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ORIGINAL ARTICLE

TERM

Local Injection of Granulocyte-Colony Stimulating Factor Accelerates Wound Healing in a Rat Excisional Wound Model

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A systemic treatment of granulocyte-colony stimulating factor (G-CSF) is known to improve healings of damaged tissues. However, recent studies suggested local actions of G-CSF on the healing processes of damaged tissues. We investigated the treatment effect of locally injected G-CSF and compared to that of systemically injected G-CSF in a rat model. A wound was created on the rat dorsum and treated either by local injection or by systemic injection of G-CSF. Wound healing rate, deposition of collagen, and gene expression were evaluated. G-CSF receptor (G-CSFR) protein was detected by Western blotting. The wound healing rate in the local injection group was significantly higher than that in the systemic injection group at days 9 and 15; it was also significantly higher than that in the control group at days 3, 9, and 15. The expression of G-CSFR protein in wound tissues was higher than in normal skin tissues. The local injection of G-CSF is more effective than systemic injection of G-CSF in promoting wound healing, which may implicate the local action of G-CSF treatment in wound healing processes.
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Key Words: Granulocyte-colony stimulating factor; Wound healing; Local injection

INTRODUCTION

The skin functions as a protective barrier against the environment. Loss of the integrity of large areas of skin may lead to increased risk of illness [1]. Wound healing is essential to prevent invasion of pathogens and to maintain the integrity of normal tissue [2], and involves several steps including inflammation, formation of granulation tissue, remodeling of connective tissue, collagenization, and formation of new blood vessels [3].

Granulocyte-colony stimulating factor (G-CSF) directly stimulates neutrophil-restricted progenitor cells into proliferation and differentiation [4]. It has been reported to be effective in the treatment of tissue repair in post-myocardial infarction by enhancing mobilization of neutrophils and macrophages from bone marrow [5]. Recently, Wang et al. [6] demonstrated that systemic injection of G-CSF can accelerate wound healing in mice. They suggested that G-CSF repairs wounds through mobilization of bone marrow derived cells (BMDCs) and up-regulation of growth factors.

Recent findings, however, have indicated that G-CSF acts directly on cardiomyocytes and monocytes by binding to a receptor expressed on these cells [7-9]. Moreover, Mueller et al. [10] reported that keratinocyte express G-CSF receptors (G-CSFR). The therapeutic effects of local injection of G-CSF on wound healing in a rat wound model remain unclear.

In this study, we investigated whether local injection of G-CSF can accelerate wound healing in a rat wound model, and evaluated whether the effects of local injection of G-CSF on wound healing occurred faster than that those of systemic injection. We also investigated the presence of G-CSFRs in wound tissues.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Koatech, Pyeongtaek, Korea), 10 weeks of age and weighing 280−300 g, were used. The rats were

kept in a specific pathogen-free facility at the Hanyang University Medical School Animal Experiment Center at a controlled temperature (23±2°C) and humidity (55±5%), with a 12 hours artificial light and dark cycle. This research protocol was approved by the Hanyang University Institutional Animal Care and Use Committee, and the experiments were performed in compliance with the ARRIVE guidelines on animal research [11].

Excision wound model

The rats were anesthetized using an intramuscular injection of a mixture of zoletil 50 (30 mg/kg, Virbac SA, Carros, France) and rompun (10 mg/kg, Bayer Korea, Seoul, Korea). The dorsum was clipped free of hair, and a wound model was created as described by Morton and Malone [12]. An impression was made on the dorsum, the interscapular region 5 mm away from the ears using a circular colored rubber stamp of 30 mm diameter. Scissors were used to excise the full thickness of skin within this marking, including panniculus carnosus, to get a wound area of approximately 706 mm^2. Wounds were left undressed and rats were housed separately.

Experimental design and G-CSF injection

All rats were divided randomly into three groups: the control group (n=20) rats was introdemally with saline 50 μg (0.2 mL)/kg/point, at 8 points around the wound once weekly for 2 weeks after wound creation; the local injection group (n=20) rats was injected intradermally with G-CSF (Leucostim, Dong-A Pharmaceutical, Seoul, Korea) 50 μg (0.2 mL)/kg/point, at 8 points around the wound once weekly for 2 weeks after wound creation; and the systemic injection group (n=5) rats was injected intraperitoneally (i.p.) with G-CSF, 400 μg (1.6 mL)/kg once weekly for 2 weeks. Rats in the control group and the local injection group were randomly euthanized on days 1, 3, 9, and 15 after wound creation for histological wound comparison (Fig. 1). The entire wound, including a 2 mm margin of unwounded skin, was excised [13]. Then, the excised tissue was bisected into two parts: one was used for histological analysis and, the other was used for quantitative real-time polymerase chain reaction (qRT-PCR) examination and Western blotting.

Wound closure analysis

To record wound closure, 5 photographs were taken of randomly selected from each group at days 0, 1, 3, 9, and 15. The wound area was measured by tracing the wound margin using Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD, USA). The researcher who measured the wound was blinded to the treatment grouping. The wound healing rate was calculated as a percentage of wound closure using the Walker and Mason formula: [(day 0 area-day n area)/(day 0 area)]×100% (n=1, 3, 9, or 15) [14].

Histology

To examine the pathology of wound healing, 4% paraformaldehyde-fixed paraffin-embedded wound sections of 5 μm thickness were stained with hematoxylin and eosin (H-E) and Masson's trichrome (MT) staining [15]. Five regions from each digitized images were selected at random from the individual sections and were quantified using the Leica image analysis system (Leica DM 4000B, Wetzlar, Germany) [16]. The stained sections were photographed using a light microscope (Leica DM 4000B). All data were evaluated by a separate blinded investigator.

Deposition of collagen percentage

MT staining was used to analyze the deposition of collagen in the dermis [17]. The mean percent area occupied by MT-stained collagen fiber was calculated for 5 randomly selected fields of each wound section using the Leica image analysis sys-

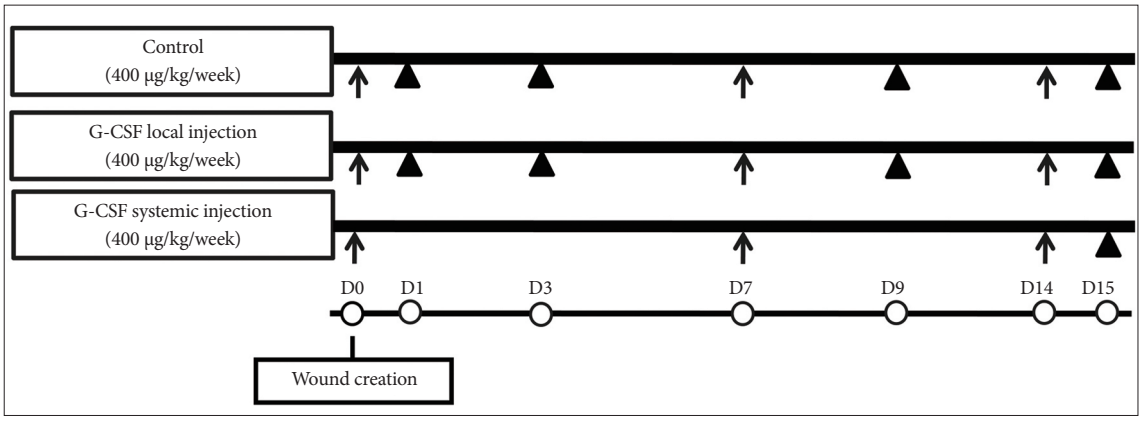

Figure 1. Schematic description of the experimental protocol. The control group (n=20) was injected with saline 400 μg/kg/week for two weeks; the local (i.d.) injection group (n=20) and systemic injection group (i.p.) (n=5) were injected with granulocyte-colony stimulating factor (G-CSF) 400 μg/kg/week for two weeks after wound creation. ↑: injection, ▲: sacrifice.

tem with magnification ×200 (Leica DM 4000B).

Western blotting for G-CSF receptor

The excised tissues were homogenized, and total proteins were extracted using protein lysis buffer (Pro-preb; iNtRON, Seongnam, Korea). Then, samples containing 100 ug of protein were transferred into sample buffer, separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (0.45 μm pore size, Bio-Rad, Hercules, CA, USA). After blocking in 5% skim milk solution for 60 minutes, the membranes were incubated with primary antibody for G-CSFR (1:250; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or glyceraldehyde-3-phosphate dehydrogenase (GADPH) (1:2000; Cell Signaling Technology, Boston, MA, USA) overnight at 4°C. Subsequently, they were incubated with HRP-conjugated anti-rabbit antibody (1:2000; Jackson Immunoresearch, West Grove, PA, USA) for 1 hour at room temperature. GAPDH was used as a protein loading control. Positive protein bands were visualized using an ECL kit (GenDEPOT, Barker, TX, USA), and the results

Table 1. Sequences of primers

Primer	Sequences	Size (bp)
VEGF	F: 5′-CTT-CCT-ATT-CCC-CTC-TTA-AAT-CGT-G-3′	102
	R: 5′-CTA-CCT-CTT-TCC-TCT-GCT-GAT-TTC-C-3′	
EGF	F: 5′-ACC-CAT-TCT-CTC-TAG-CTG-TGT-TTG-A-3′	89
	R: 5′-ACC-AGT-CCT-CTT-GTT-CAC-CCT-TAT-T-3′	
GAPDH	F: 5′-CCT-TCT-CTT-GTG-ACA-AAG-TGG-ACA-T-3′	96
	R: 5′-CGT-GGG-TAG-AGT-CAT-ACT-GGA-ACA-T-3′	

VEGF: vascular endothelial growth factor, EGF: epidermal growth factor, GAPDH: glyceraldehyde-3-phosphate dehydrogenase

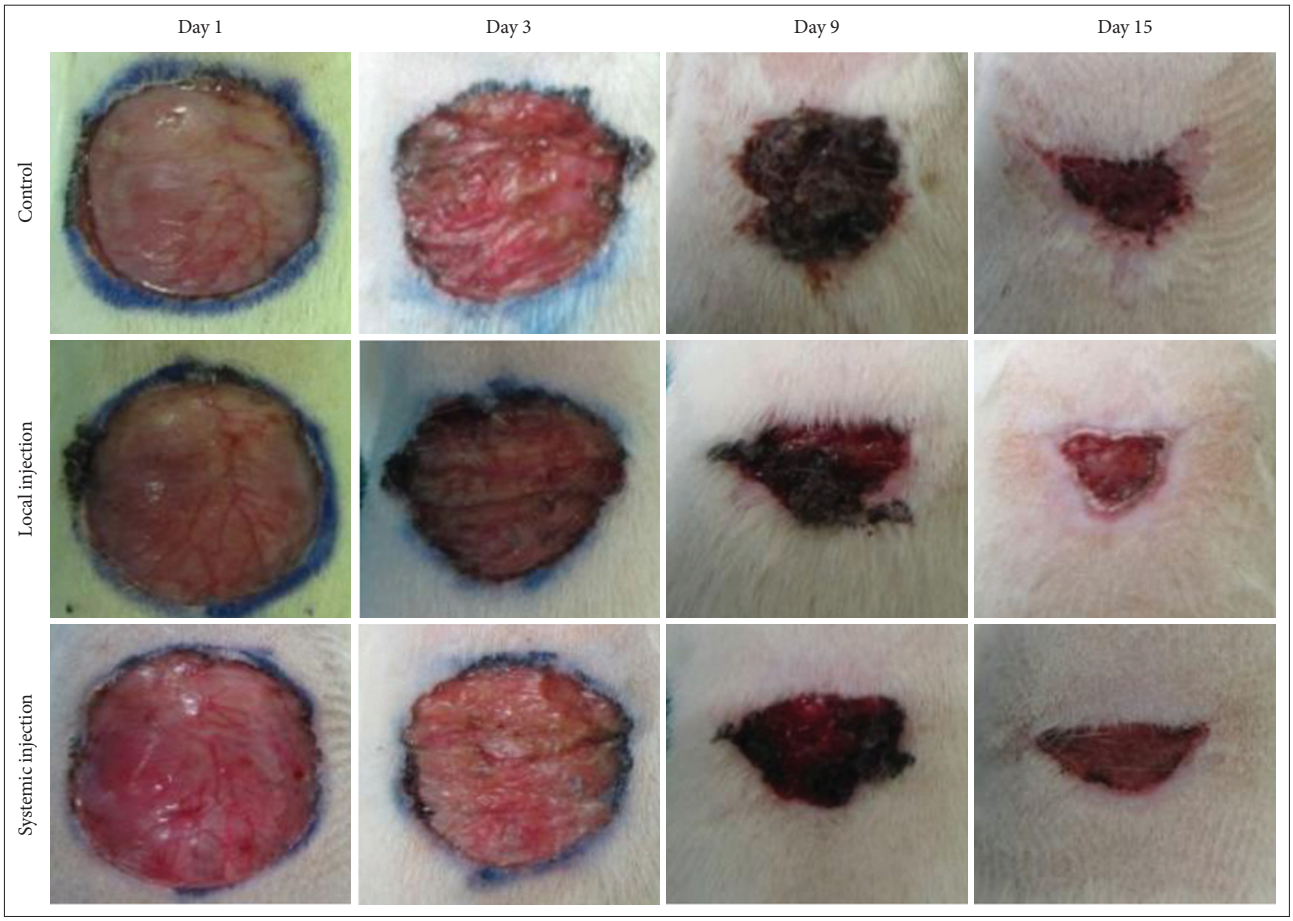

Figure 2. Visualization of wound healing. At post-wound days 1, 3, 9, and 15, the macroscopic wounds in the three groups were randomly photographed and measured. The macroscopic image in the local injection group showed that wound area decreased compared with the other groups.

were quantified with an image analyzer (Image lab 3.0, Bio-Rad, Hercules, CA, USA).

Quantitative real-time polymerase chain reaction for EGF and VEGF

Total RNA was purified using the Qiazol reagent (Qiagen, Valencia, CA, USA) following the manufacturer's instructions [15]. RNA concentrations were measured with a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific Inc., Welmington, DE, USA), and purity was determined by measuring ratios of A260 and A280, which ranged from 1.8 to 2.0. For qRT-PCR, complementary DNA was synthesized from 3 ug of RNA using Moloney Murine Leukemia virus reverse transcriptase primed with oligo (dT) (Invitrogen, Carlsbad, CA, USA). The qRT-PCR was performed using a Light Cycler 480 System (Roche, Basel, Switzerland) with a FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Indianapolis, IN, USA). qPCR amplification was performed with an initial incubation for 10 minutes at 95°C followed by 45 cycles of 10 seconds at 95°C, 10 seconds at 60°C, and 8 seconds at 72°C and then a final dissociation step at 65°C for 15 seconds. The crossing point of each sample was automatically determined by the LightCycler program. The primers used are shown in Table 1. PCR was performed in duplicate and the measured transcript levels were normalized against those of GAPDH.

Statistical analyses

For statistical analyses, the Statistical Package for the Social Sciences 21.0 software (IBM, Armonk, NY, USA) was used. All data are expressed as mean±standard deviation, except for histological data which are expressed as mean±standard error. Data were analyzed using Mann-Whitney U-tests (for single comparisons) or Kruskal-Wallis nonparametric ANOVA (for multiple comparisons). Values of $p<0.05$ were considered statistically significant.

RESULTS

Wound healing rate

Wound healing rate was calculated as a percentage of wound closure at days 1, 3, 9, and 15 (Fig. 2, Table 2). There was no difference between the three groups at day 1. The wound healing rate in the local injection group was significantly higher than that in the control group at day 3 ($p<0.05$), day 9 ($p<0.05$), and day 15 ($p<0.05$). The wound healing rate in the local injection group was significantly higher than that in the systemic in-

Table 2. Wound healing rates of the rats in Group 1, 2, and 3 at days 1, 3, 9, and 15

Group	Day 1	Day 3	Day 9	Day 15
Control	2.44±1.23	10.78±4.86	57.18±4.30	82.7±2.27
Local injection	2.67±1.43	20.04±6.20*	70.97±3.63*†	87.95±2.16*†
Systemic injection	2.57±0.69	18.05±5.39*	62.92±5.45	82.63±2.59

The wound healing rate was calculated as a percentage of wound closure: [(day 0 area-day n area)/(day 0 area)]×100%. All data were expressed as means±SD. *$p<0.05$ vs. control, †$p<0.05$ vs. systemic injection (n=5–10).

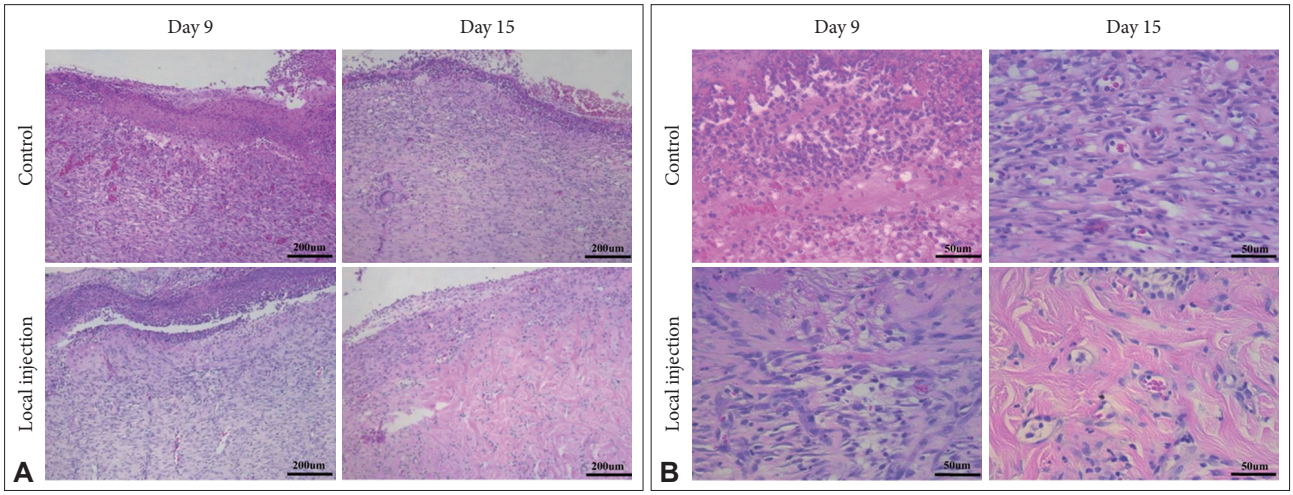

Figure 3. The pathology of wound healing. (A) Histological changes in the dermis of wounded skin (H&E stain, ×100), (B) (H&E stain, ×400). The G-CSF local (i.d.) injected group showed decreased inflammatory reaction and the presence of arranged collagen fibers at day 9. Deposition of collagen in the G-CSF local (i.d.) injected group was increased at day 15. G-CSF: granulocyte-colony stimulating factor.

jection group at day 9 (p<0.05) and day 15 (p<0.05). The wound healing rate in the systemic injection group was significantly higher than that in the control group at day 3 (p<0.05), but there were no significant differences at days 9 and 15.

Histological assessments

The process of wound healing was confirmed by H-E and MT staining. The H-E-stained sections of the local injection group showed that inflammatory reaction were decreased and dense collagen fibers were arranged in the dermis at days 9 and 15. The sections from the control group were still observed to be in the inflammatory reaction stage and loose collagen fibers were apparent at day 9 (Fig. 3). The MT-stained sections from the local injection group showed that collagen fibers were distributed compactly and regularly at days 9 and 15, but collagen fibers in the control group were loosely distributed at days 9 and 15 (Fig. 4A). The percentage deposition of collagen in the local injection group was significantly higher than that in the control group at day 9 (43.64±3.59% vs. 11.60±0.97%, p<0.05) and day 15 (51.11±1.55% vs. 25.32±1.56%, p<0.05) (Fig. 4B).

Western blotting for G-CSFR

G-CSFR expression in wound tissues was confirmed by the detection of the G-CSFR protein by Western blot analysis. Furthermore, the expression of G-CSFR protein in wound tissues was higher than that in normal skin tissues (Fig. 5).

Expression of EGF and VEGF

The average level of epidermal growth factor (EGF) in wound samples of the local injection group was significantly higher than that in the control group at day 1 (124.09±6.87% vs. 100±14.31%, p<0.05) and day 3 (94.20±19.51% vs. 65.80±8.75%, p<0.05). The level of EGF was decreased in both groups at days 9 and 15 (Fig. 6A). The levels of vascular endothelial growth factor (VEGF) increased gradually and peaked at day 9, and then decreased. The level of VEGF in the local injection group was significantly higher than that in the control group at day 9 (378.90±43.48% vs. 220.98±58.23%, p<0.05) (Fig. 6B).

DISCUSSION

Our data showed that local injection of G-CSF improved the wound healing rate more than systemic injection of G-CSF. The wound healing promoting effect of locally injected G-CSF was accompanied by accelerated collagen deposition in the dermis neighboring the wound, reduced inflammatory response and increased expression of wound healing promoting genes EGF and VEGF in wound tissues. We also confirmed that G-CSFRs are present in wound tissues.

The findings of previous studies have suggested the mecha-

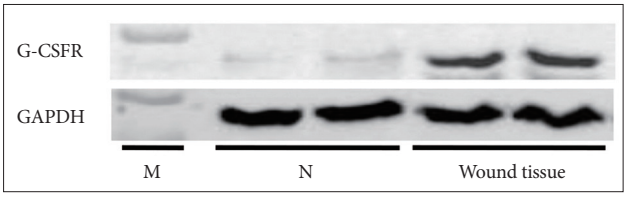

Figure 5. G-CSFR in wound tissue. Levels of G-CSFR protein in the wound were detected by Western blot. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. M: marker; N: normal skin tissue, G-CSFR: granulocyte-colony stimulating factor receptor.

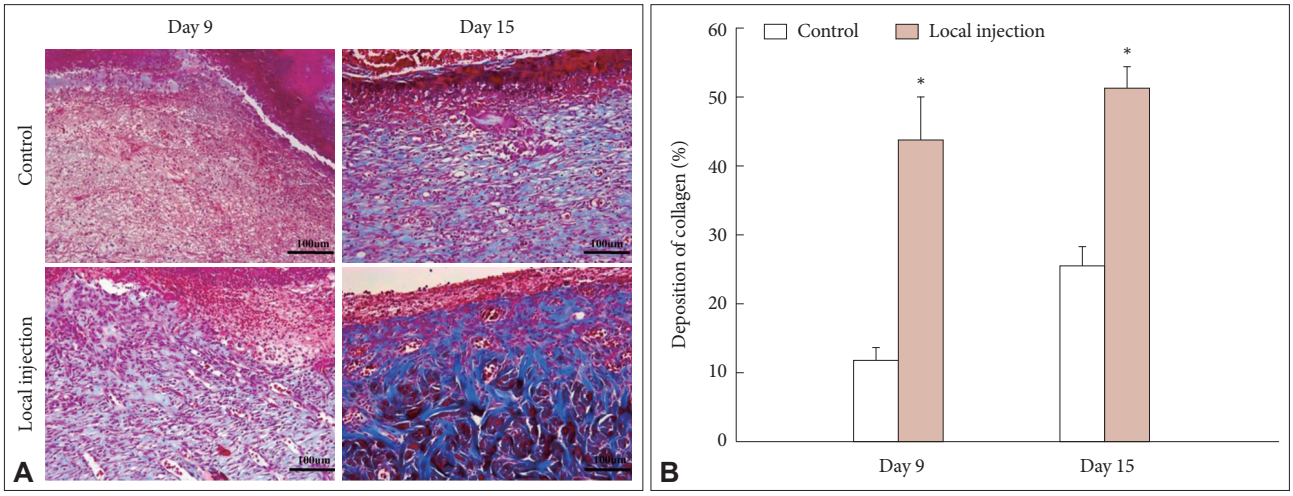

Figure 4. The pathology of wound healing. (A) Histological changes in the dermis of wound skins (MT stain, ×200). Deposition of collagen increased at days 9 and 15 in the G-CSF locally (i.d.) injected group. (B) Quantitative analysis of the deposition of collagen at day 9 and 15. The mean percent area occupied by MT stained collagen fiber density was calculated for 5 randomly selected fields of each wound section. All data are expressed as mean±SE. *p<0.05 vs. control (n=5). G-CSF: granulocyte-colony stimulating factor, MT: Masson's trichrome.

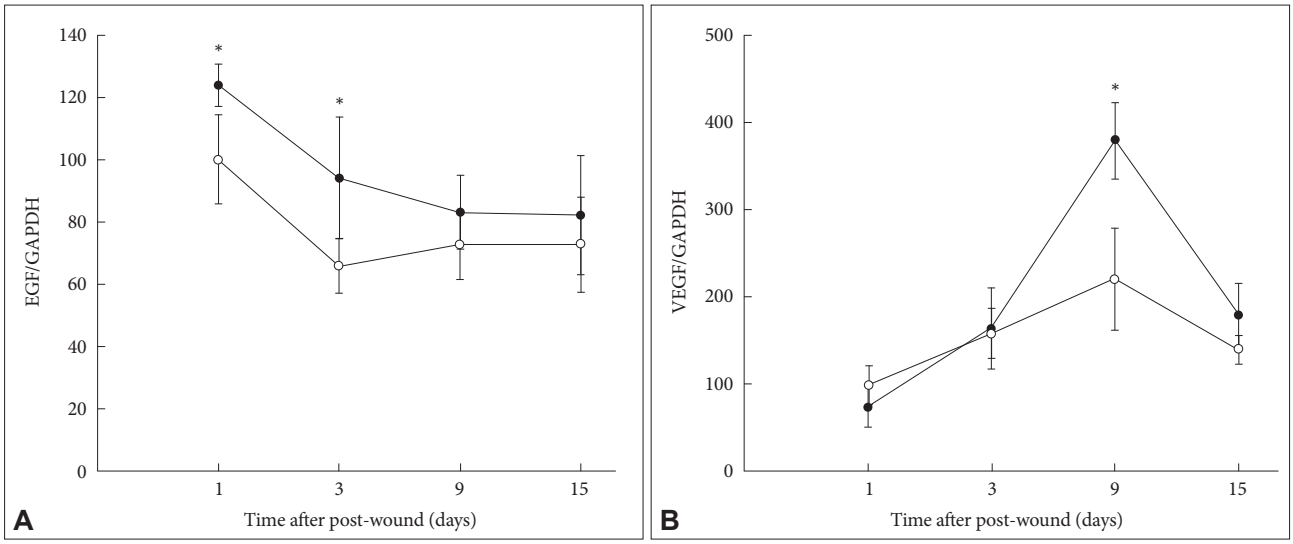

Figure 6. Time course of changes in epidermal growth factor (EGF) (A) and vascular endothelial growth factor (VEGF) (B) expression in wounds was determined by qRT-PCR. White circles indicate the control group and black circles indicate the G-CSF locally (i.d.) injected group. All data are expressed as means±SD. *p<0.05 vs. control (n=5). G-CSF: granulocyte-colony stimulating factor, qRT-PCR: quantitative real-time polymerase chain reaction.

nism for the effect of G-CSF in tissue repair. In one study, systemic injection of G-CSF increased survival after myocardial infarction by promoting BMDCs to migrate into the infarcted border zone and differentiate to cardiomyocytes [18], and Yang et al. [19] also showed that BMSCs can promote skin appendage regeneration. However, recent studies have also suggested that G-CSF exerts local actions on the healing processes in damaged tissues. Frank et al. [20] showed that the local injection of G-CSF reduced retinal ganglion cell death in a retinal ganglion cell axotomy model. We confirmed that local injection of G-CSF effectively accelerated wound healing in a rat wound model, and that local injection of G-CSF was more effective than systemic injection in accelerating wound healing. In addition, we also confirmed that G-CSFRs were present in wound tissues, which is a likely explanation of G-CSF's observed direct therapeutic effects on wound healing.

The inflammatory response following tissue injury plays an important role in wound healing. During the inflammatory phase, platelet aggregation is followed by infiltration of leukocytes into the wound site [21]. An excessive or prolonged inflammatory response, however, can result in increased tissue injury and poor healing. Successful wound repair requires the coordinate expression of both inflammation and resolution of inflammation [22]. In our study, we confirmed that the inflammatory response in the control group lasted longer than it did in the G-CSF injected group. G-CSF may promote wound healing by acceleration the inflammatory response.

Collagen is the predominant extracellular protein in granulation tissue. It provides strength and integrity to all tissues and, thus plays a vital role in wound repair, particularly in forming initial wound structure [23]. The mechanical strength of wound healing depends on the synthesis of collagen and formation of collagen fibrils and fibers. In this study, treatment of the G-CSF group increased the percentage deposition of collagen. Therefore, we confirmed that the wound healing effect related to G-CSF treatment was associated with enhanced deposition of collagen, which appeared to improve the rate of wound healing.

The wound healing process is regulated by a complex signaling network, involving various growth factors, cytokines, and chemokines [24]. EGF and VEGF are particularly important factors in the wound healing process [25]. Previous studies have shown that EGF accelerates epidermal and dermal repair through regeneration of epidermal cells, proliferation of keratinocytes, and migration of keratinocytes [26,27]. VEGF participates in wound healing through the stimulation of angiogenesis [28] and by promoting epithelialization and collagen deposition [29]. Numerous studies have demonstrated that G-CSF can increase EGF and VEGF expression [6,30-33]. Our data also indicated that the wound healing effect of G-CSF treatment was associated with increased expression of EGF and VEGF.

This study has several limitations. First, we could not rule out any potential effects of the downstream pathways activated by the binding of G-CSF to its receptor, and we could not clearly determine the major mechanism underlying the effects of local injection of G-CSF on wound healing. Second, we did not establish the optimum dosage or regimen of G-CSF injection. Third, the number of animals in the systemic injection group was small. In addition, the histological analysis and gene expression for the systemic injection group on the different stages of

wound healing were not demonstrated. Therefore, future studies should involve a larger number of animals.

In summary, local injection of G-CSF accelerated wound healing more effectively than systemic injection of G-CSF in a rat wound model. The wound healing effect of the local injection of G-CSF is associated with alleviation of local inflammation, promotion of collagen deposition and local elevation of VEGF and EGF levels, which may implicate the local actions of G-CSF in wound tissue.

Acknowledgements

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

This research protocol was approved by the Hanyang University Institutional Animal Care and Use Committee, and the experiments were performed in compliance with the ARRIVE guidelines on animal research.

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