remains liquid during the endoscopic application, forms a bioadhesive gel at body temperature, and releases EGF on the gastric ulcer. In this study, we demonstrate that thermosensitive hydrogel-containing rhEGF promotes wound healing in the rat gastric ulcer model and in cell lines.

Materials and methods

Materials

rhEGF was kindly supplied by Daewoong Pharmaceuticals Co. (Seoul, Korea). Pluronic F127 (F-127) [$(PEO)_{99}(PPO)_{69}(PEO)_{99}$] was donated by BASF Co. (Berlin, Germany). Chitosan (Mw 1–3 kDa, degree of deacetylation above 85%) was purchased from Biopolytech Co. (Seoul, South Korea). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Dulbecco's modified Eagle's medium and 0.25% Trypsin-EDTA solution were purchased from Invitrogen Life Technologies (Carlsbad, CA). All other chemicals and reagents were of analytical grade.

Preparation of rhEGF endoscopic sol–gel

rhEGF endoscopic sol–gel was prepared on a weight basis. Chitosan, pluronic F127, and glycerol were mixed and dissolved for 2 h in deionized water at 5–8°C in a cold bath. The yield concentration of each component was 2, 15–17, and 10%, respectively. Then, rhEGF was added to 0.1% (w/w) concentration in the solution. The acquired sol–gel was kept in the refrigerator to obtain a clear solution.

Estimation of temperature-dependent viscosity of rhEGF endoscopic sol–gel

The temperature-dependent viscosity of rhEGF endoscopic sol–gel with different contents of pluronic F127 (15, 16, and 17% by weight) was estimated using a Brookfield Viscometer DV-II within the temperature range of 5–70°C. rhEGF endoscopic sol–gel was loaded on the sample chamber and stabilized for at least 15 min before the measurement. The heating rate was 0.34°C/min, and the rotation of the T–F spindle was set to 0.05 cycles per minute. The thermosensitivity of sol–gel (16% pluronic F127 without chitosan) was also estimated under the same condition to see the effect of chitosan on the system.

rhEGF release study

Release of rhEGF from the endoscopic sol–gel with different contents of pluronic F127 (15, 16, and 17%) was estimated in distilled water, simulated gastric fluid, and PBS buffer, respectively. Five grams of each sol–gel was filled into dialysis tubing (MWCO 12,000–14,000 Da, 25-Å pore diameter, Spectra/Por, Spectrum Labs, CA), suspended in 200 mL of release media at 37 ± 0.5°C, and horizontally shaken at 70 rpm in a shaker (SI-300, JEIO TECH, Korea). One milliliter of medium was sampled at predetermined time intervals and replaced with fresh media. ELISA (QuantifineVR Immunoassay, R&D Systems, Minneapolis, MN) was performed to analyze drug concentration according to the manufacturer's protocol. In brief, 50-μl aliquots of the recovered samples were added to a 96-well plate coated with a mouse monoclonal antibody against rhEGF, incubated at room temperature for 2 h, and washed with 0.05% Tween 20 solution. Then, 200 μl of anti-EGF polyclonal antibody conjugated to horseradish peroxidase was added to each well and incubated at room temperature for 2 h. Solutions in each well were removed and washed with 0.05% Tween 20 solution three times. Then, 200 μL of a mixture containing hydrogen peroxide and tetramethylbenzidine was added to each well as a substrate solution, and the resultant solution was incubated at room temperature in the dark for 20 min. Fifty micrograms of 2 N sulfuric acid was finally added to each well to stop the reaction, and the absorbance was read at 450 nm on a ELISA reader (Infinite 200 PRO, Tecan Group Ltd., Switzerland). All experiments were conducted in triplicate.

In vitro wound-healing efficacy test

The effect of rhEGF endoscopic sol–gel on cellular restitution was studied on defected monolayers of MKN-28 cells (human gastric cancer cell line) following a reported method with minor modification.[26] MKN-28 cells were seeded in six-transwell-plates (Transwell®, Corning Inc., NY, USA) at a concentration of 1×10^6 cells/well and cultured until the cells reached 100% confluence. Cellular wounds were made by scratching monolayer of MKN-28 cells using a 10-μl pipette tip under microscopic observation. Then, each well was filled with 2 ml of serum-free medium containing rhEGF or rhEGF endoscopic sol–gel and incubated for 72 h. Each treatment contains 20 ng of rhEGF.

The process of migration was monitored using an inverted phase-contrast microscope at 0, 4, 24, 48, and 72 h after induction of the artificial wound. The area of the cell-free zone was captured using LEICA microscope and analyzed with Image J software (Wayne Rashband; NIH, Bethesda, MD, USA).

Animals

Male SD rats (weighing 300 g) and male CrljOri: CD-1 mice (ICR, weighing 20–25 g) were purchased from Orient Bio (Gapyeong, Gyeonggi, South Korea). All animal care and experiments were carried out in compliance with the guidelines from the Experimental Animal Research Committee of Inha University.

Gastric retention properties of rhEGF endoscopic sol–gel

Gastric retention of rhEGF endoscopic sol–gel on the stomach was assessed by fluorescent imaging in mouse. Thirty-four mice were given only water for 24 h. About 1% (v/v) fluorescence probe (methylene blue) was mixed with rhEGF endoscopic sol–gel at a concentration of 10 mg/ml and orally administered to 16 mice. For the control ($N = 16$), methylene blue in water at the same concentration was administered. As the negative control (pretreated), two mice were not treated with

methylene blue. After administration, each mouse was sacrificed at 0, 30, 60, and 120 min. The stomach was recovered, and fluorescence signal intensity (SI) of stomach was measured with an in vivo fluorescence imaging system and software (Maestro^{TM}; CRi, Inc., Waltham, MA). Additionally, mucoadhesion property of endoscopic sol–gel was estimated on the texture analyzer (see Supplemental Information).

In vivo estimation of ulcer-healing efficacy of rhEGF endoscopic sol–gel

Rat gastric ulcer was induced by gastroluminal injection of acetic acid solution based on the method described by Okabe and Amagase.[27] Rats were deprived of food for 24 h before the operation but had free access to water. At the beginning of surgery, a mixture of zoletil (15 mg/kg) and rompun (9 mg/kg) was injected to induce anesthetization. Surgery was performed on a hot pad to maintain the body temperature. The stomach was exposed by median laparotomy, and then the fundic area of the stomach was clamped with ring forceps. About 30 µl of 60% acetic acid solution was injected into the luminal side of the stomach and stood for 45 s. The initial size of the ulcer was set as the inner diameter of the ring forceps. After surgery, the abdomen was closed with a suture. The animals were randomly divided into a control group and a gel-treated group. The control group was divided into two groups: nontreated group and sol–gel without rhEGF-treated group. The treated group orally received 1 ml of rhEGF endoscopic sol–gel after ulcer induction. On days 1 and 3 after the treatment, rats were sacrificed to harvest the stomachs. The harvested stomachs were dissected along the greater curvature and fixed on a board. Whole images of the opened stomach were taken, and the ulcer area was analyzed using the Image-J program. Relative ulcer area was calculated as the ratio of the ulcer area on days 1 or 3 divided by the inner area of the ring forceps.

Histological studies

The recovered ulcer tissues were fixed in 10% neutral formalin and embedded in paraffin wax. The embedded tissues were sectioned in 3–5 µm of thickness by microtome and stained with hematoxylin-eosin (H&E). Structures of gastric mucosa and mucosal thickness at the ulcer lesion were observed on the microscope. Masson's trichrome (MT) staining and periodic acid Schiff (PAS) staining were performed to confirm fibrosis and damage to the muscle layer and to note the changes in mucosal glycoproteins.

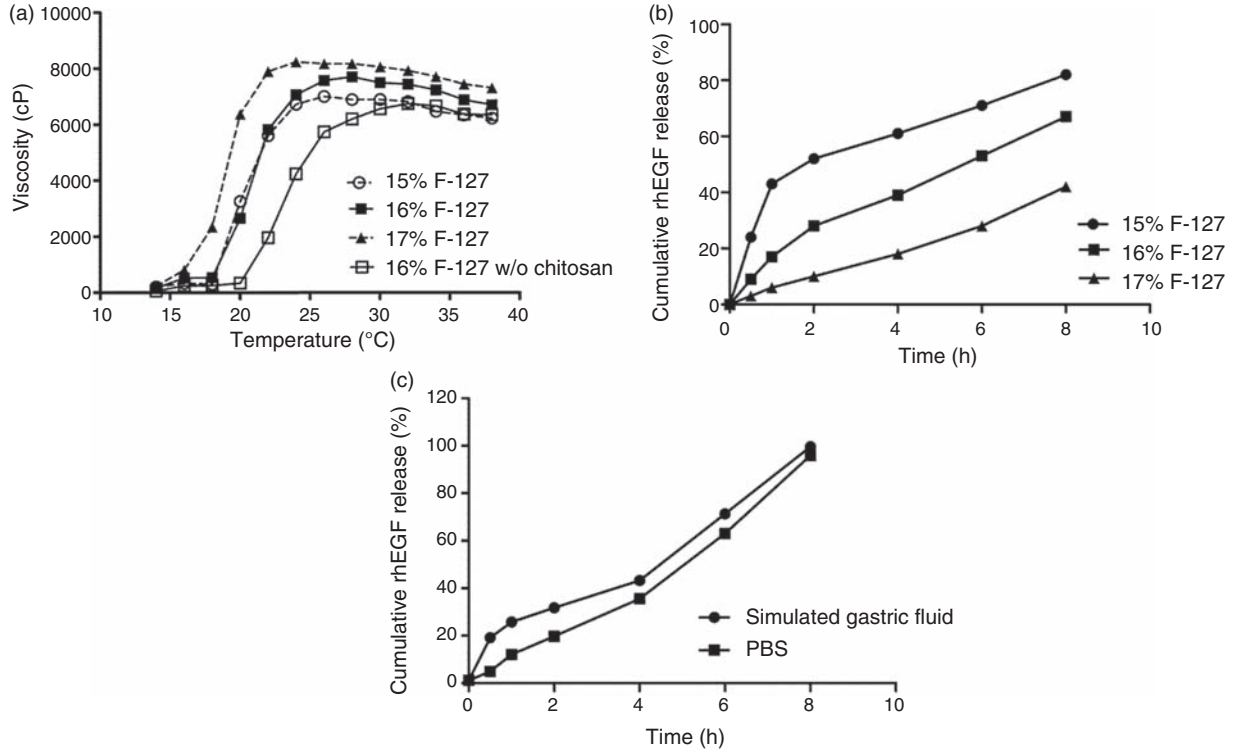

Figure 1. Temperature-dependent viscosity of rhEGF endoscopic sol–gel with different content of pluronic F127 (F-127) with or without chitosan (a) and rhEGF release profile (b, c) of rhEGF endoscopic sol–gel with different content of F-127 (b), and different release media (c).

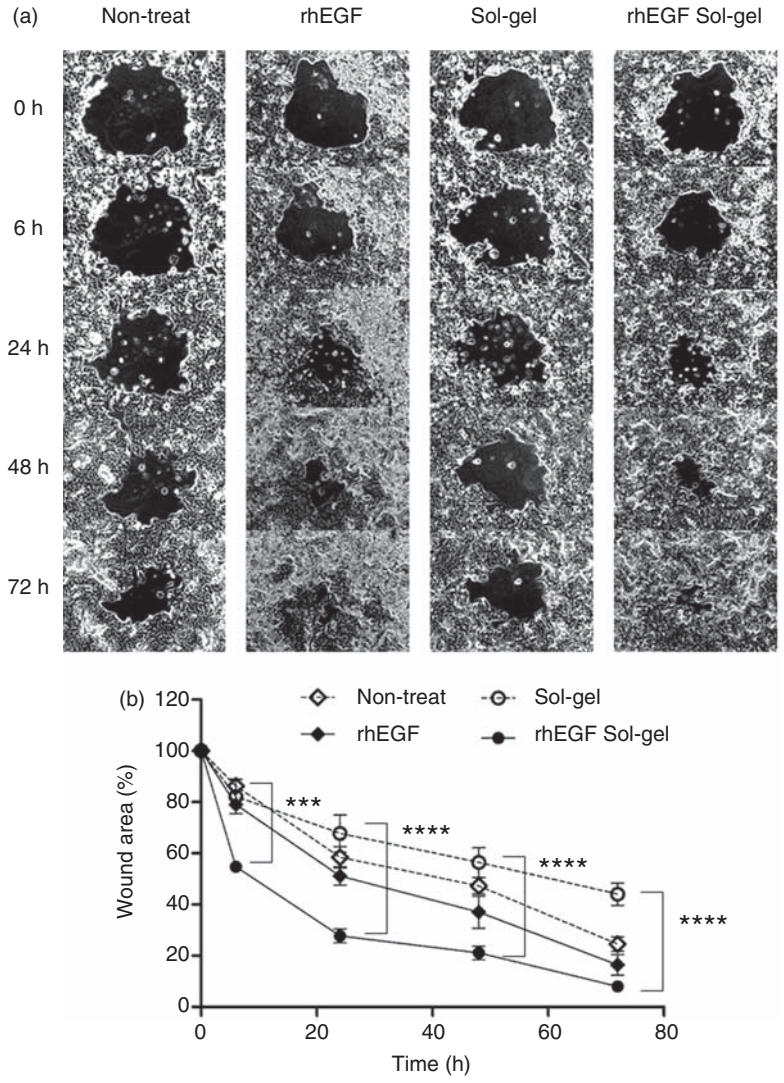

Figure 2. Restitution of wounded MKN-28 cell monolayer treated with PBS, a rhEGF solution (20 ng rhEGF), sol–gel without rhEGF and rhEGF sol–gel (20 ng rhEGF). (a) Microscopic images of cell monolayer that received each treatment (scale bar represents 200 μm). (b) Percent (%) decrease of the wound area. ***$P < 0.001$, n = 7/group.

Statistical analysis

All data are reported as mean ± SD or SEM. Statistical analysis was performed with Mann–Whitney's U test using SPSS software. P values of <0.05, <0.01, or <0.001 were considered statistically significant.

Results and discussion

Thermosensitive properties of rhEGF endoscopic sol–gel

Our sol–gel had to be delivered via endoscopic catheter that was 1.5–1.8 m in length and 1.5 mm in inner diameter. For this reason, the sol–gel transition temperature was very critical for the development (i.e. remain in sol state for easy pass-through of catheter and readily transform to gel after application on the ulcer area). Sol–gel transition temperature with different content of pluronic F127 (15, 16, and 17% by weight) was 23, 21, and 19°C, respectively (Figure 1(a)). And sol–gel (16% F-127 without chitosan) showed increased transition temperature (22–27°C) in comparison with sol–gel 16% F-127 with chitosan. The results suggested that a sol–gel with 16 wt % pluronic F127 had good feasibility for the endoscopic application.

The release of rhEGF from endoscopic sol–gel with different contents of pluronic F127 (15, 16, and 17%) was investigated. Maximum release of up to 100% of the loading dose of rhEGF in a controlled manner during the retention time would be the best method

for rhEGF delivery on the gastric ulcer. In this study, rhEGF showed sustained release from the gel for up to 8 h, and this was largely affected by pluronic F127. T_{50} (the times when 50% of the loaded dose releases) was 2, 6, and 9 h, respectively, for 15, 16, and 17% pluronic F127 contents (Figure 1(b)). Release of rhEGF was not largely affected by the release media as shown in Figure 1(c). The effect of chitosan on the release rate of rhEGF was investigated in the same method. And the result is shown in Supplemental Information Figure S1. This result suggests that chitosan does not affect the release of rhEGF, even though it affects the sol–gel transition temperature (Figure 1(a)).

Pluronics are triblock copolymers composed of a central hydrophobic chain of PPO and peripheral hydrophilic chains of PEO. The elevated temperature increases the hydrophobic interaction between PPO blocks to form 3D networks of PPO blocks.[28] This eventually increases the viscosity of the system and extends the release of rhEGF.

Cellular restitution of rhEGF sol–gel

Wound-healing efficacy of rhEGF endoscopic sol–gel was evaluated on the wounded gastric epithelial cells. Figure 2 shows that the cell-free wound area gradually decreased in a time-dependent manner. Restitution was significantly enhanced by rhEGF endoscopic sol–gel compared with other treatments (rhEGF alone, sol–gel without rhEGF). rhEGF endoscopic sol–gel showed higher efficacy than rhEGF alone, implying that endoscopic gel containing pluronic and chitosan synergically precipitated restitution and migration of the wounded cell line.

Gastric retention of rhEGF endoscopic sol–gel

Fluorescence images of stomach proved that rhEGF sol–gel was retained in the stomach for at least 2 h. The relative SI of fluorescence in the control group was dissipated within 30 min after administration. However, the relative SI in the gel-treated group was maintained up to 120 min after administration (Figure 3). Roda et al.[29] proved that the gastric-emptying time ($T_{1/2}$) of mice at fasted state was 2 ± 1 min using bioluminescence imaging. This data suggest that rhEGF sol–gel can cover the ulcer for more than 2 h; rhEGF is then released from rhEGF sol–gel, thereby facilitating ulcer healing. Mucoadhesive properties of sol–gel were estimated and shown in Supplemental Information Figure S2. In comparison with chitosan gel, which is known as typical mucoadhesive polymer, our thermosensitive sol–gel maintained adhesive property even after dilution with gastric fluids. This data suggest that gel-transformation at body temperature assists keeping adhesive property after application.

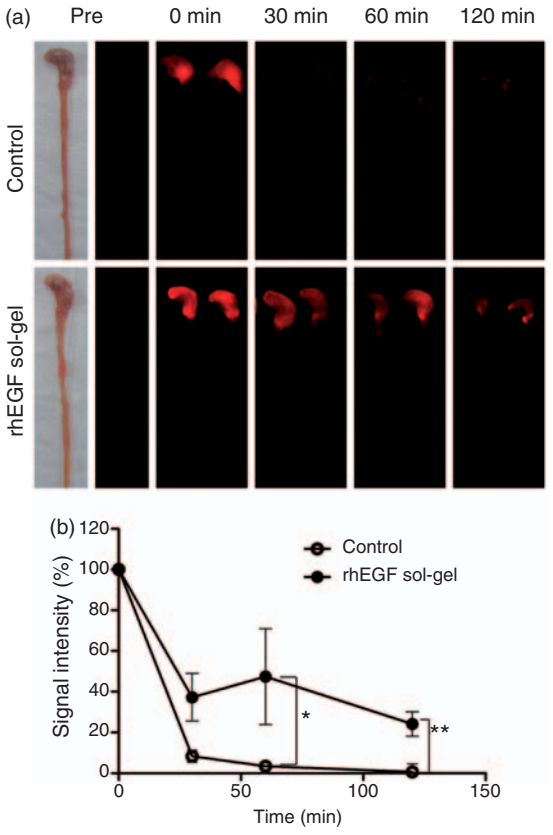

Figure 3. Retention of rhEGF endoscopic sol–gel on the gastric mucosa. (a) Macroscopic and fluorescence images of residual rhEGF sol–gel in mouse stomach. Methylene blue as a fluorescence marker was pre-mixed with water or rhEGF sol–gel and then orally administered. Mice were sacrificed 0, 30, 60, and 120 min after oral administration. (b) Fluorescent signal intensity of rhEGF sol–gel in the stomach was maintained for at least 120 min after administration. $^{**}P < 0.01$ and $^{*}P < 0.05$.

Ulcer healing of rhEGF endoscopic sol–gel in rat gastric ulcer model

Ulcer-healing efficacy of rhEGF endoscopic sol–gel was evaluated against acetic acid-induced rat gastric ulcers. One milliliter of rhEGF endoscopic sol–gel was orally administered. The inner circular area of ring forceps (32 mm^2), used for ulcer induction, was set as the initial ulcer area. One day after treatment, the ulcer areas had decreased to $74.4 \pm 3.09\%$, $54.8 \pm 5.7\%$, and $26.0 \pm 2.26\%$ of the initial area in the nontreated group, the sol–gel without rhEGF group, and the sol–gel with rhEGF group, respectively. Three days after the treatments, ulcer areas had decreased to $31.8 \pm 3.08\%$, $47.6 \pm 4.4\%$, and $15.0 \pm 3.05\%$ in the nontreated group, the sol–gel without rhEGF group, and the sol–gel with rhEGF group, respectively (Figure 4).

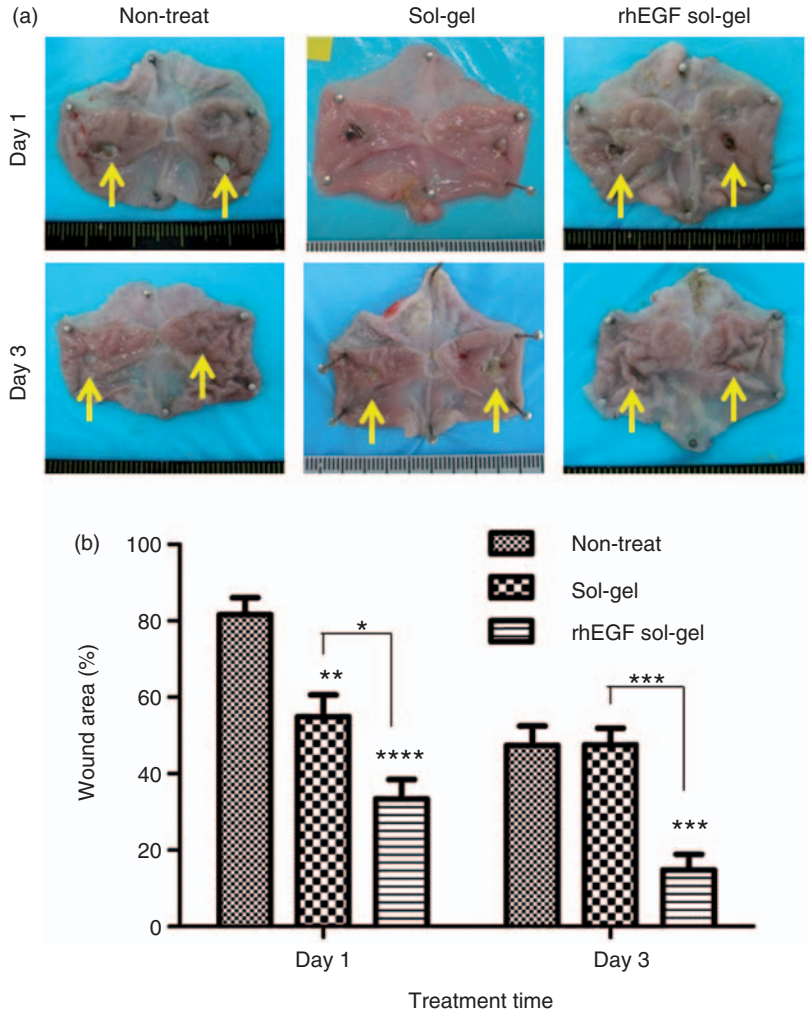

Figure 4. Efficacy of rhEGF endoscopic sol–gel for promoting ulcer healing in rat ulcer model. (a) Macroscopic images of rat stomach after ulcer induction and gel treatment. Non-treated, sol–gel (without rhEGF) treated, and rhEGF sol–gel treated gastric lesions were shown on day 1 and day 3 after ulcer induction (arrow). (b) Relative decrease (%) of the ulcer area after treatment. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$.

rhEGF sol–gel-treated rats displayed significantly facilitated ulcer healing compared with the nontreated group ($P < 0.0001$ and 0.001). In the H&E-staining images, the gastric mucosa of the nontreated group on days 1 and 3 showed deep open defect. These severe lesions were characterized by coagulative necrosis of the glands with diffuse hemorrhage of the mucosa and exhibited atrophic gastric mucosa with loss of glandular tissues. In contrast, the gel-treated group showed progressive ulcer healing with regenerating mucosa, and almost full recovery of the mucosa was observed at 3 days. Based on the microscopic images, mucosal thickness was measured and shown in Figure 5. The normal mucosal thickness was 507.97 ± 22.45 μm. On day 1 and day 3 after ulcer induction, mucosal thicknesses of the nontreated group were 180.03 ± 26.89 μm and 202.95 ± 13.41 μm, respectively. Mucosal thicknesses of the sol–gel without rhEGF group were 337.15 ± 25.29 μm and 300.86 ± 76.99 μm, respectively. In contrast, the sol–gel with rhEGF group showed rapid recovery of thicknesses, 450.73 ± 37.83 μm and 860.29 ± 98.77 μm on days 1 and 3 after ulcer treatment, respectively ($P < 0.01$ and 0.001). Overgrowth of mucosa was also observed in previous experiments and might be caused by fibrous scar formation.

Ulcer tissues were stained with MT and PAS (Figure 6). Compared to the control in MT staining, ulcer tissues treated with rhEGF endoscopic sol–gel showed rapid recovery of submucosa, muscularis muscle, and mucosa. Moreover, the gastric mucosae were stained with PAS, which stains neutral mucosubstances such as glycoproteins; based on this staining, the recovery of gastric mucosal function was confirmed. The partial mucus neck cells were stained in the

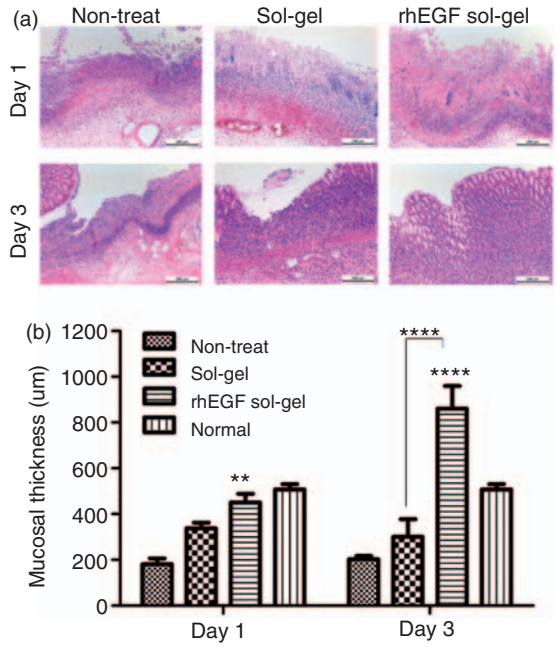

Figure 5. H&E staining and histological assessment of ulcerated mucosa in rats treated with rhEGF sol–gel. (a) Non-treated, sol–gel treated, and rhEGF sol–gel treated mucosa of rats. Mucosa in the scars from untreated ulcers contained dilated and irregular gastric glands. Well-developed glandular structures and connective tissue were found in the mucosa of the treated ulcers (scale bar represents 200 μm). (b) Thickness of the mucous layer. The mucosal thickness was increased after treatment with the gel compared to that in non-treated ulcers (n = 8/group). ****$P < 0.0001$, ***$P < 0.001$, and **$P < 0.01$.

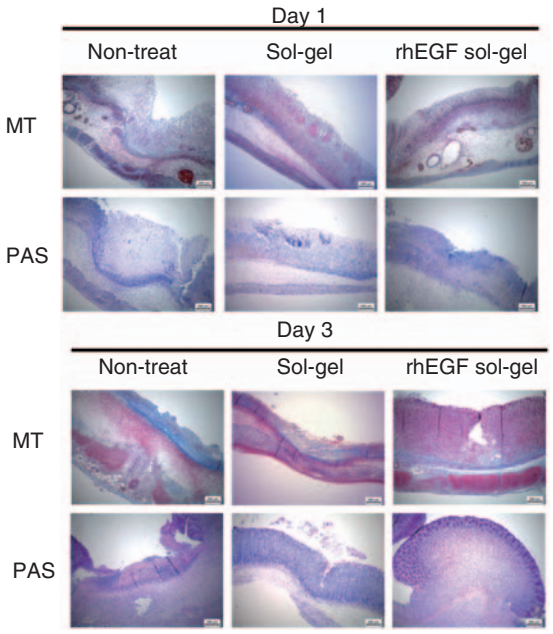

Figure 6. MT and PAS staining of non-treated, sol–gel treated, and rhEGF sol–gel-treated mucosa in rat model. Submucosal layer and muscularis mucosae in non-treated group were broken or thinning. Mucosal glycoprotein-specific staining (violet blue) was observed on the surface of the rhEGF sol–gel treated ulcers and in neck mucus cells. Scale bar represents 200 μm.

nontreated group, while the full structure of mucosae was predominantly stained in the gel-treated group on day 3. MT and PAS staining strongly supported the enhanced functional and structural recovery of gastric mucosa by the rhEGF endoscopic sol–gel.

Conclusion

Endoscopy easily reveals GI ulcers and allows exact diagnosis of their pathological status (especially, ulcer stage, bleeding, and progression to malignancy). But, to date, we do not have an endoscopic method for treating the gastrointestinal ulcer after endoscopic diagnosis.

In this study, we developed an advanced and endoscopically delivered sol–gel system (rhEGF sol–gel). Based on the temperature-dependent viscomoduli study, we found that our endoscopic polymer was able to transform to gel, covering the ulcer area and releasing more than 40% of rhEGF while it remained for 2 h in human stomach. An in vitro study showed that rhEGF sol–gel accelerated re-epithelialization and cell migration of a wounded human stomach cell line. An in vivo study in a rat ulcer model also proved that rhEGF endoscopic sol–gel accelerated the ulcer-healing process. The precipitated ulcer healing was strongly supported by histological observation. Consequently, we believe that rhEGF sol–gel can cover ulcer after delivery through an endoscopic catheter; it can stop bleeding from the ulcer, protect the ulcer from gastric fluids, and promote healing through the release of rhEGF to the damaged mucosa. Therefore, rhEGF sol–gel has potential value for the treatment of gastric ulcer via GI endoscopy. This rhEGF sol–gel could be a new therapeutic regimen of endoscopic therapy of GI ulcers.

Acknowledgements

This study was supported by a grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A111060).

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