A chitin film containing basic fibroblast growth factor with a chitin-binding domain as wound dressings

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ABSTRACT

Basic fibroblast growth factor (bFGF) can stimulate wound healing. However, consistent delivery of bFGF has many disadvantages. To decrease their instability and diffusible, we introduced chitin-binding domain (ChtBD) into bFGF. Two expression plasmids were constructed. The first one (named bFGF) contained bFGF (154 amino acids), the second (named ChtBD-bFGF) contained bFGF and the ChtBD (54 amino acids). ChtBD was derived from chitinase A1 (ChiA1) of Bacillus circulans WL-12. The recombinant protein ChtBD-bFGF had the same biological activity as bFGF in promoting fibroblast proliferation. Chitin powder was dissolved in 11 wt% NaOH and 4 wt% urea aqueous solution via the freezing/thawing method. A chitin solution was spread on a glass plate and coagulated with anhydrous alcohol. The chitin binding ability of ChtBD-bFGF was 11.4-fold higher (up to 286 μg/cm\textsuperscript{2}) than bFGF \textit{in vitro}. The immunofluorescence data indicated that the ChtBD-bFGF@chitin film promoted cell adhesion and proliferation. The ChtBD-bFGF@chitin film and bFGF@chitin films were implanted subcutaneously. Histological analysis showed that ChtBD-bFGF promoted vascularization at the implanted site more
effectively than bFGF. These results suggest that the ChtBD-bFGF@chitin film is a stabile delivery vehicle for accelerating wound healing.

Keywords: Basic fibroblast growth factor; Chitin binding domain; Chitin; Vascularization; Wound dressing

1. Introduction

The skin is the body’s largest organ and prevents pathogen invasion (Proksch, Brandner, & Jensen, 2008). When the skin is broken, wound dressings can control bleeding, prevent infection and absorb secretions to promote wound healing. Wound healing is a complicated process involving hemostasis, inflammation, proliferation, and remodeling. Minimizing bacterial infection in the wound site can reduce the number of inflammatory cells. Promoting the process of cellularization and vascularization of the wound can accelerate tissue growth. Blood clotting and inflammation play an important role in the predictable phases of wound healing.

Wound healing is a complex process and involves numerous growth factor such as basic bFGF, epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), etc. (Stadelmann, Digenis, & Tobin, 1998). The bFGF is a potent mitogen and a modulator for fibroblasts and vascular endothelial cells. In the wound site, bFGF released by platelets can promote endothelial cell proliferation and angiogenesis. This effect is important for tissue growth and tissue remodeling (Barrientos, Stojadinovic, Golinko, Brem, & Tothic-Canic, 2008). The application of a single bFGF cannot accelerate wound healing because bFGF will rapidly wash off the wound site or be absorbed into the wound dressing (Richard et al., 1995). Inappropriate wound dressings will decrease the concentration of bFGF by 50% within 4 h (Finetti & Farina, 1992).

The bFGF and wound dressing can stabilize and control the release of growth factors. Nimura et al. incorporated bFGF into chitosan solution and prepared different concentrations of bFGF-chitosan films via freeze-drying. The results of wound healing suggest that the bFGF-chitosan film was a stabile delivery vehicle for accelerating wound healing (Mizuno et al., 2003). However, growth factors are unstable and diffusible in vivo. Thus, improvements in their performance are needed for clinical applications. In recent
years, people have conjugated growth factors to materials. The modification of growth factors for immobilization on materials or for high-affinity binding to cells or scaffold biomaterials have been shown (Cao et al., 2013; Lin et al., 2012; Shiozaki et al., 2013; Wexue Zhao et al., 2008).

An ideal dressing should have surface hydrophilicity, biocompatibility, bacteriostatic properties, and gas penetration at the wound site. It should also regulate cell proliferation and accelerate wound closure. Chitin/chitosan is a naturally derived polymer that is biodegradable, biocompatible, nontoxic, antibacterial, and hydrating. In recent decades, these properties have allowed chitosan to be processed into many wound dressings (Abdel-Mohsen et al., 2016; Archana, Dutta, & Dutta, 2016). Chitin films have been used to repair a rat wound model, which displayed accelerated wound healing ability (Yusof, Wee, Lim, & Khor, 2003). Here, we introduce a chitin binding domain (ChtBD) into bFGF (named ChtBD-bFGF). The ChtBD-bFGF contains ChtBD derived from chitinase A1 (ChiA1) of Bacillus circulans WL-12. (Fig. 1). The chitin films are prepared by dissolving raw materials in urea and sodium hydroxide aqueous solution via freezing/thaw. The ChtBD-bFGF was bound onto the surface of the chitin films by immersing the chitin film in a solution of ChtBD-bFGF. The ChtBD-bFGF@chitin films were subsequently tested for their ability to promote wound healing. This study characterized the bioactivity, binding ability, cellularization, and vascularization of the ChtBD-bFGF@chitin film as a wound dressing in vitro and in vivo.

2. Materials and methods

2.1 Complex structure prediction from the separate proteins

To reveal the mechanism of ChtBD-bFGF binding to chitin, a complex was formed by the interaction between chains of ChtBD, bFGF, and N-acetylglucosamine (NAG). The complex was predicted by the HoDock1 docking method that incorporates an initial rigid docking and a refined semi-flexible docking. We generated 9,600 complex structures and scored them to identify the final correct complex structure model (Gong et al., 2010; Pronk et al., 2013). Chitin is a polymeric carbohydrate molecule composed of long chains of NAG
units bound together by glycosidic linkages. Therefore, the interaction between ChtBD, bFGF, and NAG could reveal the mechanism of ChtBD-bFGF binding to chitin.

2.2 Fabrication and Characterization of chitin films

Reagent grade chitin powders were purchased from VETEC (Shanghai, China). Chitin was purified before use by treating it with 5% wt% NaOH solution for 10 h under vigorous stirring. This suspension was then filtered and washed with distilled water. Subsequently, the chitin powder was treated with 7% (v/v) hydrochloric acid aqueous solution for 1 day. The 7 g purified chitin powder was dispersed into a 93 g mixture of NaOH, urea, and distilled water at a ratio of 11:4:85 by weight with stirring to obtain a suspension. Subsequently, the suspension was frozen at -30°C for 4 h and then thawed at room temperature. The freezing/thawing cycle was repeated twice to obtain a transparent chitin solution with a chitin concentration of 7 wt%. In this case, chitin solubility was increased with relatively pure chitin and a lower temperature. After removing the air bubbles by centrifugation, the chitin solution was spread on a glass plate as a 0.25-mm thick layer and then coagulated with anhydrous alcohol (Duan et al., 2013). Chitin films were cut into 8-mm discs for the further use.

The wide-angle X-ray diffraction (XRD) pattern of chitin powders and the dried sheets were recorded on an XRD instrument (D8 ADVANCE, BRUKER, Germany) with Cu-Kα radiation (λ = 0.154 nm). The XRD data were collected from 2θ = 5 to 35° at a scanning rate of 2° min⁻¹. Solid-state ¹³C NMR [cross-polarization, magic angle spinning, and dipolar decoupling (CP/MAS)] measurements were carried out on a Bruker AVANCE-III spectrometer operating at 75.47 MHz at room temperature. The contact time for cross-polarization was 3 ms, and the spin rate of the 4-mm rotor was 5 kHz.

2.3 Cloning ChtBD-bFGF and bFGF expression vectors

Two expression vectors were constructed. The first one contained human bFGF—a 17.2 kDa protein consisting of 154 amino acid residues. This was optimized into an E. coli codon and prepared on a DNA synthesizer. The second one contained ChtBD, TTNPGVSAWQVNTAYTAGQLVTYNGKTYKCLQPHTSLAGWEPSNVPALWQLQ
derived from ChiA1, a 52-residue. The ChtBD was amplified by PCR from the vector PTXB (purchased from NEB) containing the sequence of ChtBD (52 amino acid residues) using the forward primer (5’-CGCCATATGACGACAAATCCTGGT-3’) and the backward primer (5’-ACCTGCGCTTTGAAGCTGCCACAA-3’). The bFGF cDNA was amplified by PCR from the vector bFGF using the forward primer (5’-CAGCTTCAAGCGCCAGGTCTATT-3’) and the backward primer (5’-CCGCTCGAGACTTGGCGGACAT-3’). The ChtBD and bFGF fragments were spliced by Phusion® High-Fidelity PCR (NEB) using the forward primer (5’-CGCCATATGACGACAAATCCTGGT-3’) and backward primer (5’-CCGCTCGAGACTTGGCGGACAT-3’). The ChtBD and bFGF fragments were cloned in the expression plasmid of PET-21b. All recombinant plasmids were verified through gene sequencing.

2.4 Preparation of ChtBD-bFGF and bFGF

The recombinant plasmids bFGF and ChtBD-bFGF were transformed into the BL21 (DE3) strain of E.coli. Single clones were inoculated in 100 ml LB medium containing 100 μg/ml ampicillin in a 500 ml flask. The cultures were grown overnight at 37°C with shaking, and 80 ml was inoculated in 4 L LB medium with overnight cultures at 37°C with vigorous shaking until the OD600 was 0.5-0.7. Expression was induced by adding isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.1 mM with overnight growth. Recombinant proteins from E.coli were purified as described in the manual (Qiagen). A cleared lysate supernatant containing the 6×His-tagged proteins was loaded onto a Ni-NTA spin column. Heteroproteins were removed by washing the column with wash buffer. The bFGF and ChtBD-bFGF were eluted from the Ni-NTA spin column with elution buffer. The bFGF and ChtBD-bFGF were analyzed by SDS-PAGE.

2.5 SDS-PAGE and western blotting assays

SDS–PAGE analysis used a 15% gel according to the manual. For western blotting, proteins in the gel were transferred to a polyvinylidene difluoride membrane using an
electroblotting apparatus (Bio-Rad) at 100 mA for 30 min in 25 mM Tris–192 mM glycine. The membrane was blocked by incubating with a solution of 10% BSA for 1 h, and then incubated with the mouse anti-human bFGF monoclonal antibody (Peprotech, USA). After washing, the membrane was incubated with rabbit anti-mouse IgG conjugated to HRP (ABCAM, USA) diluted 1:2000. The bound antibody was detected using ECL.

2.6 Biological activity of ChtBD-bFGF and bFGF

The BALB/C 3T3 cells were obtained from the Cell Culture Centre of Institute of Basic Medical Sciences Chinese Academy of Medical Sciences (Shanghai, China). BALB/C 3T3 cells were cultured and expanded in basal medium containing Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 units/ml penicillin (Sigma), and 100 mg/ml streptomycin (Sigma). Cells were seeded into 96-well plates (Costar) at 1.2×10⁴ per well. The medium was replaced with DMEM containing 2% FBS in addition to ChtBD-bFGF and bFGF with serial gradient concentrations after 24 hours. The cultures were added to 1 mg/ml methylthiazol tetrazolium (MTT) and incubated for 4 h at 37°C followed by addition of 400 μl dimethyl sulfoxide after removing the media. The absorbance at 492 nm was measured immediately.

2.7 ChtBD-bFGF and Chitin films binding assay

Chitin films were immersed in ChtBD-bFGF and bFGF solutions at a concentration of 500 μg/ml at 4°C for 12 hours. Chitin films immersed in PBS at the same conditions were used as a control. The chitin films were then extensively washed with PBS 3 × 5 mins. Chitin films were incubated with rabbit anti mouse bFGF antibody (1:2000 dilution) for 2 hours at room temperature. After washing with PBS 3 × 5 mins, the chitin films were then incubated with a secondary Alexa Fluor 488 labeled goat anti rabbit IgG antibody (1:2000 dilution) for 2 hours at room temperature in the dark. The optical data on binding activities of ChtBD-bFGF and bFGF were captured by a fluorescence imaging device (CRI Maestro).
2.8 Uptake and release of ChtBD-bFGF and bFGF for chitin

The chitin films were immersed in 500 μg/mL ChtBD-bFGF and bFGF solution to obtain ChtBD-bFGF@chitin films, and bFGF@chitin films were used for the in vitro release study. Chitin films (1 mm²) incorporating ChtBD-bFGF and bFGF were incubated in 2.0 mL of phosphate-buffered saline (PBS) at 37°C under stirring at 60 rpm. At specified time intervals, 0.2 mL of the supernatant was collected. The protein content was measured using OD_{280}. The release profiles were obtained by plotting the percentage of cumulatively content of released protein against time. The experiments were performed in triplicate.

2.9 Cell proliferation

The chitin films (8 mm disk) were sterilized by immersing in 70% alcohol for 1 hour. After washing with PBS three times and immersion in ChtBD-bFGF and bFGF solutions for 12 hours at 4°C, the ChtBD-bFGF@chitin films and bFGF@chitin films were washed with PBS and placed into a 24-well plate, respectively. BALB/C 3T3 cells were seeded on different groups at initial densities of 20,000 cells/film. After 3 days of culture, actin filaments of cells were stained with Acti-stain 555 phalloidin, and the cell nucleus was stained with DAPI. The cell proliferation was evaluated via cell morphology images taken by laser scanning confocal microscope (Zeiss LSM780).

2.10 Subcutaneous implantation

Healthy 8-week-old Sprague Dawley (SD) male rats weighing ~200 g were used in this assay. Four subcutaneous pockets were made as described previously (Zhao et al., 2007). The chitin films incorporating ChtBD-bFGF, bFGF, or PBS were implanted into the pockets. After 7 days, the rats were killed with an i.p. injection of sodium pentobarbital, and the newly formed blood vessels were photographed. The skin scaffolds were excised and fixed in 4% formaldehyde for histological analysis. All animal studies were conducted in accordance with the principles and procedures outlined in “Regulations for the Administration of Affairs Concerning Laboratory Animals”, approved by the National
Council of China on October 31, 1988, and “The National Regulation of China for Care and Use of Laboratory Animals”, promulgated by the National Science and Technology Commission of China, on November 14, 1988 as Decree No. 2. Protocols were approved by the Committee of Jilin University Institutional Animal Care and Use.

2.11 Immunohistochemistry

Primary antibodies against anti-rat CD31 (Biolegend, USA) were used for ChtBD-bFGF@chitin films, and bFGF@chitin films were implanted subcutaneously to test the endothelial and proliferation cells. Briefly, formalin-fixed paraffin-embedded tissue specimens were cut into 5-μm thick sections and mounted on poly-L-lysine-coated slides. The sections were deparaffinized and rehydrated. For antigen retrieval, the slides were digested with 0.1% trypsin at 37°C for 30 min. The tissue sections were then incubated at 4°C overnight with appropriate primary antibodies (CD31, 1:2000) after eliminating endogenous peroxidase by incubating with 3% H₂O₂ for 10 min. The sections were then incubated with Alexa 488-labeled anti-rabbit IgG for 40 min at room temperature. After extensive washing, the sections were mounted on microscope slides with glue. Every section was then stained with hematoxylin and eosin (H&E). To quantify the cellularization of implanted different chitin films, we randomly photographed the areas of the around tissue of chitin films with an upright microscope (Zeiss Image A2) at 100× magnification.

2.12 Statistical analysis

The data were presented as the mean values ± standard deviations (SD). Statistical analyses were performed using Student’s t-test for paired samples and ANOVA for multiple samples. A p-value <0.05 was considered statistically significant.

3. Results and discussion

3.1 The interaction between chitin and ChtBD-bFGF

Fig. 2 shows that the complex was formed by the interaction between chains of ChtBD, bFGF, and NAG. NAG around atoms within 4Å were shown. NAG binding sites of
ChtBD-bFGF were formed by six amino acid residues. Five amino acid residues containing NAG binding sites belonged to ChtBD. Only one amino acid residue was derived from bFGF. The binding between ChtBD and NAG barely interfered the structure of bFGF. The prediction results suggest that the chitin binding sites of ChtBD-bFGF were mainly located in ChtBD, and the ChtBD-bFGF binding to chitin did not affect the bioactivity of bFGF.

3.2 Properties of pure chitin films

Structural analyses of these chitin films were carried out with 13C NMR spectra. Fig. S1 (Supplementary Materials) shows the CP/MAS 13C NMR spectra of the purified chitin powder and the chitin film, respectively. The spectrum of the chitin powder showed eight main peaks. Sharp peaks at 172.2 and 22.2 ppm were assigned to the carbonyl and methyl carbons, respectively; the peaks at 103.2, 82.1, 74.9, 72.4, 59.7 and 54.1 ppm are resonances of C1, C4, C5, C3, C6, and C2 on the N-acetyl-D-glucosamine unit of chitin, respectively. The X-ray diffraction patterns of the chitin films and purified chitin powder are shown in Fig. S2 (Supplementary Materials). All chitin samples showed six diffraction peaks at 2 \( \theta \) =9.3°, 12.8°, 19.2°, 20.7°, 23.4° and 26.4° indexed as (020), (021), (110), (120), (130), and (013), respectively, indicating the crystalline structure of \( \alpha \) -chitin. The diffraction peaks (020) and (110) of chitin films were much broader than that of the chitin powder suggesting a decrease in the crystallinity and the crystallite size. This result also caused the (021), (120), and (013) diffraction peaks of chitin to disappear, and the diffraction peaks (130) were merged into peaks (110).

3.3 Expression and purification of ChtBD-bFGF and bFGF

After induction with 0.2 mM IPTG at 22°C, the BL21(DE3) E. coli containing PET21b-ChtBD-bFGF and bFGF produced unique 23.6 and 18.3 kDa bands detected by SDS-PAGE. These bands correspond with the theoretical molecular weights, which demonstrate that the recombinant ChtBD-bFGF and bFGF could be expressed efficiently in E. coli. The recombinant ChtBD-bFGF and bFGF were purified by affinity chromatography and desalting chromatography. The purified fusion proteins showed a single and unique band by SDS-PAGE with the Coomassie brilliant blue staining in Fig. 3A. We performed
western blotting using the mouse anti-human bFGF monoclonal antibody and rabbit anti-mouse IgG conjugated to HRP. We observed the expression and purification of the target proteins as evidenced by clear single bands in the X-ray film in Fig. 3B. These data indicate that the fusion proteins present in the E. coli cell supernatants could be isolated with a high degree of purity by affinity chromatography on nickel-chelating sepharose.

Growth factors play important roles in wound healing and tissue regeneration, which can promote cell proliferation, differentiation, and maturation (Werner & Grose, 2003). However, improvements in their performance would be suitable for wound healing because of their instability and diffusible manner. Thus, the introduction of a binding domain into growth factors would be desirable for wound healing. There are two modes of action in growth factors: growth factors interact with their receptors in a diffusible manner or in a non-diffusible manner. In the diffusible manner, growth factors interact with receptors on the cell membrane and form a complex that induces phosphorylation of the receptor and triggers signal transduction in the cell. This leads to cell desensitization. On the contrary, growth factors can be presented to the cell surface or associated with some substances in a non-diffusible manner. These growth factors are barely internalized after binding to their receptors but exhibit long-term stimulation (Iwamoto & Mekada, 2000; Massague & Pandiella, 1993).

3.4 Biological activities and chitin binding abilities of ChtBD-bFGF and bFGF

Fig. 4(A) shows bell-shaped curves with the maximal activities at 10-35 pM. The ChtBD-bFGF and bFGF promoted fibroblast proliferation at suitable concentrations and inhibited them at high concentrations (>100 pM). The activity of ChtBD-bFGF showed a similar curve as the bFGF. In conclusion, ChBD did not impact activity of ChtBD-bFGF. The binding capacity of ChtBD-bFGF for chitin films was evaluated with an immunofluorescence assay using a monoclonal anti-bFGF antibody and a secondary Alexa Fluor 488-labeled anti mouse IgG antibody. Fig. 4(B) shows that the fluorescence signal of ChtBD-bFGF@chitin film was higher than bFGF@chitin film and the chitin film. These results indicated that the amount of ChtBD-bFGF on the surface of the chitin film was
higher than bFGF. The average signal of ChtBD-bFGF was 3.02-fold higher than bFGF.

The bFGF is crucial for wound healing, and it plays a role in granulation tissue formation, re-epithelialization, and tissue remodeling (Powers, McLeskey, & Wellstein, 2000). However, the expression levels of bFGF are decreased in chronic wounds. The clinical use of bFGF for treatment of wounds holds great therapeutic potential (Robson, 1997). The bFGF usually causes burst release and low accumulation at wound sites, and thus long-time and high-dose administration is required to maintain the therapeutic effect. High concentrations of bFGF will lead to cancer angiogenesis and vascular tumor (Giavazzi et al., 2003; Liekens et al., 2001). Therefore, the design and synthesis of binding bFGF was used to overcome these disadvantages. We selected chitin films because they are favorable in promoting rapid dermal regeneration and accelerating wound healing. Chitin has been prepared into various forms as a component of wound healing products (Allen & Prudden, 1966; Hoffmeister, Wenner, Wilkens, & Mukhtar, 1964; Prudden, Migel, Hanson, Friedrich, & Balassa, 1970; Yusof et al., 2003). Here, we selected chitin as a model to produce carbohydrates combined with growth factors. In future studies, we will use the same strategy for derivatives of chitin (chitosan) and other carbohydrates combined with growth factors.

3.5 Uptake and Release Kinetics of ChtBD-bFGF and bFGF

The adsorption ability of ChtBD-bFGF and bFGF binding to the chitin film are shown in Fig. 5A. More (11.4-fold) ChtBD-bFGF was absorbed than bFGF because ChtBD-bFGF contained ChtBD. Fig. 5B shows the in vitro cumulative release behaviors of ChtBD-bFGF and bFGF from the chitin films. These were characterized by the percentage release as a function of time. The bFGF without ChtBD adaptor has a high burst release (Fig. 5B); about 73% of the loaded bFGF was released within the first 48 h. The ChtBD-bFGF showed a sustained release behavior form the ChtBD-bFGF@chitin film. In detail, the ChtBD-bFGF@chitin film showed a 7.6% initial burst release within 48 h, but it was followed by a slower release over 7 days. The release kinetics showed that 90% ChtBD-bFGF was tightly bound to the chitin films after 7 days of release, and this can help the growth factors interact with receptors in a non-diffusible manner. In bFGF release
kinetics, 82% bFGF was released into incubation buffer after 7 days, and this indicated that the bFGF interacted with the receptor in a diffusible manner.

Previous studies have focused on binding bFGF on the surface of other materials. A fibrin-binding peptide Kringle1 (K1) derived from human plasminogen was fused to human bFGF. The recombinant K1bFGF showed high fibrin and plasma-clot-binding ability. On day 7, the K1bFGF remaining on the fibrin was 77.4% of the original amount—twice as much as bFGF (38.7%; p<0.005) (Wenxue Zhao et al., 2008). The collagen binding domain (CBD) of WREPSFCALS (V-bFGF) derived from von Willeband’s factor (vWF) and the TKKTLRT (C-bFGF) derived from collagenase were cloned into bFGF. The C-bFGF (25%) and V-bFGF (12%) had higher collagen binding ability than bFGF (WX Zhao et al., 2007). In our study, the binding ability of ChtBD-bFGF was 11.4-fold higher than bFGF for chitin films. This indicated that the ChtBD-bFGF had a higher binding efficiency than K1bFGF, V-bFGF, and C-bFGF.

3.6 Cell adhesion in ChtBD-bFGF@chitin Films

The BALB/C 3T3 cells were seeded and cultured in the ChtBD-bFGF@chitin films, bFGF@chitin films, and chitin films. After culture for 3 days, the morphology and adhesion of cells were observed with a confocal microscope. As shown in Fig. 6, the surface of the ChtBD-bFGF@chitin films had many cells with orderly and parallel actin filaments. On the surface of the bFGF@chitin films, the number of cells was clearly less than ChtBD-bFGF@chitin films. On the surface of the chitin films, cells were scattered on films. Actin filaments were disordered, and the cell morphology was irregular. These results indicate that the ChtBD-bFGF can change the surface performance of chitin films and promote cell proliferation.

To quantity the cellularization ability of the ChtBD-bFGF@chitin films, we randomly photographed the cells cultured on different groups on day 3. Fig. 6A showed that more cells grow on the surface of the ChtBD-bFGF@chitin films than bFGF@chitin films; there are few cells on the surface of the pure chitin films. Fig. 6B shows that the number of cells induced by ChtBD-bFGF was two-fold higher than bFGF. This result suggested that the cellularization ability was positively correlated with chitin binding ability.
3.7 Enhanced neo-vascularization during subcutaneous implantation by ChtBD-bFGF@chitin film

Accelerated wound healing by chitin films in presence of ChtBD-bFGF was evaluated in terms of increased vascularity. Neo-vascularization is a crucial and essential phase in remodeling and tissue regeneration (Wu, Chen, Scott, & Tredget, 2007). The endothelial cell-specific protein marker CD31 was used to evaluate neo-vascularization at the site of subcutaneous implantation. On day 7 after implantation, the immunofluorescence staining showed apparent neo-vascularization at the sites implanted with the ChtBD-bFGF@chitin film, bFGF@chitin film, and chitin film (Fig. 7); there was no obvious neo-vascularization in sites with the bFGF@chitin film or the chitin film. This indicated that ChtBD-bFGF@chitin film had a higher neo-vascularization ability than the bFGF@chitin film.

Neovascularization plays an important role during wound healing. An ideal wound dressings should promote angiogenesis. ChtBD-bFGF binding to chitin films continuously provides stimulation to receptors on the cell surface. Therefore, there are more new blood vessels in ChtBD-bFGF@chitin films than other groups. The ChtBD-bFGF@chitin film is a wound dressing with strong capacity for neovascularization.

3.8 The wound dressing mechanism for the ChtBD-bFGF@chitin film

Wound healing is a complex biological process. This process is regulated by different growth factors. The bFGF plays multiple roles during the various stages of wound healing. Many studies have shown that bFGF could promote epithelial healing, which is crucial for wound healing (Meduri et al., 2009). The defects in epithelial healing will result in a delayed wound healing. The process of re-epithelialization follows cell proliferation and blood vessel formation. In our study, we found that the special binding ability of ChtBD-bFGF and chitin films lead to a higher cell proliferation rate, but this is lower in the control group. In addition, the ChtBD-bFGF@chitin film has more capacity to accelerate neovascularization at the site of subcutaneous tissue than the other groups.

4. Conclusion

In this paper we developed a novel fusion protein ChtBD-bFGF with an exquisite
ability to promote cellularization and vascularization. ChtBD-bFGF can be localized longer at the surface of chitin films than bFGF. The uptake ability of chitin films for ChtBD-bFGF is 11.4-fold higher than bFGF. The ChtBD-bFG and bFGF have the same biological activity in inducing fibroblast proliferation. We found that the ChtBD-bFGF binding to chitin films could promote cell adhesion and proliferation. The histological analysis indicated that the ChtBD-bFGF@chitin films could induce vascularization. This ChtBD-bFGF@chitin film is a promising and effective strategy for wound dressings.

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References


Dermatology, 17(2), 1063-1072.


Zhao, W., Han, Q., Lin, H., Gao, Y., Sun, W., Zhao, Y., Dai, J., et al. (2008). Improved neovascularization and wound repair by targeting human basic fibroblast growth factor (bFGF) to fibrin. Journal of...
Molecular Medicine-JMM, 86(10), 1127-1138.

Fig. 1. Schematic illustration of ChtBD-bFGF binding to a chitin film for the wound healing. By applying a ChtBD-bFGF@chitin film on the skin wound site, the ChtBD-bFGF@chitin film acts as a wound dressing to promote cellularization and vascularization at the implanted site.

Fig. 2. The complex formed by the interaction between chains of ChtBD, bFGF and NAG.
Fig. 3. SDS-PAGE and western blotting of ChtBD-bFGF and bFGF fusion protein. (A) SDS-PAGE analysis of purified fusion proteins. M, protein marker; lanes 1-2, purified ChtBD-bFGF, and bFGF proteins, respectively. (B) Western blotting of purified ChtBD-bFGF and bFGF. Purified ChtBD-bFGF and bFGF proteins were analyzed by western blotting with mouse anti-bFGF and HRP-labeled rabbit anti mouse IgG antibody.

Fig. 4(A). Biological activity of bFGF and ChtBD-bFGF tested by stimulated BALB/C 3T3 cell proliferation. (B) Chitin-binding ability of bFGF and ChtBD-bFGF were detected by immunofluorescence. (a, ChtBD-bFGF@chitin; b, bFGF@chitin; c, chitin film). Error bars represent standard deviation for n=3.
Fig. 5. The adsorption ability of ChtBD-bFGF and bFGF binding to the chitin film (A). Release kinetics of ChtBD-bFGF and bFGF from the chitin film (B). Error bars represent standard deviation for $n=3$. 
Fig. 6. Cell morphology of BALB/C 3T3 cells seeded on (A) ChtBD-bFGF@chitin film, (B) bFGF@chitin film, and (C) chitin film were assayed by LSCM. The actin filaments (red) were stained by phalloidin and the cell nucleus (blue) was stained by DAPI. Scale bar 50 μm. (D) Proliferation of BALB/C cells on the surfaces of ChtBD-bFGF@chitin, bFGF@chitin, and chitin. Error bars represent standard deviation for n=3.
Fig. 7. Histological analysis of vascularization induced by ChtBD-bFGF@chitin films (A, D), bFGF@chitin films (B, E), and chitin films (C, F). Panels A, B, C are the HE stain. The white arrows indicate blood vessels. Panels D, E, F are CD31 immunofluorescence stain, and white arrows indicate endothelial (CD31) cell marker. Bar 200 μm.