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Peptide-Modified Chitosan Hydrogels Accelerate Skin Wound Healing by Promoting Fibroblast Proliferation, Migration, and Secretion

Xionglin Chen[1], Min Zhang[1], Shixuan Chen[1], Xueer Wang[1], Zhihui Tian[1], Yinghua Chen[1], Pengcheng Xu[1], Lei Zhang[1], Lu Zhang[2], and Lin Zhang[1]

Abstract

Skin wound healing is a complicated process that involves a variety of cells and cytokines. Fibroblasts play an important role in this process and participate in transformation into myofibroblasts, the synthesis of extracellular matrix (ECM) and fibers, and the secretion of a variety of growth factors. This study assessed the effects of peptide Ser-Ile-Lys-Val-Ala-Val (SIKVAV)–modified chitosan hydrogels on skin wound healing. We investigated the capability of peptide SIKVAV to promote cell proliferation and migration, the synthesis of collagen, and the secretion of a variety of growth factors using fibroblasts in vitro. We also treated skin wounds established in mice using peptide SIKVAV-modified chitosan hydrogels. Hematoxylin and eosin staining showed that peptide-modified chitosan hydrogels enhanced the reepithelialization of wounds compared with negative and positive controls. Masson's trichrome staining demonstrated that more collagen fibers were deposited in the wounds treated with peptide-modified chitosan hydrogels compared with the negative and positive controls. Immunohistochemistry revealed that the peptide-modified chitosan hydrogels promoted angiogenesis in the skin wound. Taken together, these results suggest that peptide SIKVAV-modified chitosan hydrogels may be useful in wound dressing and the treatment of skin wounds.

Keywords

SIKVAV, chitosan hydrogel, fibroblasts, wound healing, angiogenesis

Introduction

Skin wound healing is a complicated process that involves inflammation, cell proliferation, tissue formation (including angiogenesis and granulation), and tissue remodeling.[1] Various cells participate in skin wound healing, such as keratinocytes, stem cells, inflammatory cells, and fibroblasts. Fibroblasts play a critical role by differentiating into myofibroblasts, synthesizing fibers and the extracellular matrix (ECM) and proteoglycans,[2] which provide the scaffold for reepithelialization and wound healing and the secretion of a variety of growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and transforming growth factor β-1 (TGF-β1).[3] Reports have shown that EGF promoted the proliferation and migration of keratinocytes in acute skin wound healing[4]; bFGF plays a major role in granulation of tissue formation and reepithelialization[5]; TGF-β1 is involved in inflammation, angiogenesis, reepithelialization, and tissue regeneration[6]; and VEGF promotes the formation of blood vessels[7] that provide nutrition and oxygen during wound healing. Efforts have been made to design and develop systems to spontaneously deliver multiple growth factors for skin wound healing due to the insufficient

[1] Key Laboratory of Construction and Detection in Tissue Engineering of Guangdong Province, Department of Histology and Embryology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, China
[2] Key Laboratory of Functional Proteomics of Guangdong Province, Department of Pathophysiology, School of Basic Medical Sciences, Southern Medical University, Guangzhou, China

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Corresponding Authors:
Lin Zhang, Department of Histology and Embryology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China.
Lu Zhang, Department of Pathophysiology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China.
Emails: zlilyzh@126.com; zlulu70@126.com

capability of a single growth factor.[8] However, such systems are very complicated because the needs for growth factors differ according to time and location.

Chitosan is a deacetylated derivative of chitin, which is a significant component in the shells/exoskeletons of crustaceans and is one of the most abundant natural polysaccharides.[9,10] The deacetylation degree and molecular weight of chitosan are important aspects affecting its physical and biological properties.[11] Chitosan has high concentrations of hydroxyl and primary amino functional groups, which enable its various applications in drug delivery and pharmaceuticals, biotechnology, agriculture, and environmental protection due to its excellent adsorption, carrier, and antibacterial capabilities.[9,12,13,14] Due to its good biocompatibility, easy processing and good physical and chemical stability, chitosan has also been extensively investigated for skin wounds in the form of composite films.[15] A recent study by Lefler and Ghanem[16] showed that bFGF/chitosan matrices promoted reepithelialization and inhibited bacterial growth.

The ECM is composed of various biomacromolecules, such as fibronectin, elastin, proteoglycans, and laminin. Among these, laminin promotes the proliferation and migration of cells, regulates the maturation and differentiation of cells, and is also involved in signal transduction.[17] Laminin is a Y-shaped protein with 1 α peptide chain and 2 β peptide chains conjugated via sulfate ester bonds.[18] Its peptide chain contains a peptide sequence of Ser-Ile-Lys-Val-Ala-Val (SIKVAV). This small peptide sequence of SIKVAV has been recognized to promote the initial adhesion, proliferation, and migration of lung fibroblasts, vascular smooth muscle cells, and endothelial cells.[19,20] The peptide chain of SIKVAV also promoted the adhesion of bone marrow-derived mesenchymal stem cells (BM-MSCs) and their differentiation into adipocytes and osteoblasts.[21] He et al.[22] demonstrated that the peptide SIKVAV covalently conjugated to poly-L-lactic acid accelerated the neurite outgrowth in neonatal mouse cerebellum C17.2 stem cells. Hashimoto et al.[20] reported that a peptide SIKVAV-modified alginate dressing promoted the reepithelialization in the wound of a rabbit ear and accelerated wound healing. Kibbey et al.[23] demonstrated that peptide SIKVAV promoted tumor cell growth and angiogenesis. In vitro, peptide SIKVAV also promoted endothelial cells to form an endothelial tubular structure[24] and facilitated angiogenesis. Therefore, peptide SIKVAV may represent a useful therapeutic agent in skin wound healing.

In this study, we integrated peptide chain SIKVAV and chitosan into a single piece of peptide-modified chitosan hydrogels for skin wound healing. In vitro studies were conducted to investigate the capability of peptide SIKVAV to promote fibroblast proliferation and migration and the secretion of various biomacromolecules. In vivo studies in mice were performed to determine the effects of peptide-modified chitosan hydrogels in skin wound healing by examining wound closure, reepithelialization, collagen deposition, and angiogenesis at skin wound sites. The results suggest that peptide SIKVAV-modified chitosan hydrogels may be useful in the treatment of skin wound healing due to their action in enhancing the proliferation and migration of fibroblasts, collagen deposition, and growth factors secretion.

Materials and Methods

Materials

Chitosan (85% deacetylation degree, molecular weight 100,000) was purchased from Golden-Shell Pharmaceutical Co. Ltd. (Yuhuan, Zhejiang, China). Methacrylic anhydride was purchased from APC Chemicals Company (Montreal, Canada). 3-(maleimid)opropionic acid *n*-hydroxysuccinimide ester (SMP; 97%) was purchased from Polysciences Corporation (Taipai, Taiwan). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulfate (APS), and dimethylformamide were purchased from Sigma-Aldrich (Guangzhou, China). Peptide SIKVAV was purchased from Peptide Biotech Co. Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, penicillin, streptomycin, and trypsin were purchased from Invitrogen (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) test kits for VEGF, EGF, TGF-β1, bFGF, and collagen I and III were purchased from Shanghai Lichen Biotechnology (Shanghai, China). A cell counting assay kit-8 (CCK-8) was purchased from Biotium Inc (Beijing, China). Sodium pentobarbital was purchased from Aladdin (Shanghai, China). Platelet endothelial cell adhesion molecule-1 (CD31) polyclonal antibody, biotinylated goat anti-rabbit secondary antibody, and streptavidin–biotin complex (SABC) detection kits were purchased from Wuhan Boster Biological Engineering Co. Ltd. (Wuhan, China).

Synthesis of Peptide-Modified Chitosan Hydrogels

Peptide-modified chitosan hydrogels were prepared as described in our previous report.[25] Briefly, methacrylic anhydride was reacted with chitosan in 3% acetic acid solution. The resulting mixture after the reaction was dialyzed against water, and chitosan macromers that carried polymerizable double bonds due to the methacrylamide groups were obtained as a dry powder after lyophilization. Chitosan macromers were reacted with SMP via an amide coupling reaction. The resulting mixture was dialyzed against water to obtain maleimidopropionic-conjugated chitosan macromers after lyophilization for 24 hours under −20 °C by freeze dryer (EPSILON 2-10D LSC, Marin Christ Corporation, Osterode, Germany). Maleimidopropionic-conjugated chitosan macromers were further reacted with peptide SIKVAV under nitrogen at room temperature. Peptide SIKVAV-modified chitosan macromers were obtained after dialyzing the reaction mixture against water followed by freeze-drying. Peptide-modified chitosan hydrogels were synthesized from peptide-modified chitosan macromers via free radical polymerization with APS and TEMED as the initiators and catalyst, respectively. For comparison, chitosan hydrogels without peptide SIKVAV were synthesized from the initial chitosan macromers carrying polymerizable double bonds using the same protocol for

synthesizing peptide-modified chitosan hydrogels. The animal studies in this work were approved by the Institutional Animal Care and Use Committee (IACUC).

Fibroblast Primary Culture

Primary fibroblasts were obtained from neonatal C57BL/6 mice via trypsin digestion and tissue adherence. The skin was peeled off using curved tweezers after the removal of the head, limbs, and tail from the sacrificed mouse. The resulting skin was rinsed with Hank's balanced salt solution (HBSS) (Wuhan Boster Biological Engineering Co. Ltd.) and then trypsinized for 30 min. The skin was shredded into pieces with curved tweezers. The skin pieces were transferred to a 10-cm dish filled with the medium and incubated in humidified atmosphere containing 5% CO_2 at 37 °C. These skin pieces were discarded after fibroblasts grew out from the pieces in 24 h. The collected fibroblasts were cultured to 80% to 90% confluence and passed into new dishes at a split of 1:2.

CCK-8 assay

Peptide SIKVAV solutions were prepared at 0 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, and 400 μg/mL in DMEM. The passage 3 fibroblasts were resuspended at 2.5×10^4 cells per mL and seeded into 96-well plates at 100 μL cell suspension per well. After 24 h of adherence, the fibroblast culture was continued in DMEM supplemented with 10% FBM and peptide SIKVAV at various concentrations for 1, 2, or 3 d. The absorbance for each well of the 96-well plate was read at 450 nm using an Epoch automatic microplate reader (BioTek Corporation, Winooski, VT, USA) after incubations for 1, 2, or 3 d.

Transwell Test

The passage 3 fibroblasts were cultured in serum-free medium for 12 h. The cells were harvested and resuspended in serum-free DMEM to a density of 1×10^5 cell per mL after washing with HBSS. For each transwell of a 24-well plate, 500 μL of serum-free DMEM medium supplemented with peptide SIKVAV was added to the lower chamber, and 100 μL of the cell suspension was added to the transwell upper chamber. After 24-h incubation at 5% CO_2 and 37 °C, the transwell upper chamber was taken out and washed with phosphate-buffered solution (PBS) before being fixed using 4% paraformaldehyde (PFA) for 30 min. The cells that adhered to the lower side of the transwell membrane were stained with 0.5% crystal violet (Tianjin Zhiyuan Chemical Reagent Co., Ltd., Tianjin, China) for 30 min after the nonmigrated cells were carefully removed from the upper side of the transwell membrane using cotton swabs. Five images were captured randomly for each transwell membrane using an inverted microscope (Leica Microsystems, Guangzhou, China).

ELISA Assay

The harvested passage 3 fibroblasts were resuspended in DMEM with 10% FBS to a density of 5×10^4 cells per mL and seeded to a 96-well plate at a 100-μL cell suspension per well. After the seeded cells were incubated for 12 h at 5% CO_2 and 37 °C, the medium in each well was replaced with 100 μL DMEM with 10% FBS supplemented with peptide SIKVAV at various concentrations. The cells were cultured for another 1, 2, or 3 d before the supernatant was measured for EGF, bFGF, VEGF, TGF-β1, and collagen I and III using the corresponding ELISA kit assays according to the manufacturer's instructions.

In Vivo Studies of Peptide-Modified Chitosan Hydrogels on Skin Wound Healing in Mice

Seventy-two female C57BL/6 mice aged 8 to 12 wk were purchased from the Experimental Animal Center of Southern Medical University. The experiment was approved by the Southern Medical University Ethics Committee and strictly complied with the National Institutes of Health (NIH) safety guidelines. After the mice were anesthetized via an intraperitoneal (i.p.) injection of 1% pentobarbital sodium at 0.01 mL/g body weight, the hairs were removed at the unilateral sites of the backs of mice. The wounds were created at these sites at a depth of 6 mm to establish wound models. The mice were then randomly divided into 4 groups, including a negative control group that received no treatments, 2 positive control groups that received treatments of either the injection of peptide SIKVAV solution (peptide group) or a dressing of chitosan hydrogels (chitosan group) at the wound sites, or an experimental group that received a treatment of a dressing with peptide-modified chitosan hydrogels (chitosan + peptide group) at the wound site. After the treatment operation, the treated or nontreated wounds were further covered with Tegaderm tape (Anji Bofeng Medical Supplies Co., Ltd., Huzhou, China) to protect the wounds. The mice were then individually housed and fed ad libitum. A digital camera was used to record the wound of each mouse at 3, 5, and 7 d postoperation and before they were sacrificed. The residual wound percentage was calculated using Equation (1).

$$\text{The residual wound percentage}(\%) = \frac{S_t}{S_0} \times 100\%, \quad (1)$$

where S_0 was the initial wound area for a mouse, and S_t was the residual wound area at the indicated time for that mouse.

Histological Observations

The wounds with surrounding tissues were harvested from the mice at 3, 5, or 7 d posttreatment. The harvested tissues were rinsed with PBS and then fixed using 4% PFA solution. After dehydrating with a series of gradient ethanol (increased from 70% to 100%), the tissues were embedded in paraffin (Guangzhou Mayor Rong Chemical Co., Ltd.,

Guangzhou, China). Thin tissue slices (5 μm) were prepared from the embedded tissue samples, followed by staining with either hematoxylin and eosin (H&E) or Masson's trichrome (Beijing Noroblide Technology Co., Ltd., Beijing, China) according to the corresponding standard procedures. The rate of reepithelialization in the skin wounds was calculated using Equation (2).

$$\text{The rate of reepithelialization } (\%) = \frac{S_t}{S_0} \times 100\%, \quad (2)$$

where S_0 was the initial wound distance for a slice, and S_t was the keratinocytes covering the wound distance at the indicated time for that slice.

Immunohistochemistry Assay

The thin tissue slices were deparaffinized and then rehydrated to retrieve antigen using 0.1 M citrate buffer via incubation in 5% bovine serum albumin (BSA) for 2 h. The slices were then incubated with polyclonal rabbit antimouse CD31 antibody at 4 °C overnight. The resulting tissue slices were rinsed with PBS, followed by incubation with biotinylated goat antirabbit secondary antibody for 2 h. The tissue slices were reacted with SABC for 20 min, colored with 3,3′-diaminobenzidine (DAB), stained with hematoxylin, and dehydrated with a gradient ethanol series. The dehydrated tissue slices were soaked with xylene (Guangzhou Chongwen Chemical Co., Ltd. Guangzhou, China) and then sealed with resin (Guangzhou City Kay Show Trade Co., Ltd. Guangzhou, China). Each tissue slice was observed via microscope (400×) at 5 randomly selected locations to count the new capillaries with endothelial cells positive for CD31 at the indicated time points.

Statistical Analysis

Numerical data were expressed as the means ± standard deviation. Statistical differences between the groups were assessed using one-way analysis of variance (ANOVA) in SPSS20.0 software (International Business Machines Corporation, Guangzhou, China). A P value of less than 0.05 ($^*P < 0.05$) indicates a statistical difference between the groups. A P value less than 0.01 ($^{**}P < 0.01$) indicates a statistically significant difference between the groups. Experiments were performed in at least triplicates.

Results

Peptide-Modified Chitosan Hydrogels Promoted Skin Wound Healing and Reepithelialization in Mouse Models

First, we incorporated peptide SIKVAV into chitosan hydrogels as a wound healing dressing and tested their effectiveness in skin wound healing in mouse models. Surprisingly, treatment with the injection of peptide SIKVAV did not positively impact skin wound closure compared with the negative group until 7 d following the treatment (Fig. 1A and B). The 2 groups treated with chitosan or peptide-modified chitosan hydrogels demonstrated enhanced wound closure at the skin wounds at 3, 5, and 7 d after initial treatment compared with the negative control (Fig. 1A and B). More importantly, peptide-modified chitosan hydrogels statistically enhanced

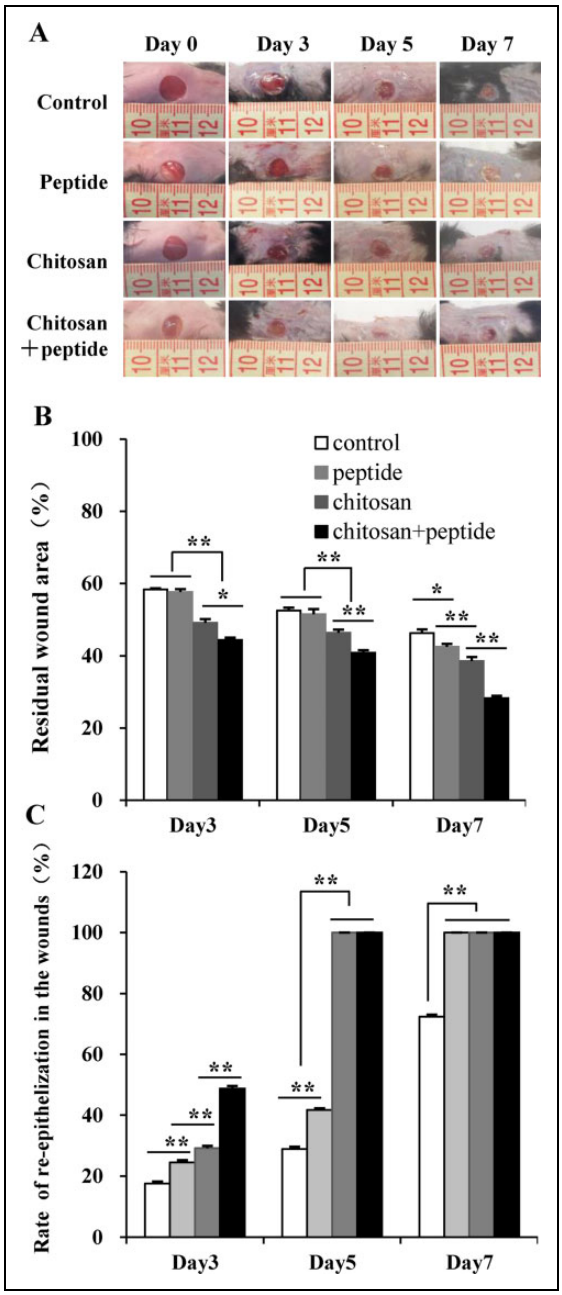

Fig. 1. Peptide-modified chitosan hydrogels significantly promoted skin wound healing in mice: (A) typical images of wounds in the 4 groups of mice at 3, 5, or 7 d following initial treatment; (B) the residual wound percentage for the 4 groups of mice ($n = 6$, $^*P < 0.05$, and $^{**}P < 0.01$). (C) Peptide-modified chitosan hydrogels promoted the reepithelialization process of wounds in the 4 groups of mice ($n = 6$, $^*P < 0.05$, and $^{**}P < 0.01$).

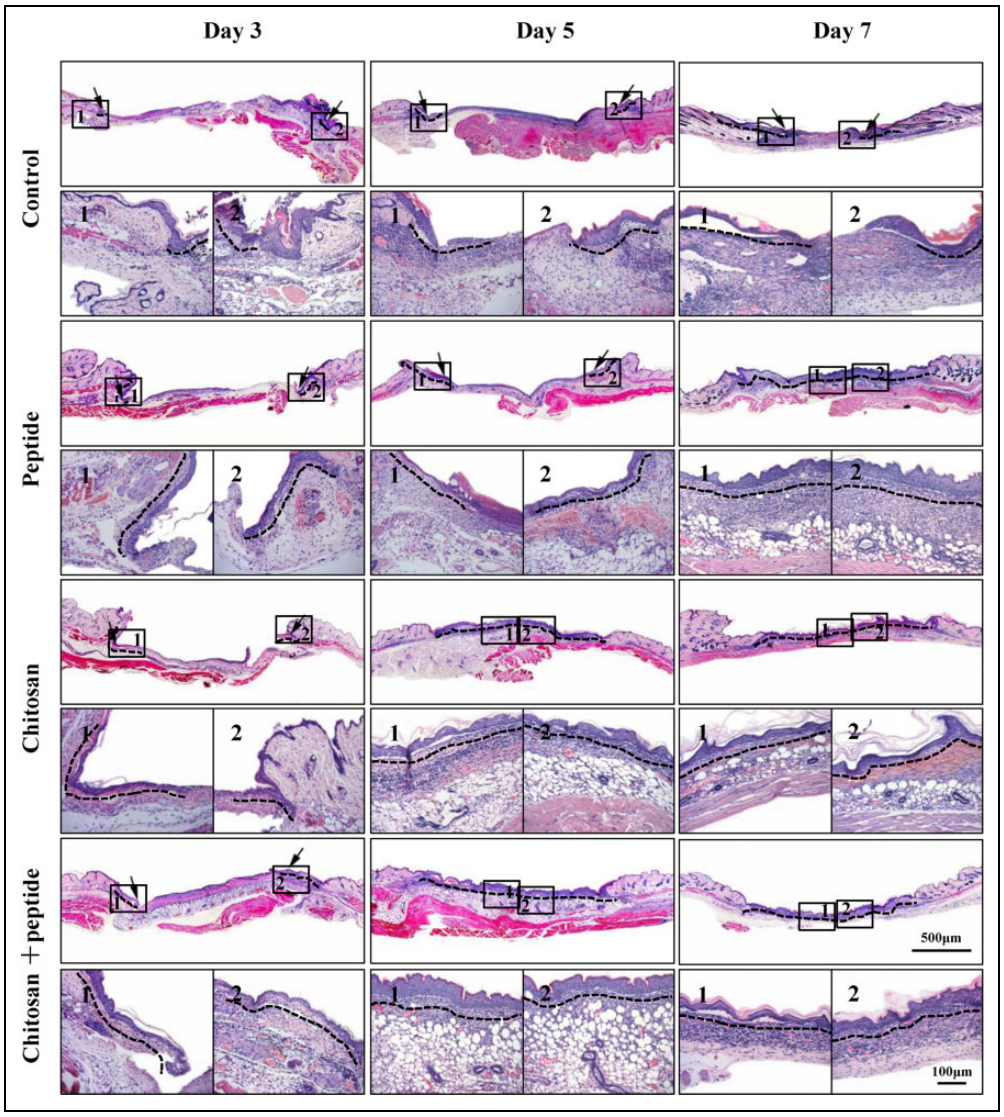

Fig. 2. Hematoxylin and eosin staining showing the reepithelialization process of wounds in the 4 groups of mice.

the wound closure compared with chitosan hydrogels alone at all of the tested time points.

Fig. 2 shows the results from H&E staining, and Fig. 1C shows the reepithelialization process of wounds in all groups. The peptide-modified chitosan hydrogels promoted the reepithelialization of the skin wound. Keratinocytes migrated only somewhat from the wound edge in the negative control group, peptide group and chitosan group at 3 d after the treatment. However, at the same time point, keratinocytes migrated a substantially longer distance in the chitosan + peptide group. Five days after the treatment operations, both the chitosan and chitosan + peptide groups finished reepithelialization. However, keratinocytes had not covered the wounds in the other 2 groups, especially in the negative control group. The peptide group completed reepithelialization at 7 d after the treatment operation; at the same time point, the keratinocytes did not completely cover the wound in the negative control group.

Peptide-Modified Chitosan Hydrogels Promoted Collagen Deposition at the Skin Wounds

We used Masson's trichrome staining to determine the collagen fibers that were newly deposited at the wounds after various treatments (Fig. 3A). At 3 d following the initial treatment, few newly formed collagen fibers were found at the wounds for the 3 control groups including negative control, peptide and chitosan; however, the chitosan + peptide group had more collagen fibers deposited than the other 3 groups. At 5 d after the initial treatment, small amounts of newly formed collagen fibers were observed at the wounds of the 3 control groups, whereas collagen fibers had continuously deposited in the wounds of the chitosan + peptide group. At 7 d following the initial treatment, the collagen fibers completely filled the wounds in the chitosan + peptide group, whereas the amounts of newly formed collagen fibers only increased in the wounds in the peptide and chitosan

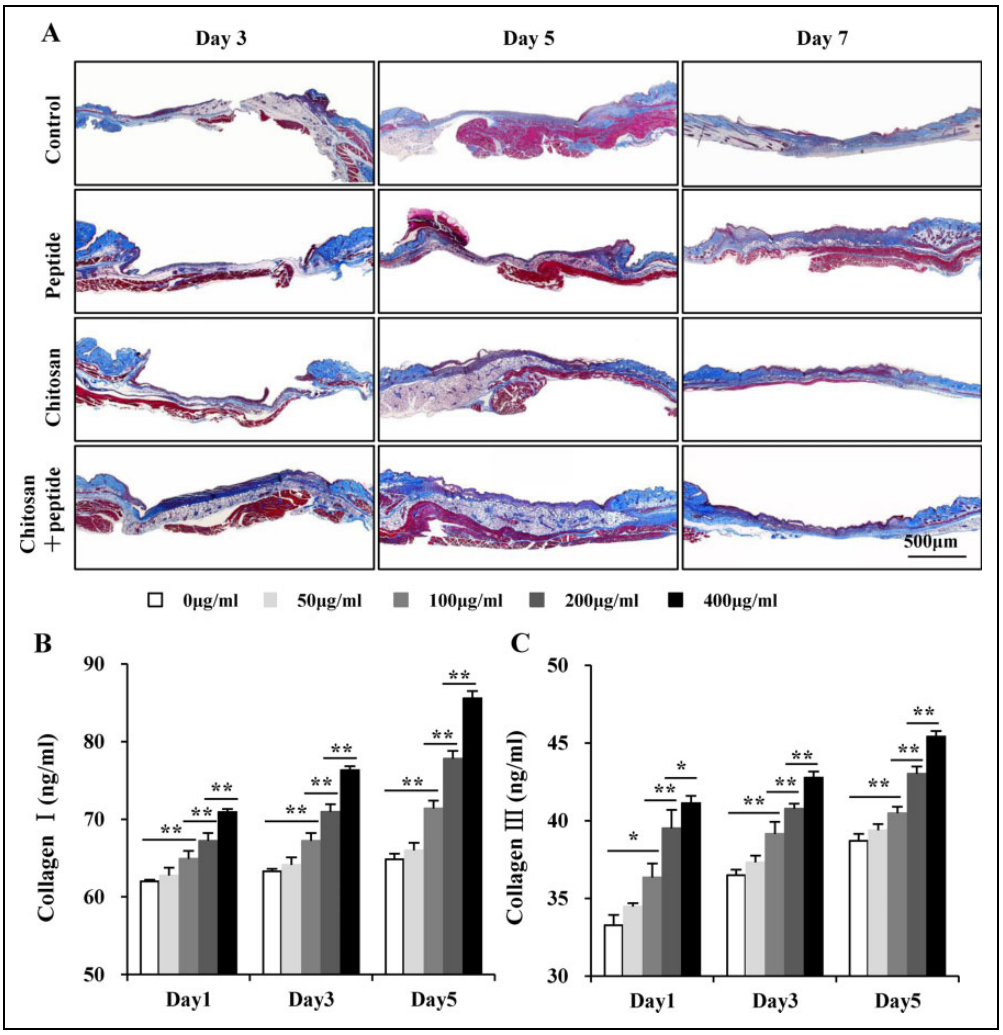

Fig. 3. Peptide-modified chitosan hydrogel significantly promoted the deposition of collagen in skin wounds at 3, 5, and 7 d following initial treatments (A, Masson's trichrome staining) and the synthesis of collagen I (B) and collagen III (C) by fibroblasts via incubation at 0, 50, 100, 200, and 400 μg/mL for 24, 48, or 72 h ($n = 3$, * $P < 0.05$ and ** $P < 0.01$).

groups. In the negative control group, fewer collagen fibers were observed in some wound areas. Further study found that fibroblasts synthesized more collagen I (Fig. 3B) and collagen III (Fig. 3C) when incubated with peptide SIKVAV at concentrations of 100 μg/mL or above. Statistically significant differences were observed between the groups of 0 μg/mL peptide and 100 μg/mL peptide ($P < 0.01$) or above ($P < 0.01$) at all 3 time points studied.

Peptide-Modified Chitosan Hydrogels Promoted Angiogenesis at the Skin Wounds

An immunohistochemistry assay was used to determine the CD31 expression in capillary endothelial cells at the wounds in all 4 groups (Fig. 4A). Although the angiogenesis of the wounds had no significant difference between the groups at 3 d after the initial treatments ($P > 0.05$), when the skin wounds received treatments of either peptide injection ($P < 0.01$) or chitosan hydrogels ($P < 0.01$), statistically significant angiogenesis was observed at 5 and 7 d after the initial treatments compared with that in the negative control group. Moreover, the wounds treated with peptide-modified chitosan hydrogels ($P < 0.01$) had even more significant angiogenesis than the peptide or chitosan groups (Fig. 4B).

Peptide SIKVAV Promoted the Proliferation and Migration of Fibroblasts and Enhanced the Secretion of Biomolecules by Fibroblasts

A CCK-8 assay (Fig. 5C) showed that although the proliferation of fibroblasts showed no significant difference between the groups after 24 h of incubation ($P > 0.05$), peptide SIKVAV significantly promoted the proliferation of fibroblasts at concentrations of 100 μg/mL ($P < 0.05$) or above ($P < 0.01$) after 48 and 72 h of incubation. A migration test using the transwell method also demonstrated that peptide SIKVAV significantly promoted the migration of

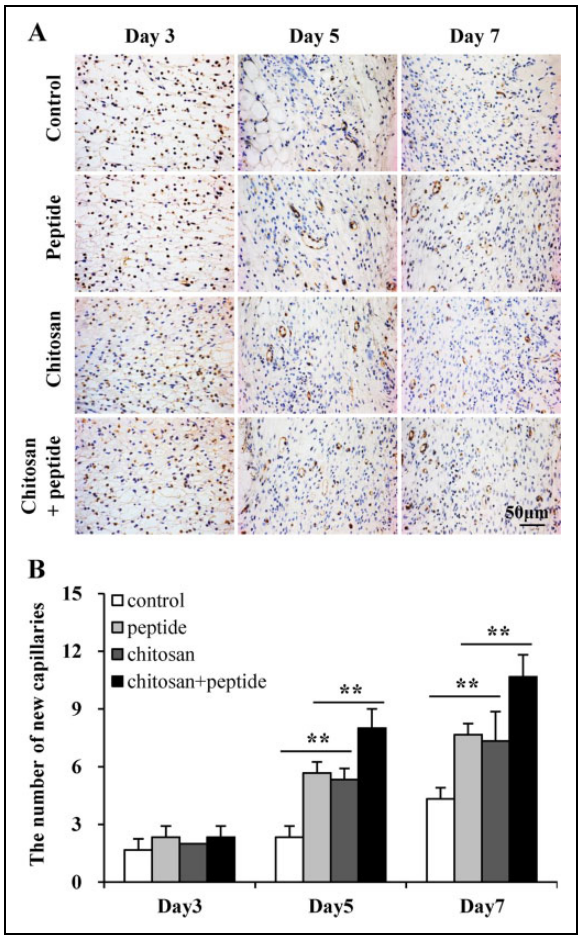

Fig. 4. Peptide-modified chitosan hydrogels promoted angiogenesis in skin wounds: (A) immunohistochemical staining for vascular endothelial CD31 expression at 3, 5 and 7 d following initial treatments. (B) New blood capillary density ($n = 6$, * $P < 0.05$, and ** $P < 0.01$).

fibroblasts at concentrations of 100 μg/mL ($P < 0.01$) or above ($P < 0.01$; Fig. 5A and B). Fig. 5A clearly shows that the peptide SIKVAV-induced migration of fibroblasts was concentration dependent.

Fig. 5 D to G shows the secretion of 4 important growth factors by fibroblasts treated with peptide SIKVAV at various concentrations. There were significant differences in the amounts of the 4 growth factors secreted by fibroblasts at any time point in 3, 5, and 7 d between those treated with peptide concentrations of 100 μg/mL ($P < 0.05$) or above ($P < 0.01$) and those that were untreated. Moreover, peptide SIKVAV at 400 μg/mL significantly increased the fibroblast secretion of all 4 growth factors at any time point ($P < 0.01$).

Discussion

Wound healing is a complicated biological process involving in a variety of cells and many growth factors for the synthesis of the extracellular matrix and the proliferation and migration of cells. TGF-β1, bFGF, EGF, VEGF, and PDGF were involved in wound healing, as demonstrated in in vivo animal studies.[3] Fibroblasts play an important role in this complicated process. Fibroblasts are transformed into myofibroblasts, which participate in wound contraction, and fibroblasts also synthesize the extracellular matrix and secrete a variety of growth factors.[3] Peptide SIKVAV has been reported to have the biological activity of growth factors and promote the adhesion, proliferation, and migration of cells.[20] Thus, in this work, we investigated the effects of peptide SIKVAV on the in vitro cultured fibroblasts. Peptide SIKVAV accelerated fibroblast proliferation and migration to some extent in a concentration-dependent manner. Significant promotion effects were observed when a peptide SIKVAV concentration of 100 μg/mL or higher was used to induce fibroblasts (Fig. 5A to C). This is consistent with the report from Hashimoto et al.[20] Our previous studies also showed that peptide SIKVAV-modified chitosan hydrogels could promote the adhesion and proliferation of BM-MSCs in vitro.[25] Peptide SIKVAV affected the secretion of TGF-β1, bFGF, EGF, and VEGF by fibroblasts in a concentration-dependent manner. Significant amounts of TGF-β1, bFGF, EGF, and VEGF were secreted by fibroblast cells incubated with peptide SIKVAV at a concentration of 100 μg/mL or higher (Figs. 5D to G). These growth factors enhanced the proliferation and migration of fibroblasts in addition to the synthesis of fibers and ECM, which could accelerate wound healing.[3]

In particular, TGF-β1 is able to promote the proliferation of fibroblasts and their transformation into myofibroblasts, the formation of connective tissue including fibers and ECM, and the reepithelialization process.[26,27] Dermal collagen is primarily composed of collagen I and III. Newly formed collagen fibers provide scaffold for cell proliferation and angiogenesis and facilitate epidermal cell migration at wound sites. The Masson's trichrome staining results demonstrated that the mice treated with peptide-modified chitosan hydrogels had more newborn collagen fibers at their wound sites than the other groups and fibroblasts synthesized more collagen I and collagen III when incubated with peptide SIKVAV in vitro. The reduced effectiveness of peptide SIKVAV injection on the deposition of collagen I and III in wound models may be partially attributed to the fact that the injected peptides were not limited to the wound sites after injection. In the in vitro studies and peptide-modified chitosan hydrogels, the peptides were limited to the local cells.

bFGF promoted the formation of connective tissue and reepithelialization but also inhibited scar formation. bFGF accelerated angiogenesis by enhancing the proliferation and migration of endothelial cells.[27,28] EGF played a major role in the reepithelialization of wounds by promoting epidermal cells around the wound to proliferate and migrate to the wound center.[29,30] In vivo, our studies showed that peptide-modified chitosan hydrogels had better effects on reepithelialization at the wounds than other groups according to H&E staining. This may be due to the enhanced

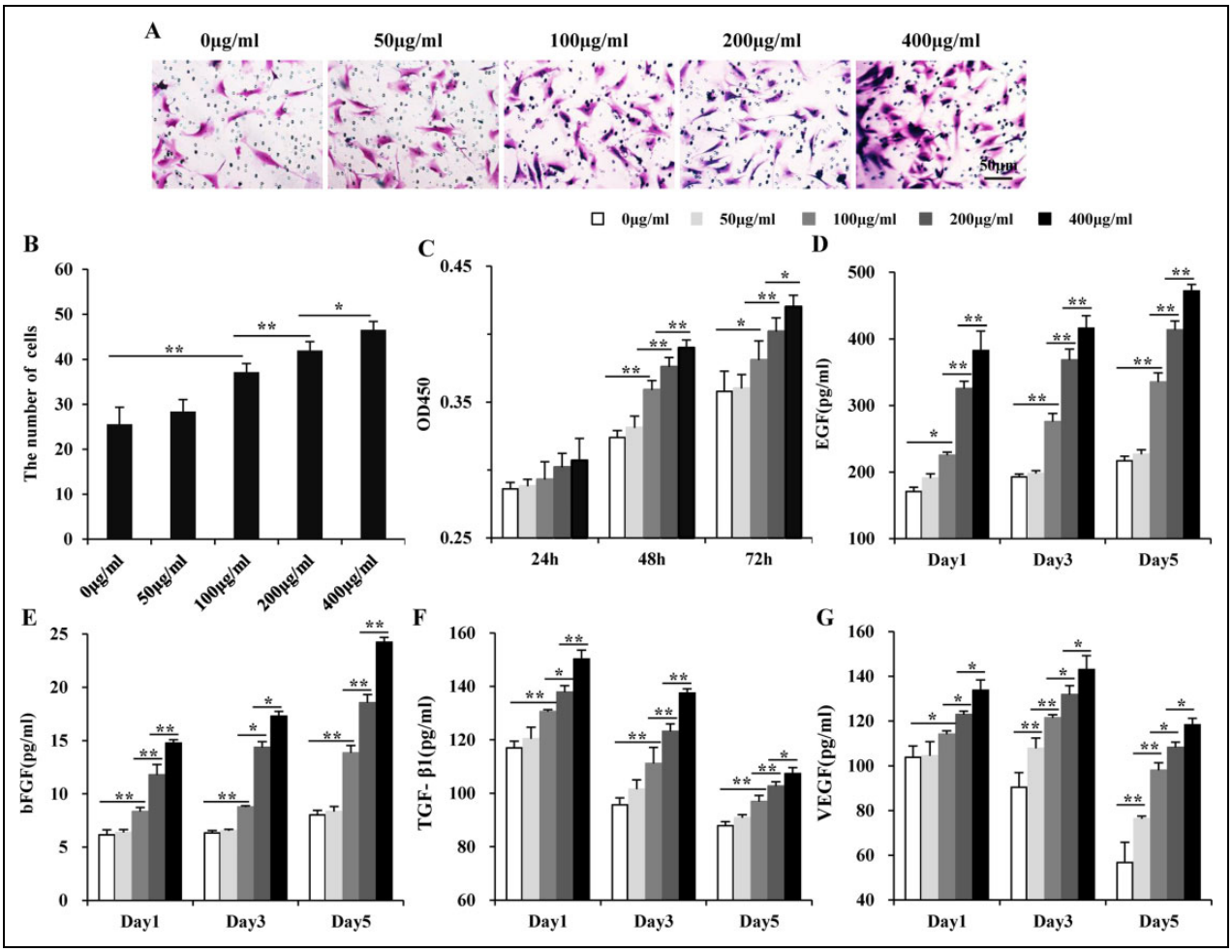

Fig. 5. Peptide Ser-Ile-Lys-Val-Ala-Val (SIKVAV) affects the proliferation and migration of fibroblasts in a concentration-dependent manner: (A) typical microscope images and (C) the number of migrated fibroblast cells after induction by peptide SIKVAV at 0, 50, 100, 200, and 400 μg/mL for 24 h ($n = 3$, * $P < 0.05$, and ** $P < 0.01$.), scale = 50 μm. (B) The proliferation of fibroblasts incubated with peptide SIKVAV at 0, 50, 100, 200, and 400 μg/mL for 24, 48, or 72 h ($n = 4$, *$P < 0.05$, and ** $P < 0.01$). Peptide SIKVAV affects the secretion of epidermal growth factor (EGF) (D), basic fibroblast growth factor (bFGF) (E), transforming growth factor β-1 (TGF-β1) (F), and vascular endothelial growth factor (VEGF) (G) by fibroblasts via incubation at 0, 50, 100, 200, and 400 μg/mL for 24, 48, or 72 h ($n = 3$, * $P < 0.05$ and ** $P < 0.01$.).

secretion of bFGF and EGF by fibroblasts at the wounds treated with peptide-modified chitosan hydrogels, as demonstrated in the in vitro studies.

Immunohistochemistry staining of CD31 markers in vascular endothelial cells at the wound sites showed that angiogenesis in the chitosan + peptide group was significantly increased compared with those in the other 3 groups. This may be attributed to the enhanced secretion of VEGF by fibroblasts at the wounds treated with peptide-modified chitosan hydrogels. However, the 2 groups treated with the injection of peptide or chitosan hydrogels had significantly more angiogenesis than the negative control group. Granulation tissue and new capillaries began to form at the early stages of wound healing. Newly formed blood vessels in wounds are essential for supplying nutrition to granulation tissue and the epidermis. This study demonstrated that the peptide SIKVAV-modified chitosan hydrogels synergistically accelerated vascularization. This may be because peptide SIKVAV itself promotes endothelial cell migration and enhances the secretion of endogenous growth factors, such as VEGF[3] and bFGF by fibroblasts. This is consistent with a report from another group that peptide SIKVAV promoted the adhesion and migration of vascular endothelial cells, thereby enhancing angiogenesis.[24]

The peptide-modified chitosan hydrogels were effective for promoting reepithelialization, collagen deposition and angiogenesis in the skin wound compared with the other 3 groups. This indicated that the in vitro activities of the novel peptide reflected those in vivo, even after the peptide linking chitosan. This finding also suggested that the fibroblasts within the wound had sufficiently attached to the peptide-modified chitosan hydrogels, were activated by the peptide SIKVAV and transferred these signals to neighboring cells with the following epithelialization by epithelial cells. Angiogenesis by endothelial cells and collagen deposition synthesized by fibroblasts were also promoted.

Due to its good biocompatibility, easy processing and good physical and chemical stability, chitosan has also been extensively investigated for skin wounds in the form of composite films.[31] Chitosan hydrogels exhibit excellent water absorption capacity, which provides a moist environment for wound healing[32] and prevents the accumulation of fluid in wounds. Chitosan hydrogel could effectively reduce the loss of water and electrolytes from the wound in the skin. The peptide-modified chitosan hydrogels had the aforementioned features and contained peptide SIKVAV, which could accelerate the reepithelialization shown in Fig. 2, collagen deposition and angiogenesis (Figures 2-4) in the skin wounds in vivo.

In conclusion, peptide SIKVAV promoted the proliferation and migration of fibroblasts and the synthesis of EGF, bFGF, VEGF, platelet-derived growth factor (PDGF), TGF-β1, and collagen I and III by in vitro cultured fibroblasts in a concentration-dependent manner. The in vivo studies on skin wounds established in mice showed that the injection of peptide SIKVAV or chitosan hydrogels enhanced skin wound healing and that peptide-modified chitosan hydrogels further improved wound healing in a synergistic manner. Therefore, peptide-modified chitosan hydrogels may have utility as an effective wound dressing to treat skin wounds.

Authors' Note

Xionglin Chen and Min Zhang contributed equally to this work.

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Ethical Approval

This study was approved by the Institutional Animal Care and Use Committee.

Statement of Human and Animal Rights

The animal studies in this work were approved by the Institutional Animal Care and Use Committee.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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