An extract of the root of *Lithospermun erythrorhison* accelerates wound healing

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SUMMARY

Metabolic disease such as diabetes, which is caused by stress or imbalanced diet, has been increasing. A diabetic tend to suffer from a delay or difficulty of wound healing. The extract of SHIKON (SK), that is the root of *Lithospermun erythrorhison*, has been reported to have an effect on healing for normal wound, but has never studies for intractable wound so far. Therefore we examined the effect of SK extract on wound healing with healing impaired mouse model.

Full-thickness round wounds were created on the backs of *db/db* mice and applied SK, and we observed neovascularization and collagen synthesis, distribution of apoptotic cells, and vascular endothelial growth factor (VEGF)- positive cells in granulation tissue.

After two weeks, a number of capillary vessel and collagen synthesis were increased in SK-treated wounds. Infiltration of VEGF-positive neutrophils was also seen in the wound, besides apoptotic fibroblasts and endothelial cells were appeared in the granulation tissue. After three weeks, the wound closed completely with SK-treated but not in control.

These results suggest that SK enhanced neovascularization by VEGF and this kind of apoptosis process makes the scar smooth. In this study, it is obvious that SK also accelerates healing of intractable wound.

INTRODUCTION

Over the past few years, the scientific studies of traditional medicine have been carried out increasingly on the ground of great interest in possibility of the natural-oriented substances. As for the cosmetics, a lot of botanical ingredients have been investigate of its efficacy and application. "SHIKON" (SK), the root of a *Lithospermun erythrorhison* SIEB. *et* Zucc., is the main component of Japanese traditional ointment called "SHIUNKO", which is well known as a remedy for skin disease, burns and incised wound. Some experimental and clinical studies indicated that SK also has anti-inflammatory [1-3], anti-bacterial [4] and accelerate granulation tissue formation [5]. We have been devoted to the study of SK, which we have been used as a cosmetic active ingredient for a long time, and found that SK was effective for wound healing [6]. Recently, metabolic disease such as diabetes, which is caused by stress or imbalanced diet has been increasing. A diabetic tends to suffer from a delay or difficulty of wound healing. Although studies have been carried out for normal wound, we do not know SK would accelerate intractable wound. Therefore we examined the effect of SK extract on wound healing with healing impaired diabetic (*db/db*) mouse, which is a good model for the investigation of intractable wound.

In the process of wound healing, it is said that there are three phases such as the

inflammatory phase, the proliferative phase, and the maturation phase [7, 8]. Especially, granulation tissue formation during the proliferative phase is important for reconstruction of tissue in the wound healing process. The granulation tissue is increased during proliferation phase and then decreased with the progression of epithelialization on the wound bed by apoptosis of its own cellularity [9]. The SK extract enhances granulation tissue formation through mechanisms such as VEGF secretion by infiltrated cells [10].

In this study, we focus on granulation tissue formation and evaluate the effect of extract of SK by means of several indexes such as VEGF secretion, apoptosis, collagen synthesis, and neovascularization with *db/db* mice.

MATERIALS AND METHODS

Animals

Female 8-week-old diabetic mice, C57BL/ksJ *db/db* Jcl (Clea Japan, Inc), were used in this study. The mice were maintained in individual cages during the experiment.

Preparation of Samples

SK extract was obtained from the root of *Lithospermun erythrorhison* SIEB. *et* Zucc.,

(Koshiro Company Ltd., Osaka, Japan). Cutting SK was reflux with diethyl ether for three hours and the solvent was removed. The extracted SK was diluted with isopropyl lauroyl sarcosinate (Eldew® SL-205 Ajinomoto Co., Inc Tokyo Japan).

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 mice with skin biopsy punch (6 mm diameter; Acu Punch®, Acuderm Inc., USA). After the operation, 10 μl of vehicle or test solution (0.2% or 2% SK) were soaked into filter paper discs.

The filter paper was covered with the Finn Chamber® (Epitest Ltd Oy, Tuusula, Finland) and Scanpor® tape (Alpharma AS Norway), and the tape was covered with film dressing (Bioclusive®, Johnson and Johnson, USA). Every week, we reapplied the samples and changed the film dressing.

Evaluation of Wound Closure

The wound edges were traced onto polyethylene sheets and scanned it into a computer. The wound area was measured using an image analyzing soft (Mac SCOPE, Mitani Co. Japan).

Histology

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

VEGF Immunohistochemistry

Cryosections and deparaffinized sections of formalin-fixed tissue were used. Endogenous peroxidase activity was blocked by means of 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 non-specific 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) for 30 min at room temperature, followed by incubation with an 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'-diaminobenzine 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. The variety of apoptotic cells was distinguished with H-E stain of serial section and with immunohistochemistry.

Detection of Apoptotic Cells with *in situ* End Labeling of Fragmented DNA

Cryosections and deparaffinized sections of formalin-fixed tissue were used. Apoptotic cells were determined with an *in situ* apoptosis detection kit, Apop Tag® (Oncor, Gaithersburg, Germany), which is based on the TUNEL assay. Sections were digested with proteinase K (20 μg/ml) for 15 min at room temperature and then washed with dH_2O. 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 sec 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 for 1 min with using the same method as for VEGF immunohistochemistry. Nuclei were counterstained with hematoxylin.

Statistical Analysis

For the statistical evaluation, Stat View® 5.0 (SAS Institute Inc., Cary, N.C.) on a Power Macintosh G3 was used for analysis of the data on wound closure, capillary number, and collagen in the granulation area to express as the mean ± SD or SEM. 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 three weeks to observe that SK has dose dependence on its efficiency for the progress of wound healing. After two weeks, the decrease in wound area and wound closure had been accelerated by 0.2% SK (Table I). 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 control. 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 wound rather than at the edge of wound in 0.2% SK-treated mice (Table I).

Optimum Concentration of SK Accelerates Wound Closure and Granulation Tissue Formation, and Leads to the Infiltration of VEGF-positive Cells

Since 0.2%SK accelerated wound healing, we observed the healing process visually. After a week with treatment of 0.2%SK, wound fluid was secreted and granulation tissue formed from the edge of the wound. Incrustation occurred in wounds treated with 0.2% SK, and the crust covered the entire wound area. In contrast, the crust covered only a part of the

wound in controls. After two weeks, 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 a part of the wound edge. After three weeks, the 0.2% SK-treated wounds had closed, but wound closure had not yet finished in the controls, where extensive granulation tissue was began to form at this time. Fig. 2 shows that the reduction of the wound area occurred earlier in 0.2% SK-treated mice than in controls.

We also evaluated the process of wound healing histologically. After two weeks, the amount of granulation tissue had remarkably increased in 0.2% SK-treated wounds. Fig. 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. 3-B, F). After three weeks, epithelialization was not complete in the control (Fig. 3-C), but was complete in 0.2% SK-treated wounds. Cell numbers had also decreased in the SK-treated granulation tissue (Fig. 3-D). We counted the capillary vessels in H-E–stained histological sections (Fig. 4). It was clear that the number of capillary vessels was increased in the center of the SK-treated wounds (Fig. 4-B, Table I) compared to the center of control wounds at the same time (Fig.4-A, Table I). 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. 4–A, B Upper and Lower, Table I). After three weeks, the number of capillaries had decreased in 0.2% SK-treated wounds (Fig. 4-D) but the number of capillaries in the controls tended to increase (Fig. 4-C) from the second week. Collagen synthesis surrounded the capillary vessels in the granulation tissue significantly increased with SK treatment compare to control (Fig.5). However, the cellularity was decreased after three weeks in 0.2% SK-treated mice (Figs. 3-C, D).

As capillaries and cells were decreased with progress of epithelializtion, we consider that these cells would be involved in apoptosis. Therefore we investigated cell density to detect the induction of apoptosis. After two weeks, a few macrophages and lymphocytes were detected as apoptotic cells in the controls (Fig. 6-A), whereas a lot of fibroblasts, myofibroblasts, macrophages and endothelial cells were detected in 0.2% SK-treated mice (Fig. 6-B). Apoptotic cells of fibroblasts and endothelial cells had been increasing after three weeks in control (Fig. 6-C), on the other hand apoptotic cells had decreased in 0.2% SK-treated wounds (Fig. 6-D).

Our results show that SK accelerates the onset of apoptosis in fibroblasts and endothelial cells, and it suggests shortening the inflammation phase and starting the

maturation phase earlier. Next, we investigated the distribution of VEGF, which is one of the cytokine related to granulation tissue formation. In the center of the wound area, VEGF was mainly expressed in giant cells in the controls (Fig. 7-A), but was also slightly expressed in infiltrated neutrophils. VEGF was distributed in the granulation tissue in 0.2% SK-treated wounds after two weeks (Fig.7-B). After three weeks, however, there was little strong expression of VEGF in the controls, and none in 0.2% SK-treated wounds (Figs. 7-C, D).

DISCUSSION

This study showed that SK accelerates wound healing in *db/db* mouse. Diabetic mice have suppressed granulation tissue formation and allograft rejection [10], and reduced induction of keratinocyte growth factor (KGF) and vascular endothelial growth factor (VEGF)[11, 12]. Mutant diabetic mice (*db/db*) show a delay of healing response in the dermis relative to normal mice (*db/+*). It is reported that it takes 11 days for non-diabetic mice to reach 80% of wound healing, while *db/db* mice take 18 days [13]. At first, we studied the dose dependence of SK to heal the intractable wound with using these mice. It was clarified that the SK does not work on dose dependent, but has an optimum

concentration. This result suggest that higher doses of SK extract will overstimulate the wound, while in lower doses it is possible to say that SK will not sufficient for pharmacological function.

We observed the time course of wound healing on optimum concentration, epithelialization was completed in SK-treated, whereas not finished in control after three weeks. Then, we investigated about the function of accelerating wound healing histologically. The granulation tissue formation was occurred in early phase in SK-treated than control. Especially, a lot of capillary vessels and collagen was recognized in the center of wound after two weeks. (Figs. 4-B, 5, Table I) . However, after three weeks, fibroblasts and capillary vessels in granulation tissue were decreased. (Fig. 4-D, Table I). These changes indicate that the process of wound healing progress from the proliferative phase to the maturation phase. That means SK treatment make the wound to move maturation phase earlier than control that is still decreasing capillary vessels in the third week. SK also accelerates scar formation. Decreasing cells in granulation tissue would be involved elimination of these cells by apoptosis. In this study, it is observed that the apoptotic fibroblasts and endothelial cells appear in the center of SK-treated wound earlier than vehicle-treated.

Ordinary, appearance of apoptotic cells during wound healing is well known. The distribution of apoptotic cells in open wound are initially observed in the wound edge mainly but as the wound is getting better, distribution gradually appear to the center area of wound. On the other hand, in *db/db* mice, it was reported that the appearance of the apoptotic pattern is significantly delayed[14] and inflammatory cells are decreased . Considering these facts, SK would accelerate to shift the stage of healing from proliferation phase to maturation phase in *db/db* mice.

We kept the wound wet by covering it with a film dressing. This made the wound keep warm and clean, and it's also helpful for the wound to keep the wound fluid containing growth factor. However, over-secretion of wound fluid is sometimes impediment for smooth epithelialization. We therefore absorbed the excess amounts of secreted fluid with Scampor® tape or paper discs. Consequently, there was a suitable quantity of moisture and growth factors, such as VEGF, on the wound. VEGF is produced from neutrophils and macrophages [6, 15], and has an effect on the proliferation of endothelial cells and involved angiogenesis [16, 17]. The result of observation about distribution of VEGF, in early phase, VEGF-positive neutrophils were infiltrated into granulation tissue in SK-treated wound (Fig.7-B). On the contrary, in control, VEGF is mainly expressed in

macrophages, and the wound indicates the aspect of the inflammatory phase (Fig.7-A). After three weeks, however, there was none expression in SK-treated wounds (Figs. 7-D). These results suggest that the change of appearance of the VEGF positive cells was reflected to the phase of wound healing, and SK shortened inflammatory phase and shifted to proliferation phase earlier than control.

These results lead us to the conclusion that SK makes the cells surrounded the wound stimulate and consequently accelerate the production of cytokines or chemokines, and then neutrophils would respond to the signal and migrate to the wound. Neutrophils have a possibility to stimulus the production of VEGF due to the phagocytosis for the scrap of wound. The neutrophils would undergo apoptosis after they clean the wound, then macrophages would infiltrate the wound to phagocytose the apoptotic neutrophils, and that makes macrophages produce more amount of VEGF. VEGF produced from these cells may enhance growing of granulation tissue, and also neutrophils that infiltrated into the granulation tissue secrete VEGF and would accelerate granulation tissue formation further. 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, we consider the mechanism of SK-accelerated wound healing. As SK also has antibacterial activity, it has a great expectation that SK is useful and practical for both normal and intractable wound healing.

CONCLUSION

In this study we found that SK shortened the inflammatory phase, and advanced the proliferative and maturation phases. It has been known that SK also has anti-bacterial activity. Therefore, we conclude that SK is useful for wound healing, and could potentially help people with intractable wounds and other chronic ulcers.

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Table I Effect of SK at Two Weeks after Preparation of Wound

treated sample	n	wound area (mm^2)	wound closure(%)	capillary number			area of collagen synthesis(%)
				upper ?	center ?	lower	
control	6	27.6? 3.9	2.3? 2.6	16.5? 10.2	12.2? 11.5	23.6? 5.1	24.6? 2.1
0.2% SK	6	21.0? 2.4*	25.7? 3.4*	19.9? 5.8	39.6? 12.1*	21.2? 5.7	47.2? 9.9*
2% SK	6	31.7? 5.5	**-12.2? 2.1	24.0? 13.7	8.0? 8.5	26.3? 8.6	13.5? 4.7

* p <0.05 *versus* control
** Wound area was larger than the size of wound that prepaird when observation started.

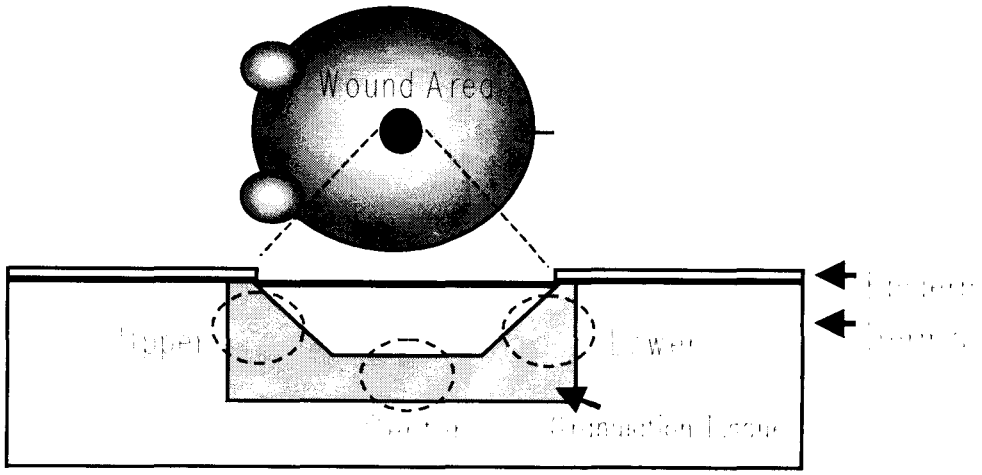

Figure 1. Evaluation of Wound Healing.

Full-thickness wounds were prepared on the shaved backs of mice. The wound area was measured by tracing the edges of the wound over the film dressing. The capillary number was counted in three sections of granulation tissue. Upper: the anterior wound edge; Center: middle of wound; Lower: the posterior wound edge.

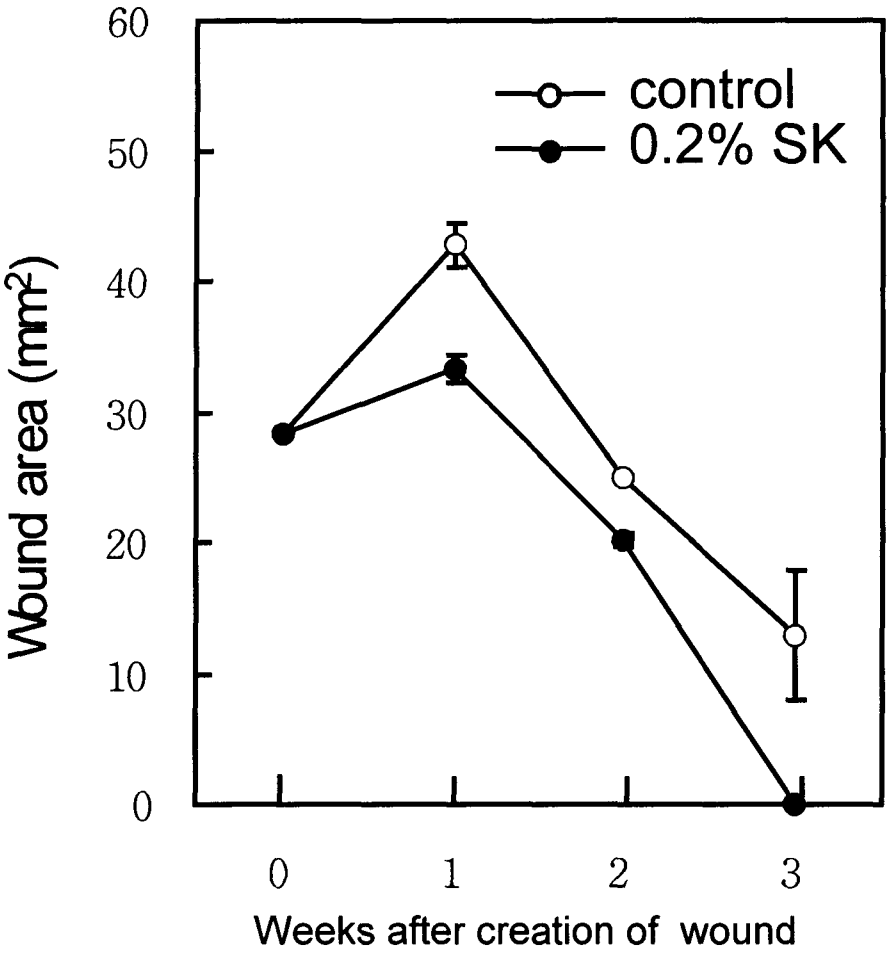

Figure 2. Time Course of Wound Area Following 0.2% SK Treatment.

The reduction of the wound area was earlier in 0.2% SK-treated (-●-) than control (-○-) . The wound closed completely by the third week in 0.2%SK-treated mice. The difference between control and 0.2%SK-treated from first week to third week was significant by Fisher's PLSD post hoc test. $p<0.05$ was evaluates to be significant. Data are represented as the mean ± the SEM (n=6).

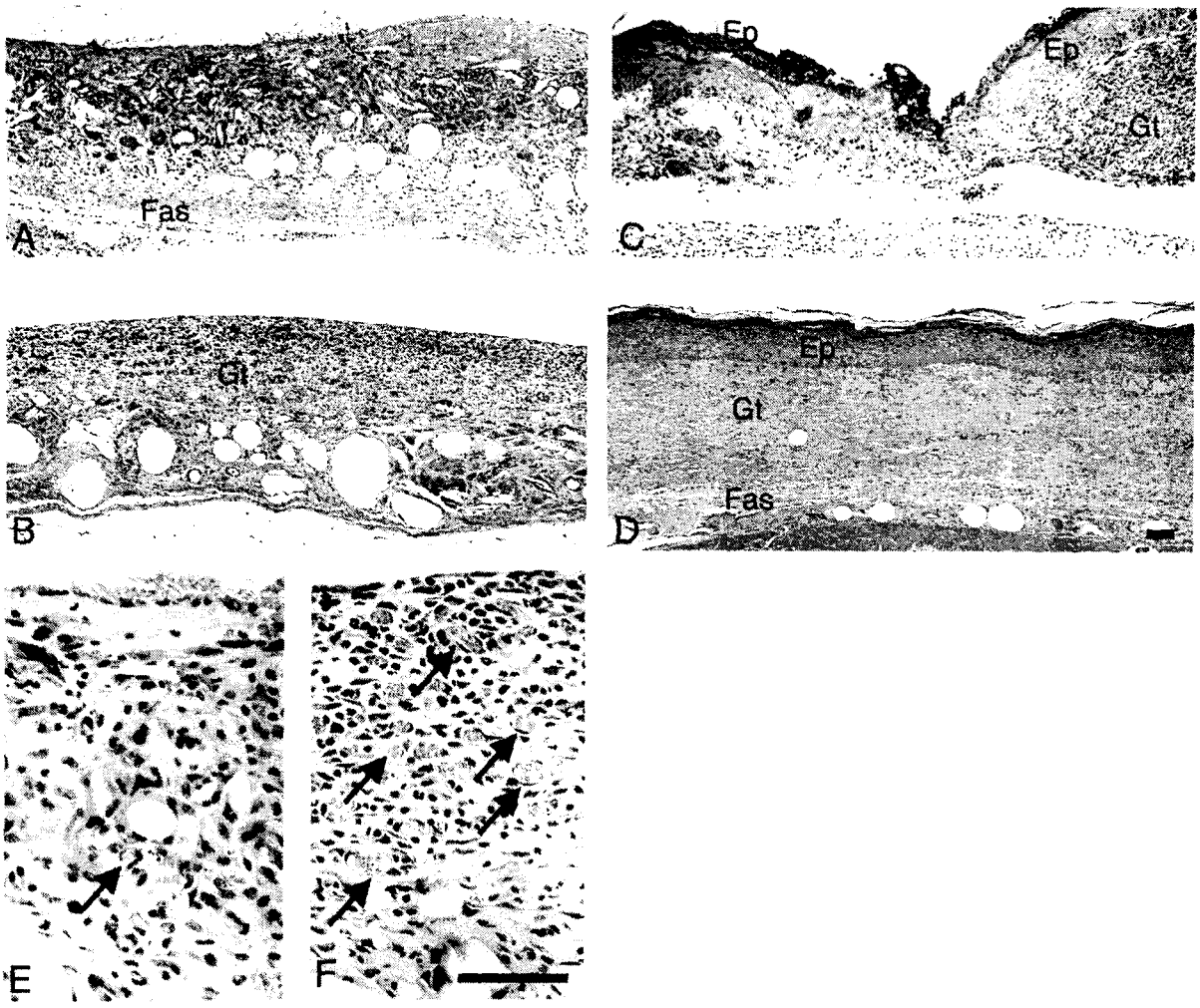

Figure 3. Histology of a Central Part of the Wound (H-E Stain).

At the second week, granulation tissue has increased in 0.2% SK-treated wounds (B), but not in control (A). The granulation tissue has many new capillary vessels (arrow) in the 0.2% SK-treated wounds (F), and few vessels in the control (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). There are a few inflammatory cells around the area that is not covered with epithelium in the control (C). Gt: granulation tissue, Ep: epithelium, Fas: fascia (D) Bar=50 μm, (F) Bar=30 μm.

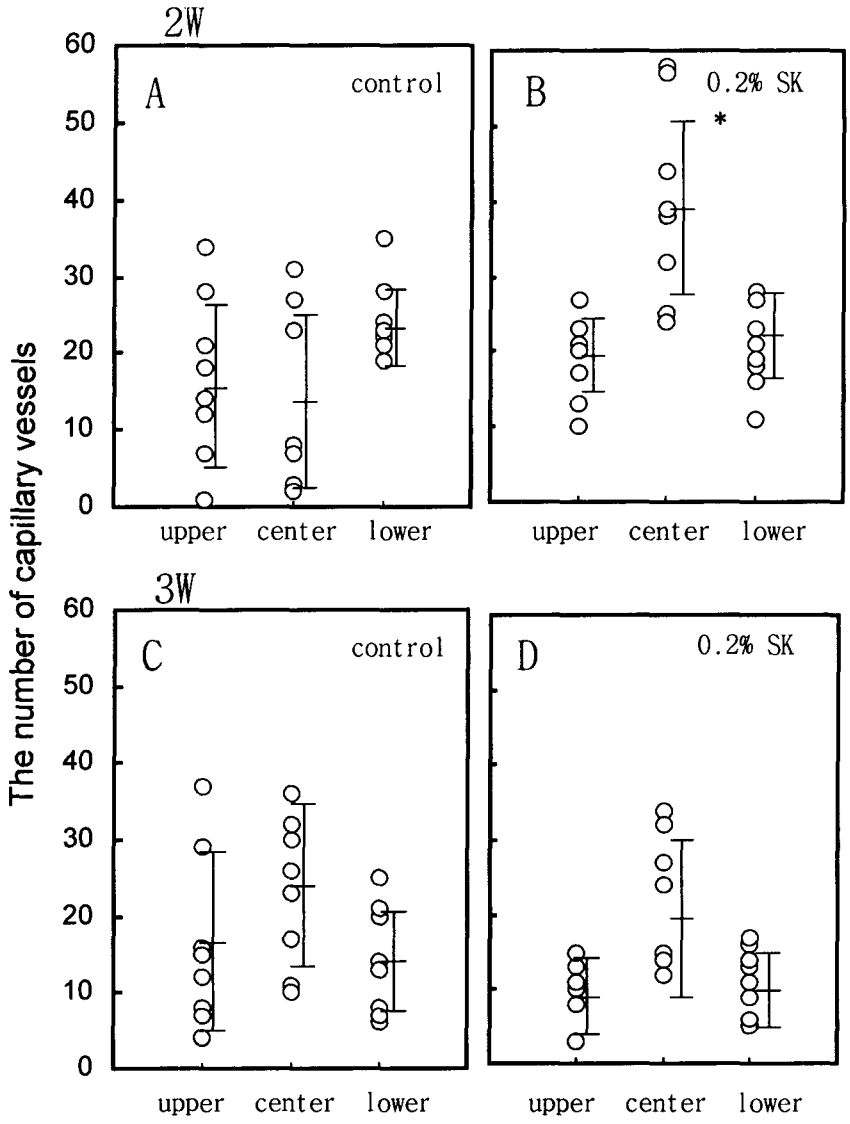

Figure 4. The Number of Capillary Vessels in the Wound was Counted in H-E–Stained Sections.

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 represented as the mean ± S.D. (n=9). *p<0.001 *versus* control.

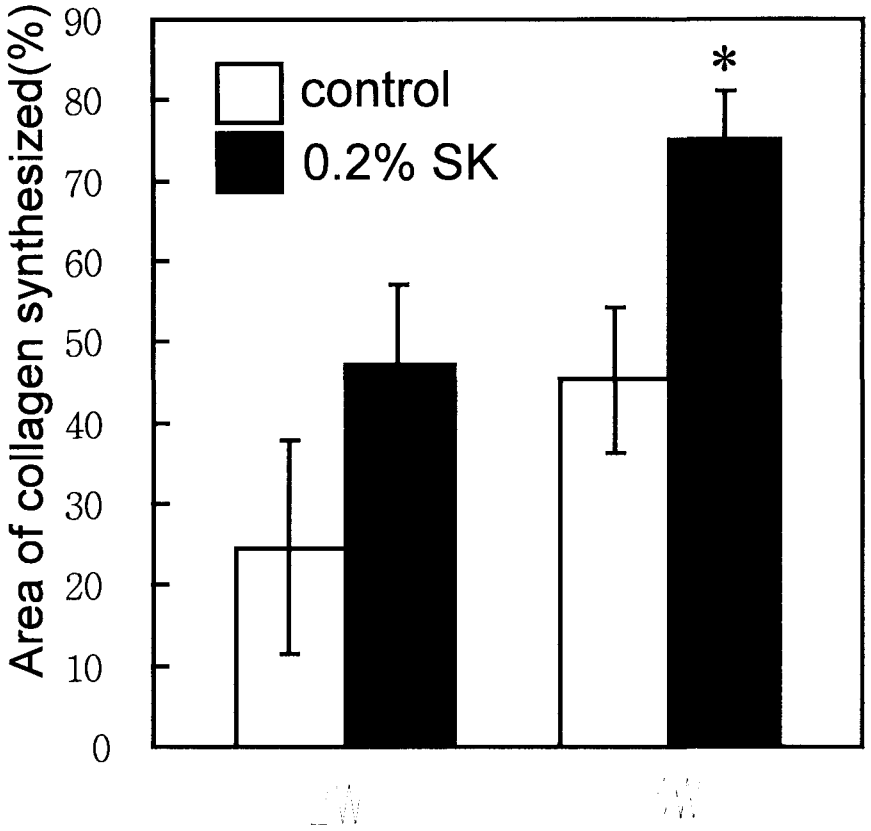

Figure 5. The Effect of SK on Collagen Synthesis.

A greater percentage of area has collagen in 0.2% SK-treated wounds (▨) than in control wounds (□). 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.

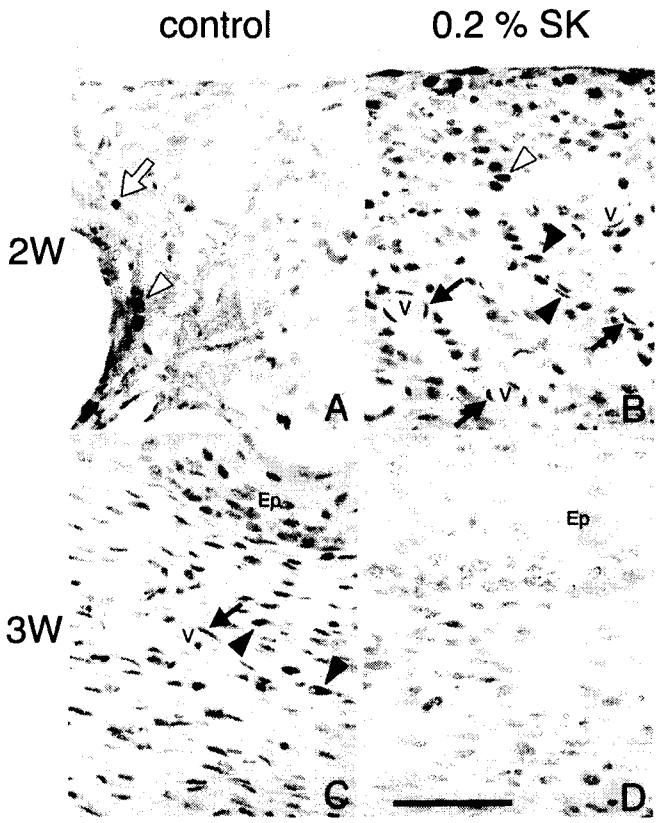

Figure 6. Distribution of Apoptotic Cells in the Central Part of the Wound.

The distribution and variety of apoptotic cells changed as healing progressed. In the control, apoptotic lymphocytes (open arrow) and macrophages (open arrowhead) can be seen inside the wound at the second week (A). At the third week, the majority of apoptotic cells were fibroblasts (arrowhead) and endothelial cells (arrow). In 0.2% SK-treated wounds, the majority of apoptotic cells were fibroblasts and endothelial cells at the second week (B), and very few apoptotic cells were seen at the third week (D). V: vessel, Ep: epithelium. (D) Bar=50 μm.

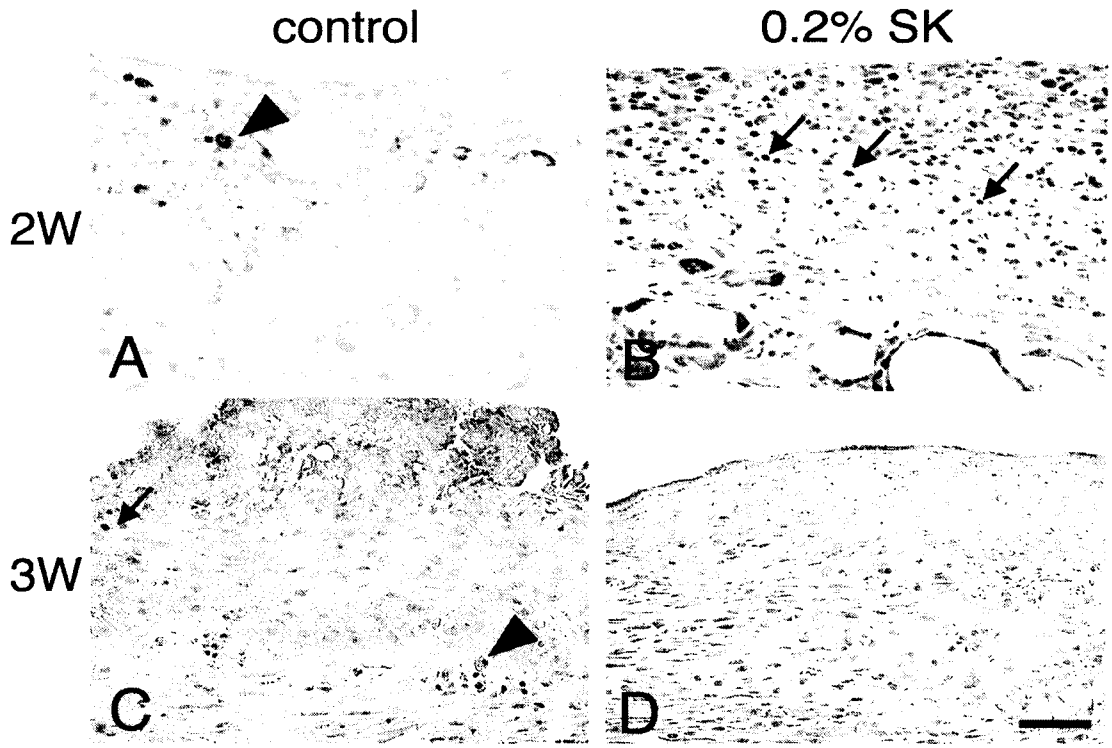

Figure 7. Immunohistochemistry for VEGF in Infiltrated Cells in a Central Part of the Wound.

The distribution and variety of VEGF-positive cells changed as healing progressed. At the second week, VEGF-positive macrophages (arrowhead) are seen inside of the wound (A), and many VEGF-positive neutrophils (arrow) have infiltrated the granulation tissue in 0.2% SK-treated wounds (B). At the third week, VEGF-positive macrophages and neutrophils are seen around the crust in the control (C), and there are few VEGF-positive cells in the 0.2% SK group (D). Bar=50 μm.