An Extract of the Root of Lithospermum erythrorhizon Accelerates Wound Healing in Diabetic Mice

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Many people suffer from intractable bedsores, which sometimes develop because of chronic metabolic failure in patients. An extract of the root of Lithospermum erythrorhizon (SK) has been reported to have an effect on wound healing. However, the effects of SK have not been studied in chronic wounds, such as bedsores. The healing-impaired diabetic (db/db) mouse is a good model for the investigation of clinical healing therapies. Therefore, we examined whether SK accelerates wound healing in db/db mice. Full-thickness round wounds of 6-mm diameter were created on the backs of mice. After applying SK, we covered the wound with a film dressing to keep it moist. At three weeks, wound closure was complete in SK-treated mice but not in controls. Capillary vessel number and collagen synthesis increased earlier in wound healing in SK-treated wounds. At this time, vascular endothelial growth factor (VEGF)-positive neutrophils had infiltrated the wound and the appearance of apoptotic fibroblasts and endothelial cells in the granulation tissue was more advanced than in the controls. Where the wound was covered with epithelium, there tended to be less infiltration of VEGF-positive cells and apoptotic cells. These results suggest that the inflammatory phase was shortened, and the proliferative and maturation phases were advanced by SK. It is known that SK also has antibacterial activity. Therefore, we conclude that SK is useful for wound healing in db/db mice, and could potentially help patients with intractable bedsores.

Key words Lithospermum erythrorhizon; wound healing; db/db mouse; vascular endothelial growth factor (VEGF); granulation tissue; apoptosis

Ulcers are open wounds on skin or mucosa that have a deficiency of surface structure. In the skin, ulcers are easily caused by burns, venous stasis, and bedsores. People who develop skin ulcers from bedsores tend to also have other diseases and therefore may be bedridden all day. Because some of these patients also have chronic metabolic failure, curing the ulcers can be difficult.1) Impairments of wound healing have been studied using healing-impaired animal models, such as aged animals,2) or those with low nutrition,3) circulation failure,4) or diabetes.5) Various materials for accelerating wound healing have also been investigated with such animal models.

“Shikon” is an old Japanese herbal medicine that is thought to accelerate wound healing. “Shikon,” the root of a Lithospermum erythrorhizon Sieb. et Zucc., is the main component of “SHUNKO,” which is used as an ointment. It has been reported that the ether extract of “Shikon,” which contains shikonin and acetylshikonin with pigment ingredients, inhibits blood vessel permeability6–8) and delayed-type hypersensitivity,9) accelerates granulation tissue formation,10) and has antitumor11) and antibacterial activity.12) In addition, when the “Shikon” ointment is applied to a wound, it accelerates wound healing and inhibits capillary vessel permeability in rats.6–8)

Although several studies have examined wound healing with “Shikon” in normal animals, no studies have been performed in a healing-impaired mouse model, which may be more representative of certain patient populations. Diabetic mice have suppressed granuloma formation and allograft rejection,13) and reduced induction of keratinocyte growth factor (KGF) and vascular endothelial growth factor (VEGF).14,15) Mutant diabetic mice (db/db) show a delayed healing response in the dermis relative to normal mice; for example, nondiabetic mice take 11 d for a wound to reach 80% of healing, while db/db mice take 18 d.16) In open wounds, apoptosis is initially mainly limited to the wound edge and follows the advancing epithelial edge toward the center of the wound as healing progresses. The appearance of the apoptotic pattern is significantly delayed17) and inflammatory cells are decreased in the healing wound in db/db mice. Wet conditions are also considered to be more effective than dry conditions for healing wounds.18) To keep the wound moist, we generally use an occlusive dressing. The advantages of using a dressing include the reduction of acute inflammation, heating, the prevention of minor trauma, and the retention of cytokines or growth factors on the wound bed. Consequently, the use of a dressing accelerates wound closure.

Granulation tissue formation is necessary for the wound healing process. The formation of the granulation tissue decreases with the progression of the epithelialization of the wound bed. The reduction of cellularity in the granulation tissue reflects apoptosis, and the number of apoptotic cells peaks at 20 d after wound creation.19) The “Shikon” extract enhances granulation tissue formation through mechanisms involving VEGF secretion from infiltrated cells.20) In this study, we have examined whether wound closure in db/db mice is accelerated by the ether extract of “Shikon” (SK), focusing on granulation tissue formation, VEGF secretion, apoptosis, collagen synthesis, and neovascularization.

MATERIALS AND METHODS

Animals Female 8-week-old diabetic mice, C57BL/ksJ db/db Jcl, were purchased from Clea Japan, Inc. The mice were maintained in individual cages during the experiment.
Preparation of Samples  SK was extracted from the root of *Lithospermum erythrorhizon* Sieb. et Zucc. (Koshiro Company Ltd., Osaka, Japan) with ether under reflux and the solvent was removed. The extracted SK was diluted with isopropyl lauroyl sarcosinate (Eldew® SL-205, Ajinomoto Co., Inc., Tokyo).

Creation of Wounds and Application of Samples  Mice were anesthetized with sodium pentobarbital solution, and their dorsal hair was shaved. A full-thickness round wound was prepared on the back of the mice with a skin biopsy punch (6 mm diameter; Acu Punch®, Acuderm Inc., U.S.A.). After the operation, 10 μl of vehicle or test solution (0.2% or 2% SK) was soaked into filter paper discs for a Finn Chamber® (Epitest Ltd. Oy, Tuusula, Finland) and placed on the wound. The filter paper was covered with the Finn Chamber® and Scanpor® tape (Alpharma AS, Norway), and the tape was covered with film dressing (Bioclusive®, Johnson and Johnson, U.S.A.). The sample was reapplied and the wound covered again with film dressing at one week intervals.

Evaluation of Wound Closure  Wound area was measured as follows. The wound edges were traced onto polyethylene sheets on the days when the dressing were changed and the traced picture was scanned into a computer. The wound area was measured using image analyzing software (Mac SCOPE, Mitani Co., Japan).

Histology  Two or three weeks after the creation of the wound, the mice were killed and the wound area excised. The tissue was fixed in 10% phosphate buffered formalin solution. The formalin-fixed tissues were dehydrated, embedded in paraffin, and cut into 4 μm sections. The sections were stained with hematoxylin and eosin (H-E), and Azan. The number of capillaries was counted in the H-E stained sections, and the collagen area in the granulation tissue was measured in Azan-stained sections. The number of capillaries was counted in three parts of the granulation tissue (upper, center, and lower) (Fig. 1). The area of collagen synthesis was derived from the area stained blue by the Azan stain.

VEGF Immunohistochemistry  Cryosections and deparaffinized sections of formalin-fixed tissue were used. Endogenous peroxidase activity was blocked by incubating the sections in methanol containing 0.3% hydrogen peroxide for 20 min at room temperature. After washing with phosphate buffered saline (PBS), the sections were treated with normal swine serum (DAKO, Glostrup, Denmark) for 30 min at room temperature to block nonspecific reactions. The sections were incubated in rabbit anti-human VEGF (V3) antibody (1:100, IBL, Fujioka, Japan) overnight at 4 °C. After washing with PBS, they were incubated with biotinylated swine anti-rabbit IgG antibody (DAKO, Glostrup, Denmark) overnight at 4 °C. After washing with PBS, the sections were incubated with avidin–biotin peroxidase complex (Vector Laboratories, Peterborough, U.K.) for 30 min at room temperature, followed by working strength TdT enzyme was applied for 1 h at 37 °C, working strength stop/wash buffer for 15 s and washing with PBS, they were incubated with biotinylated swine anti-rabbit IgG antibody (DAKO, Glostrup, Denmark) overnight at 4 °C. After washing with PBS, the sections were incubated in rabbit anti-human VEGF (V3) antibody (1:100, IBL, Fujioka, Japan) overnight at 4 °C. After washing with PBS, they were incubated with biotinylated swine anti-rabbit IgG antibody (DAKO, Glostrup, Denmark) overnight at 4 °C. After washing with PBS, the sections were incubated with avidin–biotin peroxidase complex (Vector Laboratories, Peterborough, U.K.) for 30 min at room temperature. Immunoreactions were visualized by treating the sections with 0.25 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.05 M Tris-buffered saline (pH 7.4) in the presence of 0.003% hydrogen peroxide for 3 to 5 min. Nuclei were counterstained with hematoxylin.

Detection of Apoptotic Cells with *in Situ* End Labeling of Fragmented DNA  Cryosections and deparaffinized sections of formalin-fixed tissue were used. Sections were digested with proteinase K (20 μg/ml) for 15 min at room temperature and then washed with dH2O. Apoptotic cells were determined with an *in situ* apoptosis detection kit, ApopTag™ (Oncor, Gaithersburg, Germany), which is based on the TUNEL assay. The sections were incubated in PBS containing 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. After washing with PBS, equilibration buffer was applied and the sections were incubated for 1 min at room temperature. The excess liquid was tapped off and working strength TdT enzyme was applied for 1 h at 37 °C, followed by working strength stop/wash buffer for 15 s and incubation for 10 min at room temperature. The sections were washed with PBS and anti-digoxigenin peroxidase conjugate was applied for 30 min at room temperature. Finally, the color was developed in DAB solution (using the same method as for VEGF immunohistochemistry) for 1 min. Nuclei were counterstained with hematoxylin. The apoptotic cell type was determined by H-E staining of serial sections and immunohistochemistry.

Statistical Analysis  Data on wound closure, capillary number, and collagen in the granulation area were analyzed with Stat View® 5.0 (SAS Institute Inc., Cary, N.C.) on a Power Macintosh G3 and expressed as the mean ± S.D. or S.E.M. Significant differences were evaluated according to the Bonferroni–Dunn method, Fisher’s PLSD method, and Student’s *t*-test.

RESULTS

Effect of SK Concentration on Wound Healing  SK (0.2% or 2%) or vehicle was applied to 6-mm wounds created on the backs of db/db mice. We measured wound area over a period of two weeks to determine whether the concentration of SK affects the progress of wound healing. At two weeks, the decrease in wound area and wound closure had been accelerated by 0.2% SK (Table 1). In contrast, treatment with 2% SK delayed wound closure relative to treatment with vehicle. Complete wound healing also occurred earlier in 0.2% SK-treated wounds than in controls. There was a greater number of capillary vessels in 0.2% SK-treated than in 2% SK-treated wounds, and the capillaries were distributed at the center of a wound rather than at the edge of a wound in 0.2% SK-treated mice (Table 1). These results clearly indicate that the effects of SK are not dose-dependent, but rather that 0.2% is the optimum concentration.

Optimum Concentration of SK Accelerates Wound Healing  SK (0.2% or 2%) or vehicle was applied to 6-mm wounds created on the backs of db/db mice. We measured wound area over a period of two weeks to determine whether the concentration of SK affects the progress of wound healing. At two weeks, the decrease in wound area and wound closure had been accelerated by 0.2% SK (Table 1). In contrast, treatment with 2% SK delayed wound closure relative to treatment with vehicle. Complete wound healing also occurred earlier in 0.2% SK-treated wounds than in controls. There was a greater number of capillary vessels in 0.2% SK-treated than in 2% SK-treated wounds, and the capillaries were distributed at the center of a wound rather than at the edge of a wound in 0.2% SK-treated mice (Table 1). These results clearly indicate that the effects of SK are not dose-dependent, but rather that 0.2% is the optimum concentration.

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Closure and Granulation Tissue Formation, and Leads to the Infiltration of VEGF-Positive Cells

Little bleeding occurred when we created wounds on the backs of db/db mice. In the first week, wound fluid was secreted and granulation tissue formed from the edge of the wound (Fig. 2). Incrustation occurred in wounds treated with 0.2% SK, and the crust covered the entire wound area. In contrast, the crust covered only part of the wound in controls. At the second week, granulation tissue had formed in the 0.2% SK-treated wounds, whereas in the controls, the crust covered the entire wound area and the granulation tissue was seen only in part of the wound edge. At the third week, the 0.2% SK-treated wounds had closed, but wound closure had not yet finished in the controls, where extensive granulation tissue was forming only at this time. Figure 3 shows that the reduction of the wound area occurred earlier in 0.2% SK-treated mice than in the controls.

Next, we evaluated the process of wound healing histologically. At the second week, the amount of granulation tissue had increased remarkably in 0.2% SK-treated wounds. Figure 4 shows the neovascularization of the granulation tissue. The granulation tissue at the center of the wound in 0.2% SK-treated mice was filled with fibroblasts, endothelial cells, and collagen. New capillary vessels had also increased (Figs. 4B, F). At the third week, epithelialization was not complete in the controls (Fig. 4C), but was complete in 0.2% SK-treated wounds. Cell numbers had also decreased in the SK-treated granulation tissue (Fig. 4D). We counted the capillary vessels in H-E-stained histological sections (Fig. 5). It was clear that the number of capillary vessels was increased in the center of the SK-treated wounds (Fig. 5B, Table 1) compared to the center of control wounds at the same time (Fig. 5A, Table 1). However, there was no difference in the number of capillary vessels between control and 0.2% SK-treated wounds at the edge of the wound (Figs. 5A, B Upper and Lower, Table 1). At the third week, the number of capillaries had decreased in 0.2% SK-treated wounds (Fig. 5D), while the number of capillaries in the controls increased slightly (Fig. 5C) beginning March 2003

### Table 1. Effect of SK at Two Weeks after Preparation of Wound

<table>
<thead>
<tr>
<th>Treated sample</th>
<th>n</th>
<th>Wound area (mm²)</th>
<th>Wound closure (%)</th>
<th>Capillary number</th>
<th>Area of collagen synthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Upper</td>
<td>Center</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>27.6±3.9</td>
<td>2.3±2.6</td>
<td>16.5±10.2</td>
<td>12.2±11.5</td>
</tr>
<tr>
<td>0.2% SK</td>
<td>6</td>
<td>21.0±2.4*</td>
<td>25.7±3.4*</td>
<td>19.9±5.8</td>
<td>39.6±12.1*</td>
</tr>
<tr>
<td>2% SK</td>
<td>6</td>
<td>31.7±5.5</td>
<td>−12.2±2.1*</td>
<td>24.0±13.7</td>
<td>8.0±8.5</td>
</tr>
</tbody>
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* p<0.05 versus control.

Wound area was larger than the size of the original wound when observation standard.

![Fig. 2. Time Course of Wound Healing in Control and 0.2% SK-Treated Mice](image)

Upper panels: control; lower panels: 0.2% SK-treated. Wound closure appeared earlier in the 0.2% SK-treated mice than in the controls.

![Fig. 3. Time Course of Wound Area Following 0.2% SK Treatment](image)

The reduction of the wound area was earlier in 0.2% SK-treated (○—○) than controls (□—□). The wound closed completely by the third week in 0.2% SK-treated mice. The difference between controls and 0.2% SK-treated mice from the first week to third week was significant by Fisher’s PLSD post hoc test. A p<0.05 was considered significant. Data are presented as the mean±S.E.M. (n=6).
in the second week. Collagen synthesis surrounded the capillary vessels in the granulation tissue. The synthesis of collagen was higher in SK-treated wounds than in controls (Fig. 6). However, the cell density decreased at the third week in 0.2% SK-treated mice (Figs. 4C, D). We investigated whether the decrease in cell density could have been caused by the induction of apoptosis by detecting apoptotic cells at the center of the wound. At the second week, macrophages

Fig. 4. Histology of Central Part of the Wound (H-E Stain)

At the second week, granulation tissue had increased in 0.2% SK-treated wounds (B), but not in controls (A). The granulation tissue has many new capillary vessels (arrow) in the 0.2% SK-treated wounds (F), and few vessels in the controls (E). At the third week, the wound was covered with epithelium and the cellularity tended to decrease in the granulation tissue in 0.2% SK-treated mice (D). A few inflammatory cells around the area that is not covered with epithelium can be seen in the controls (C). Gt: granulation tissue, Ep: epithelium, Fas: fascia (D) bar=50 μm, (F) bar=30 μm.

Fig. 5. The Number of Capillary Vessels in the Wound was Counted in H-E-Stained Sections

At the second week, the number of capillary vessels increased in the center of the 0.2% SK-treated wound (B) relative to controls (A). At the third week, the number of capillary vessels decreased in 0.2% SK-treated wounds (D), but increased in controls (C). Data are presented as the mean±S.D. (n=9). *p<0.001 versus control.

Fig. 6. The Effect of SK on Collagen Synthesis

A greater percentage of area has collagen in 0.2% SK-treated wounds (B) than in control wounds (C). These bars show the ratio of collagen area to granulation tissue measured. The amount of collagen is derived from the area of blue color on an Azan-stained section. Data are represented as the mean±S.D. (n=6). *p<0.01 versus control.
and lymphocytes were detected as apoptotic cells in the controls (Fig. 7A), whereas fibroblasts or myofibroblasts, macrophages, and endothelial cells were detected in 0.2% SK-treated mice (Fig. 7B). At the third week, fibroblasts and endothelial cells were detected as apoptotic cells only in controls (Fig. 7C) and not in 0.2% SK-treated wounds (Fig. 7D). Lymphocytes were defined as having uninuclear and round shapes, while macrophages had protean shapes and were F4/80 positive. Spindle-shaped cells that were α-smooth muscle actin positive were defined as myofibroblasts, while other spindle-shaped cells were regarded as fibroblasts (data not shown).

In the center of the wound area, VEGF was mainly expressed in giant cells in the controls (Fig. 8A), but was also slightly expressed in infiltrated neutrophils. VEGF was distributed in the granulation tissue in 0.2% SK-treated wounds at the second week (Fig. 8B). At the third week, however, there was little strong expression of VEGF in the controls, and none in 0.2% SK-treated wounds (Figs. 7C, D).

DISCUSSION

The results of the present study indicate that SK is capable of accelerating wound healing in db/db mice. db/db Mice are characterized by a reduction in growth factors,14,15) so that wound healing is delayed compared to normal mice. Hayashi8) reported that an ether extract of “Shikon” decreases vascular permeability, forms granulation tissue, and accelerates wound healing in rats when used at an optimum concentration of 0.1—0.2%. Higher doses than this concentration will overstimulate the wound, while lower doses will not have an adequate pharmacological action. Although normal animals have been used in previous studies on the effects of “Shikon” on wounds, in the present study, db/db mice were used. Because previous reports used different experimental conditions, we attempted to confirm the optimum concentration in these mice. Previous studies reported that the ether extract of “Shikon” is hard to dissolve in solvents,6) therefore, in this study, we used a high polarity solvent, isopropyl lauroyl sarcosinate, for the sample base. First, we examined wound closure using two concentrations of SK, 0.2%
and 2%, for 2 weeks. As was found in previous studies, wound closure was accelerated at the lower concentration but not the higher.\textsuperscript{19} In the remaining experiments, we examined complete healing and granulation tissue formation using 0.2% SK, which resulted in wound closure at 3 weeks after SK application. These results for wound closure were compared with past studies of bFGF, an ulcer remedy, that was administered to \textit{db/db} mice. Although the wounds were closed by bFGF but not SK in the second week, the results in the third week were virtually identical (at second week; bFGF 25%, SK 81%, at third week; bFGF <0.001%, SK 0%). The approximate ratio of wound area to control).\textsuperscript{21} To study wound repair, wound healing is often subdivided into three phases: an inflammatory phase, a proliferative phase, and a maturation phase.\textsuperscript{22,23} We tried to determine if SK shortened these phases. In the beginning of the histological analysis, we found that the granulation tissue was increased in SK-treated wounds. Therefore, we focused on the center of the wound. We found that capillary vessels were distributed among most of the granulation tissue, but increased at the center of the wound, especially at the second week. At the third week, the percentage of collagen increased more gradually. These changes indicate a shift in wound healing from the proliferative phase to the maturation phase. In an open wound, all three phases co-exist together, with the center area of the wound the last to be repaired. SK also accelerates scar formation.

The decrease in capillary number is related to the elimination of cells through apoptosis, a function that is necessary for the process of wound healing. Apoptosis is related to the elimination of inflammatory cells\textsuperscript{24} in the early phase, and to the elimination of both fibroblasts and endothelial cells during the transition between granulation tissue and scar.\textsuperscript{19} We examined the pattern of apoptotic cells in the wound bed during the proliferative and maturation phases, and found at the second week, in addition to macrophages, that apoptotic fibroblasts were already seen in the granulation tissue in SK-treated wounds, while macrophages and lymphocytes distributed in the wound bed were still the majority of apoptotic cells in control wounds, indicating that SK advances wound healing. The distribution of apoptotic cells changed as epithelialization advanced. Apoptotic cells tended to increase in the areas where epithelialization was advanced and the proliferative phase had shifted to the maturation phase. Brown, \textit{et al.}\textsuperscript{17} have shown that apoptosis during wound healing is delayed in diabetic mice relative to nondiabetic mice. Our results indicate that SK accelerates the onset of apoptosis in fibroblasts and endothelial cells, and suggesting a shortening of the inflammation phase and an earlier start of the maturation phase.

The wound was kept moist by covering it with a film dressing, which also kept the various growth factors in the wound fluid close to the wound. However, oversecretion of wound fluid is sometimes deleterious for smooth epithelialization. We therefore absorbed excess secreted fluid with Scampor tape or paper discs. Consequently, there was a suitable quantity of moisture and growth factors, including VEGF, on the wound. VEGF is produced by neutrophils and macrophages,\textsuperscript{20,25} and has an effect on the proliferation of endothelial cells and plays a role in angiogenesis.\textsuperscript{26,27} The fact that we found VEGF was produced not only by neutrophils but also by macrophages and endothelial cells at the second week (Fig. 7B) suggests that the distribution and variety of VEGF-positive cells contributes to the phase of wound healing.

VEGF from macrophages was reduced in \textit{db/db} mice.\textsuperscript{28} VEGF-positive neutrophils increase in the necrotic tissue around the pouch wall one day after injection of SK into the pouch of ICR mice.\textsuperscript{29} SK on the wound, therefore, would appear to accelerate the infiltration of neutrophils during the inflammation phase into the healing wounds of \textit{db/db} mice. Cytokines or chemokines would be produced by cells stimulated by SK, and then neutrophils would migrate to the wound. Neutrophils are responsible for cleaning the wound by phagocytosis, and would also produce VEGF concurrently. The neutrophils would undergo apoptosis after they clean the wound. Next, macrophages would infiltrate the wound to phagocytose the apoptotic neutrophils, and produce growth factors such as VEGF. VEGF from these cells may enhance the growing of granulation tissue and also neutrophils infiltrated there secrete it. Finally, in the area of increased granulation, fibroblasts and endothelial cells gradually start to undergo apoptosis, and the granulation tissue is filled with collagen. When the wound is epithelialized completely, the cellular component decreases and VEGF secretion is suppressed. Thus, SK accelerates wound healing, and it also has antibacterial activity. These facts suggest that SK is useful for wound healing in \textit{db/db} mice, and could potentially help patients with intractable bedsores and other chronic ulcers.

REFERENCES


