

## Enhancement of Gastric Ulcer Healing and Angiogenesis by Cochinchina *Momordica* Seed Extract in Rats

Cochinchina momordica seed is the dried ripe seed of *Momordica cochinchinensis*, a perennial vine. The antiulcer effect of an extract from cochinchina momordica seeds (SK-MS10) was evaluated in a rat model of acetic acid-induced gastric ulcers. Gastric ulcers were produced by subserosal injection of acetic acid. SK-MS10 (200 mg/kg) or vehicle was administered orally once per day for 14 days after the acetic acid injection. The stomach was removed and the ulcer size measured at day 7 and 14 of the treatment. Expression of vascular endothelial growth factor (VEGF) was assessed by real-time RT-PCR and Western blot analysis. In addition, the microvasculature density (MVD) adjacent to the ulcer margin was examined by immunohistochemistry. The treatment with SK-MS10 for 7 and 14 days significantly accelerated ulcer healing and increased the expression of mRNA (at day 7) as well as VEGF protein (at day 14) compared to the vehicle-treated rats. The MVD for factor VIII was also higher in the SK-MS10 treatment group compared to the vehicle-treated rats; however, these differences were not statistically significant. These results suggest that SK-MS10 treatment accelerates the healing of gastric ulcers via upregulation of VEGF and angiogenesis in an acetic acid rat model.

**Key Words :** Cochinchina *Momordica* Seed Extract; Acetic Acid; Stomach Ulcer; Vascular Endothelial Growth Factor; Angiogenesis

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## INTRODUCTION

Peptic ulcers affect a large portion of the population worldwide and are commonly induced by *Helicobacter pylori* infection or one of several other factors including stress, smoking, and the ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) (1, 2). Currently, the relatively higher incidence of gastric ulcers in the elderly and the expanded use of NSAIDs, along with alcohol ingestion and stress have been shown to increase with aging (3). The approach to the treatment of peptic ulcer disease includes inhibition of gastric acid secretion by H<sub>2</sub> receptor blockers or proton pump inhibitors (PPIs) as well as eradication of *H. pylori*. However, development of tolerance to such treatments and the incidence of relapse as well as side effects interfere with their clinical usefulness. Therefore, new antiulcer drugs are under investigation.

Cochinchina momordica is the dried ripe seed of *Momordica cochinchinensis*, a perennial vine that grows in southern China and Vietnam, and is known for its anti-inflammatory activi-

ty against suppurative skin infections (4). Chemical analysis shows that the cochinchina momordica seeds are composed of compounds including fatty acids, saponins, proteins,  $\alpha$ -spinasterol, oleanolic acid, and momordica acid (4). Among these compounds, momordica saponin I, glycoside, a triterpenoid saponin containing disaccharide chain, has been found to be a major active ingredient (5). Recently, we reported that SK-MS10, an extract from cochinchina momordica seeds, has gastroprotective effects against acute gastric mucosal damage by suppressing proinflammatory cytokines, down-regulating cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), 5-lipoxygenase (5-LOX), and increasing the synthesis of mucus in an acute gastric mucosal damage model, using ethanol and water immersion restraint stress (WRS) (6). Furthermore, we demonstrated that the calcitonin gene-related peptide (CGRP)-nitric oxide (NO) pathway played an important role in the gastroprotective effects of SK-MS10 (6).

The so-called acetic acid ulcer model has been shown to be a useful model for investigating the pathophysiology of gas-

tric ulcers and the efficacy of antiulcer drugs (7). This model mimics human ulcers in terms of both pathophysiological features and healing mechanisms. To investigate whether SK-MS10 improves the healing of gastric ulcers, and to evaluate the mechanisms involved, we used the acetic acid-induced ulcer rat model in this study. The goal of this study was to investigate the effects of SK-MS10 on angiogenic responses such as the microvasculature density, and the expression of vascular endothelial growth factor (VEGF).

## MATERIALS AND METHODS

### Preparation and composition of SK-MS10

Five liter of aqueous ethanol solution was added to one kg (dry weight) of cochinchina momordica, purchased at an herb market in Korea. Extraction was performed for 4 hr at 80°C, and this process was performed twice. The extract was filtered and concentrated under reduced pressure at 60°C using a rotary evaporator. After complete removal of the solvent in a vacuum oven, 60 g of ethanol extract in powder form (SK-MS10) was obtained. SK-MS10 was dissolved in the carboxymethylcellulose (CMC) during the experiment.

### Animals

Seven-week-old male Sprague-Dawley rats (Orient Co. Ltd., Seoul, Korea) were housed in a cage maintained at 23°C, 12/12-hr light/dark cycles under specific pathogen-free conditions. After 1 week of adaptation, the 8-week-old rats weighing 250-300 g were used for the experiments. The rats were starved but allowed water for 12 hr prior to the experiments. All experimental procedures described here were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU-070419-2).

### Induction of the gastric ulcer

Gastric ulcers were induced with acetic acid treatment, according to a previously described method (8). Briefly, the fasting rats were anesthetized with an intramuscular injection of 80 mg/kg of ketamine, and then their stomachs were exposed via a midline incision. Acetic acid (20%, 30  $\mu$ L) was injected into the subserosal layer at the junction of the anterior wall of the antrum and corpus using a microsyringe (Hamilton Co., Reno, NV, USA). The abdominal incision was then sutured closed. From day 1 after ulcer induction, the rats were treated with SK-MS10 (200 mg/kg) or a vehicle by gavage. The rats were sacrificed on day 7 or 14. Each experimental group consisted of six animals. After measuring the size of the ulcer, the ulcer tissue was cut in half. One half was used for histological and immunohistochemical examination, while the other was used for mRNA level assays. As soon as the

mucosa was collected, it was stored in liquid nitrogen and the samples were kept in a -80°C freezer until used for the experiments.

### Ulcerated area determination

After the animals were sacrificed, the isolated stomachs were cut open along the greater curvature and washed in ice-cold saline. To assess the degree of gross mucosal damage, the mucosal sides of the stomachs were photographed using a digital camera, and part of the mucosa was immediately fixed with a 10% formalin solution. After fixed in formalin overnight, the stomach was opened along the greater curvature and spread out with pins on a cork board, and then photographed. The ulcerated area ( $\text{mm}^2$ ) was quantified using the following equation:  $S = \pi (d1/2) \times (d2/2)$ , where  $S$  represented the ulcerated area ( $\text{mm}^2$ ),  $d1$  and  $d2$  the longest longitudinal and transverse diameters of the ulcer.

### Immunohistochemical staining for von willebrand factor

For the angiogenesis studies, the sections were incubated with an antibody for the von Willebrand factor (factor VIII-related endothelial antigen; Chemicon International, Temecula, CA, USA) after deactivation of endogenous peroxidase with 0.3%  $\text{H}_2\text{O}_2$  and blocking of nonspecific binding sites. The microvasculature was visualized by the avidin-biotin-peroxidase complex method. The degree of microvasculature found in the ulcer base granulation tissue was determined in three randomly chosen 1  $\text{mm}^2$  fields. The microvasculature density was expressed as the number of vessels per  $\text{mm}^2$  of the ulcer base.

### Western blotting for VEGF

The gastric mucosa was homogenized with lysis buffer containing 25 mM Tris-HCl (pH 7.4), EGTA (1 mM), DTT (1 mM), leupeptin (10  $\mu\text{g}/\text{mL}$ ), aprotinin (10  $\mu\text{g}/\text{mL}$ ), PMSF (1 mM), and Triton X-100 (0.1%). Briefly, the proteins (each sample, 30  $\mu\text{g}$ ) were separated by SDS-PAGE (7.5% wt/wt gel) and transferred to nitrocellulose membranes. All procedures were carried out in Tris buffer (40 mM, pH 7.55) containing 0.3 M of NaCl and 0.3% Tween 20. The membranes were then blocked with dried milk (6% wt/vol), and subsequently incubated with VEGF antibody (mouse monoclonal antibody, 1:500; Chemicon International) at 4°C overnight. The blots were incubated with secondary antibody (goat anti-mouse polyclonal antibody, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an imaging analyzer was used to measure the band densities.

### Real time PCR for VEGF

RNA was extracted from the gastric mucosa using the

RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA samples were diluted to a final concentration of 0.5 mg/mL in RNase-free water and stored at  $-80^{\circ}\text{C}$  until use. Synthesis of the cDNA was performed with 1 mg of total RNA with M-MLV Reverse Transcription Reagents (Invitrogen, Carlsbad, CA, USA). The 20- $\mu\text{L}$  reverse transcription reaction consisted of 4  $\mu\text{L}$  of First-strand buffer, 500 mM deoxynucleoside triphosphate mixture, 2.5 mM oligo (dT)<sub>12-18</sub> primer, 0.4 U/mL ribonuclease inhibitor, and 1.25 U/mL Moloney murine leukemia virus reverse transcriptase (Invitrogen). The thermal cycling parameters for the reverse transcription were 5 min at  $65^{\circ}\text{C}$ , 50 min at  $37^{\circ}\text{C}$  and 15 min at  $70^{\circ}\text{C}$ . Real time PCR amplification and determination were performed using the ABI PRISM 7000 Sequence Detection System, TaqMan universal PCR master mix, commercially available predesigned, gene specific primers, and FAM-labelled probe sets for quantitative gene expression (TaqMan Gene Expression Assays, rodent VEGF, mouse  $\beta$ -actin; Applied Biosystems, Foster City, CA, USA). All of the probes used in these experiments spanned an exon-intron boundary. The VEGF and  $\beta$ -actin mRNA was quantified by parallel estimation. The thermal cycler conditions were 2-min hold at  $50^{\circ}\text{C}$  and 10-min hold at  $95^{\circ}\text{C}$ , followed by 40 cycles of 15 s at  $95^{\circ}\text{C}$  and 1 min at  $60^{\circ}\text{C}$ .

### Statistical analysis

All statistical calculations were performed using SPSS software (version 12.0; SPSS Inc., Chicago, IL, USA). The results were compared using the Mann-Whitney U test and the Wil-

coxon rank sum test. All values are reported as means  $\pm$  standard errors. Statistical significance was set at  $P$  value  $<0.05$ .

## RESULTS

### Gastric ulcer healing with SK-MS10

SK-MS10 significantly accelerated ulcer healing by day 7 and day 14. That is, compared to the vehicle-treated group (Fig. 1A, B) the mean ulcer size in the SK-MS10-treated group (Fig. 1C, D) was significantly smaller by day 7 and 14 after ulcer induction. Numerically, the ulcer area 7 and 14 days after SK-MS10 treatment was 33.2 mm<sup>2</sup> and 9.3 mm<sup>2</sup>, respectively, which was smaller than the 52.6 mm<sup>2</sup> and 32.3 mm<sup>2</sup> size of the vehicle treated group (Fig. 1E).

### Expression of factor VIII in the ulcer mucosa

As shown in Fig. 2, microvessels were stained brown using the von Willebrand factor antibody. The microvasculature density (MVD) in the SK-MS10 treated group was increased compared to the vehicle treated group; however, this difference did not reach statistical significance. That is, the microvessel densities in the ulcer granulation tissues of the SK-MS10 treated rats on day 7 and 14 were 40.8 vessels/mm<sup>2</sup> and 36.8 vessels/mm<sup>2</sup>, respectively, which was higher than in the vehicle treated rats (32.4 vessels/mm<sup>2</sup> and 24.2 vessels/mm<sup>2</sup>, respectively).

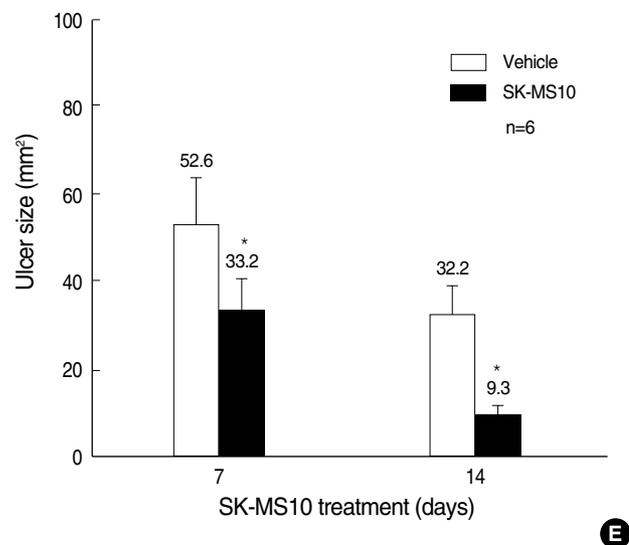
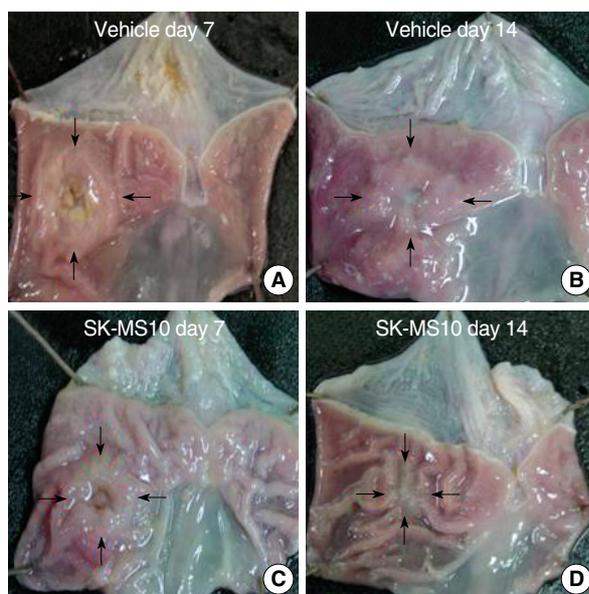
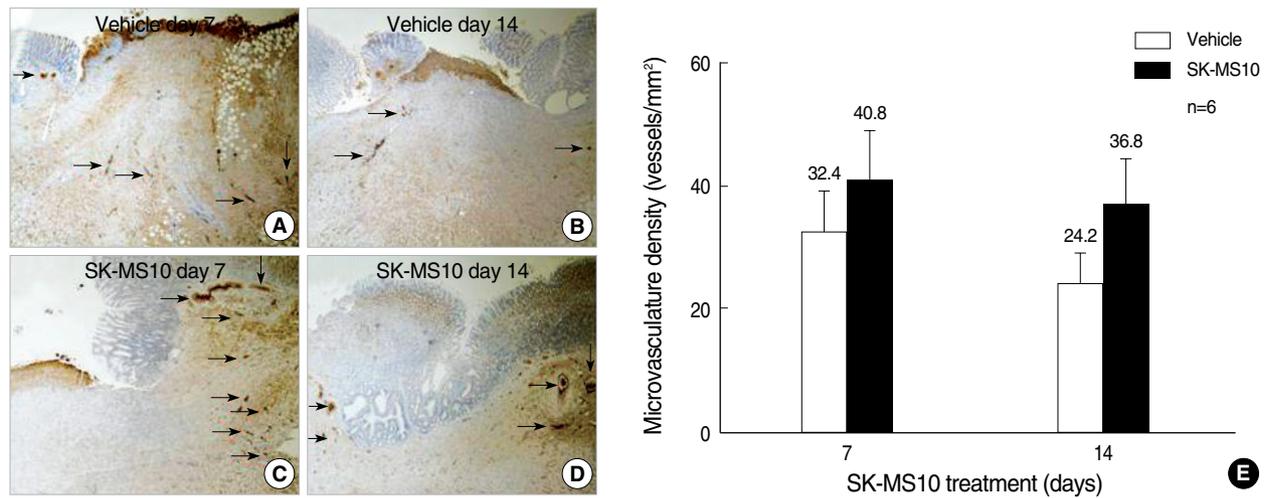
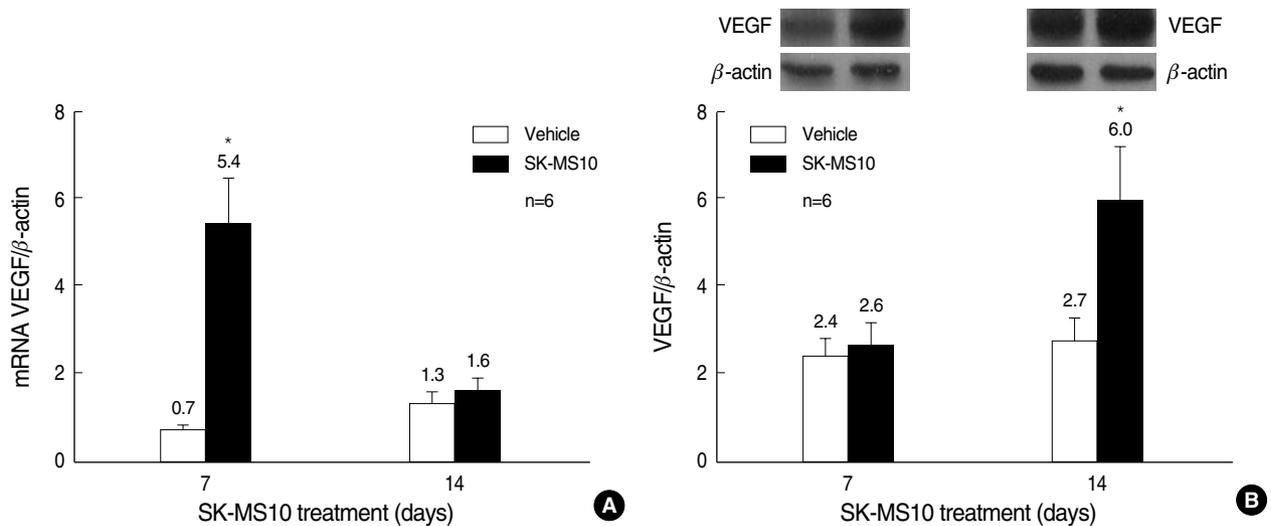


Fig. 1. Effects of SK-MS10 on the healing of gastric ulcers. Macroscopic appearance of ulcers generated at the gastric mucosa in the vehicle treated group at 7 and 14 days (A, B) and the SK-MS10 treated group at 7 and 14 days (C, D). Arrows indicate ulcer. (E) Summarized results on changes of the ulcer area in the vehicle and SK-MS10 treated groups. Results are the mean  $\pm$  SE in 6 animals per group. \* $P$  value  $<0.05$  when compared with the vehicle treated group.



**Fig. 2.** Effects of SK-MS10 on angiogenesis in the gastric ulcer. Immunohistochemical staining of microvessels with the von Willebrand factor in the ulcer bases of rats in the vehicle treated group at 7 and 14 days (A, B) and the SK-MS10 treated group at 7 and 14 days (C, D). Note the von Willebrand factor positive cells (dark brown spots indicated by arrows). (E) The number of microvessels at the gastric ulcer bases of the rats in the vehicle and the SK-MS10 treated groups. Results are mean  $\pm$  SE in 6 animals per group. \*P value <0.05 when compared with the vehicle treated group.



**Fig. 3.** Effects of SK-MS10 on the expression of VEGF. (A) The relative mRNA expression of VEGF. (B) Western blot analysis for VEGF. Results are mean  $\pm$  SE in 6 animals per group. \*P value <0.05 when compared with the vehicle treated group.

**Expression of VEGF in the ulcer mucosa**

The mRNA expression of VEGF after 7 days of SK-MS10 treatment was significantly higher than in the vehicle treated group (0.7 vs. 5.4 for VEGF/ $\beta$ -actin, respectively Fig. 3A). However, the mRNA expression of VEGF after 14 days of SK-MS10 treatment was not significantly different from the vehicle treated group (Fig. 3A). On Western blot analysis, the expression of VEGF proteins 14 days after SK-MS10 treatment was significantly higher than in the vehicle group (2.7 vs. 6.0 for VEGF/ $\beta$ -actin, respectively Fig. 3B). However, the protein expression of VEGF 7 days after SK-MS10 treat-

ment was not significantly different from the vehicle treated group (Fig. 3B).

**DISCUSSION**

Since introduced in 1969 by Takagi et al. (9), the acetic acid-induced gastric ulcer model has proved useful for investigating the pathophysiology of gastric ulcer disease and the efficacy of antiulcer drugs (7). The reasons for the usefulness of this model include the following. First, the ulcer induction procedure is simple, readily resulting in ulcers of consistent

size and severity with a 100% success rate. Second, the acetic acid-induced ulcers resemble human peptic ulcers both macroscopically and histologically. Indeed, spontaneous relapse of healed ulcers is frequently observed, just as in patients with peptic ulcer disease. Finally, the ulcers respond well to various anti-ulcer drugs, such as PPI, sucralfate and several growth factors. In this study, we investigated the effects of SK-MS10 on the healing of this type of experimental ulcer in the rat, and found that oral administration of SK-MS10, at a dose of 200 mg/kg for 7 and 14 days, accelerated the healing of the gastric mucosa in the rat model.

The regrowth of blood vessels into the ulcerated area, i.e., angiogenesis, plays a pivotal role in the acceleration of the healing of ulcers since the neovasculature promotes nutrient supply to the healing tissue (10). This complex sequence of events requires a high degree of coordination among different cell types, which is regulated by several factors. Among them, growth factors such as the vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and their receptors are known to play an important role in tissue repair and the healing of ulcers (11, 12). VEGF, previously described as a vascular permeability factor, is also known as a growth factor that is involved in the mucosal protection and the angiogenic response that occurs during the healing of ulcers (13). Specific expression of VEGF has been demonstrated at the ulcer margin in humans (14). In addition, exogenous VEGF has been reported to significantly enhance the healing of acute gastric mucosal injury induced by ethanol and duodenal ulceration (15). Furthermore, local injection of plasmid-DNA encoding VEGF has been shown to stimulate the healing of ulcers in the rat (16). By contrast, NSAIDs, cyclooxygenase-2 (COX-2) inhibitors, and alendronate have been shown to delay the healing of ulcers, and impair angiogenesis and the down regulation of growth factors such as bFGF and VEGF (17-19). Recently, the saponins isolated from Red Ginseng exhibited wound healing of burns in mice and promoted angiogenesis via the stimulation of VEGF production (20). In the present study we found that SK-MS10, with momordica saponin as a major component, enhanced the expression of VEGF by stimulation of angiogenesis in the gastric mucosa of the rat, similar to the effects of Red Ginseng. This expression of VEGF has been reported to be upregulated by COX-2 derived prostaglandin  $E_2$  (PGE<sub>2</sub>) (21). Thus, it might be valuable to investigate whether SK-MS10 could induce COX-2/PGE<sub>2</sub> production in acetic acid induced ulcer. Besides some bioactive components, *cochinchina momordica* are also rich in protease inhibitors. Several trypsin inhibitors and chymotrypsin inhibitor have been isolated (22-25). Protease inhibitor from *cochinchina momordica* has been reported to possess antioxidative activities in the rat hepatocyte (26). In addition, high carotenoid contents such as carotene, lycopene are known to be detected in the active fraction of *cochinchina momordica* seed membrane (27). Carotenoids also are known to potent antioxidative activity and have been reported to have anti-

ulcerogenic effect on ulcer models in rat (28). These antioxidative components of the seeds may provide a favorable environment for ulcer healing and the anti-ulcerogenic effect. However, further studies with these isolates from *cochinchina momordica* and phytochemical analysis are needed to clarify the main contributor of the mechanism on ulcer healing. Interestingly, the duration of treatment for the detection of a significant difference was different for the mRNA and the protein expression. That is, the mRNA expression of VEGF 7 days after SK-MS10 treatment, and the expression of VEGF proteins 14 days after SK-MS10 treatment were significantly higher than in the control group. This discrepancy might be due to the lag time between the translation of mRNA and the expression of the protein. In addition, it is possible that the VEGF expression is regulated at the post-translational level, although further studies are necessary to clarify this.

MVD assessment is the most commonly used technique to quantify angiogenesis. Initially, we used antibodies directed against platelet endothelial cell adhesion molecules, CD31 and CD34; commonly used for the assessment of angiogenesis in several prior studies (29-31). However, these antibodies did not work properly for staining the microvessels even after several trials. As an alternative marker, we used an antibody against the factor VIII-related antigen, also known as the von Willebrand factor, staining mainly the mature vessels and cross reacting lymphatic endothelium (32). This marker, in the SK-MS10 treated group, was increased compared to the vehicle treated group; however, the difference did not reach statistical significance. The relatively small sample size (n=6) might have limited the power to detect a significant difference between the two groups. This finding may also be partly due to the variation in the assessment of the MVD, depending on the fields examined, and the experience of the pathologist. So, further study extending the number of animals is needed to clarify the effect on the angiogenesis of SK-MS10 for strengthening the statistical power. However, the increasing tendency of the MVD with SK-MS10 treatment was supported by the results of enhanced expression of VEGF by using the real-time PCR and Western blot analysis.

Several studies have provided evidence for a role of sensory neuron in gastric ulcer healing. Sensory nerve ablation by high dose capsaicin impaired the ulcer healing (33). Recently, Ohno et al. has demonstrated that CGRP had proangiogenic activity associated with the enhancement of ulcer healing using CGRP knockout mice (30). We previously reported that SK-MS10 has a gastroprotective effect against acute gastric mucosal damage using a model with ethanol and WRS (6). These gastroprotective effects were found to be mediated by the upregulation of CGRP. Thus CGRP might play a role in the ulcer healing by SK-MS10 in the acetic acid-induced ulcer model. Actually CGRP has been known to be the major transmitter from afferent nerve fibers and suppress acid output (34). Thus, there is a possibility of the stimulation of CGRP by SK-MS10 could contribute to the antisecretory

effect. By contrast, proinflammatory cytokines play an important role in impairing ulcer healing (19, 35). IL-1 $\beta$  has been used in a model of induction of gastric ulcer recurrence; approximately 80-100% of healed ulcers recur at the sites of scarred mucosa within 48 h after injection of IL-1 $\beta$  (36). In a previous study, we demonstrated that SK-MS10 reduced the increases of mucosal myeloperoxidase, IL-1 $\beta$ , and TNF $\alpha$  levels in an acute mucosal damage model using ethanol and WRS (6). This result further suggested that SK-MS10 have potent inhibitory effect on inflammation, which may also contribute to the enhancement of ulcer healing. In addition to this gastroprotective effect of SK-MS10 we identified an additional mechanism of VEGF expression associated with the anti-ulcer effects of SK-MS10 in the chronic ulcer model using acetic acid in the rat.

In conclusion, the results of this study suggest that SK-MS10 accelerates the healing of acetic acid induced gastric ulcers in rats by enhancing angiogenesis and the expression of the angiogenic growth factor, VEGF. These findings suggest that SK-MS10 might be an alternative treatment of gastric ulcer disease in humans sometime in the near future. In addition, acetic acid ulcer models appear to be quite useful for pharmacological studies of anti-ulcer drug.

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