Accelerated Vascularization of Silk Fibroin Scaffolds Through Immobilized Basic Fibroblast Growth Factor (bFGF)

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ABSTRACT

Angiogenesis of an implanted tissue is one of the most important issues in skin repairment. Porous bFGF-immobilized silk fibroin (bSF) scaffolds was fabricated by freeze drying using EDC agent crosslinking bFGF and SF with an aim of controlling bFGF delivery for the revascularization improvement. The bSF scaffolds retained a porous structure with 200~300 μm diameter and 85~91% porosity. The seeded L929 cells had notably improved growth and proliferation on bSF scaffolds, suggesting bFGF sustained release from SF scaffolds and remained bioactivity. After implantation into the skin defect of sprague-dawley (SD) rat dorsum, the bSF scaffolds revealed significantly higher wound recovery rate with complete reepithelialization and regeneration of skin appendages compared with SF scaffolds. The new vessels density and collagen deposition were also higher in bSF scaffolds after 4-week treatment, which provided the therapeutic potentials of the SF scaffolds in the regeneration of wounded tissue.

INTRODUCTION

Most skin wounds can heal naturally, but severe wounds are difficult to heal, owing to the lack of cellular and molecular signals to promote the angiogenesis which were required for normal wound repair. Various materials have been adopted to improve the wound skin recovery, including sponge, hydrogel and microspheres [1]. An ideal scaffold material as skin substitutes should provide a combination of suitable mechanical properties along with biological signals, which is required for successful wound skin regeneration in tissue engineering strategies [2].

Silk fibroin (SF) has been used as clinical sutures for centuries because of the impressive mechanical properties and controllable biodegradability [3]. SF materials support the attachment, proliferation and differentiation of many types of cell lines [4]. Due to the excellent biocompatibility, SF scaffolds as a popular starting material for tissue engineering have been used in the repair of many tissues, including skin, nerve and cartilage [5]. But pure SF porous scaffolds are usually
accompanied by scanty angiogenesis ability that prevents its graft application in skin. Basic fibroblast growth factor (bFGF) is a multifunctional polypeptide, which promotes growth and differentiation of a broad spectrum of cell types for wound healing, including dermal fibroblasts, endothelial cells and so on [6]. Because of the angiogenic and mitogenic properties, bFGF has been proved the stimulative effects in the regeneration of number of tissues such as cartilage, blood vessel, skin and bone [7]. In addition, the gradual release of bFGF remaining bioactivity in the later phase of wound healing process is essential to modulate the cell growth and extracellular matrix (ECM) remodeling [8]. However, topical application of bFGF directly to stimulate wound healing have been explored without significant improvement as its rapid diffusion and degradation in the open wound or injected into the body in soluble form [9]. Moreover, the long-term efficacy, stability and dose selection of those exogenous growth factors have prevented the therapy from becoming a common practice in treating. Therefore, prolongation, stabilization and localization of the bioactivity of bFGF over an extended time period are required in wound repair practically.

Controlled release or immobilization is very important for protecting and delivering growth factors. Immobilization protects growth factors against cellular inactivation and internalization of the receptor/ligand complex, thus prolonging bioactivity of growth factors [10]. Moreover, the covalent immobilization is preferred method over controlled delivery because the effect of growth factors is to be seen locally within the biomaterials rather than in the surrounding tissue [11]. As such, covalent immobilization of bFGF is an attractive approach to induce vascularization for tissue engineered constructs.

Thus we developed new sustained release system of bFGF using SF scaffolds as a carrier by covalent immobilization. In this study, the bSF (bSF) scaffolds with stabilized bioactivity of bFGF was prepared through freeze-drying method using EDC as corsslinking agent. The bSF scaffolds could be a valuable modality in promoting skin tissue regeneration as the release bFGF for a long period in a controlled manner increased its efficacy for improving the angiogenesis in the wound healing process.

**EXPERIMENTAL PROCEDURE**

**Preparation of regenerated SF scaffolds.** *Bombyx mori* silk fibroin (SF) solutions were prepared according to previous published procedures [12]. Briefly, it was degummed in a solution containing 0.2 wt.% Na₂CO₃ at 90~100°C for 60 min and repeated 3 times. The degummed SF was dissolved in a mixture of CaCl₂/CH₃CH₂OH/H₂O (1:2:8 molar ratio) at 72 °C for 1 h. The SF solution was obtained by dialyzing for 4 days to remove the excess salts. The EDC, NHS and MES were then added into the solution account for 20 %, 10 % and 20 % weight ratio against the total weight of SF in solution [13]. The bFGF solution (10 μg/mL) of 2 mL was added into SF solution of 40 mL. Then the mixed solution with continuously stirring at ice-water bath for 6 h and reacted at 4 °C overnight. The solution were collected from the column and then repeatedly washed with a centriprep (Millipore, M.W. 10000) by centrifugation to purify the product. After all, the mixed solution was poured into stainless steel dish, frozen at -40 °C for 12 h, followed by lyophilization for 48 h.
**Pore characteristics.** The scaffolds were imaged using a Hitachi S-4800 scanning electron microscope (SEM). The border of each pore in the top layer of SF scaffolds was defined according to a gradient method [14]. The images of the top layer of the cross-sections were obtained. Each pore area \( x_1, x_2, \ldots \) and the average pore diameter \( \bar{d} \) (μm) and porosity \( P \) (%) are given by Eqs.

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\begin{align*}
\bar{d} &= \frac{\sum_{i=1}^{n} \left( \frac{4x_i}{\pi} \right)}{n} \quad \text{(1)} \\
P &= \frac{\sum_{i=1}^{n} s_i}{S} \quad \text{(2)}
\end{align*}
\]

**Cell viability.** The scaffolds prepared as above section were cut into circular discs with the diameter of 15 mm and placed in 24-well plates, sterilized by \( \gamma \)-ray irradiation followed by rinsing with PBS prior to cells seeding. L929 cells at a density of \( 3 \times 10^5 \) cells/well were seeded onto the scaffolds. The growth and proliferation assay of bFGF responsive L929 cells was carried out and investigated by SEM and MTT assay. Cultures were selected for MTT assay on days 1, 3, 5, 7 and 9 d. Six MTT assay replicates were performed for each formulation and culture period.

**Grafting in vivo.** Sprague-Dawley (SD) rats (180~200 g, SPF grade, male) were maintained under general anesthesia during surgery. The full-thickness wounds (10×10 mm) were created on the upper back of each rat using a pair of sharp scissors and a scalpel. The full-thickness skins were separated by drum dermatome to obtain thin split thickness skin grafts. The bSF scaffolds as dermal substitutes were implanted into the defect sites followed by covering with thin split-thickness skin grafts. The surgical groups with SF scaffolds were used as controls. The wounds were covered by vaseline carbasus and dry carbasus, and then treated with circular bandages. After surgery, each rat was caged individually with free access to water and food.

**Graft viability.** The wound area was examined and photographed after treatment for 0, 2 and 4 weeks. The transplantation sites and boundaries were determined by scar lines and the absence of hair. The viable graft had an appearance similar to normal skin, whereas the necrotic tissue appeared brown and black, and even detached from the transplanted sites. The graft viability was assessed post grafting 2 and 4 weeks and determined according to the following equation [15]: graft viability = [(total area of skin graft)-(area of graft necrosis)]/total area of skin graft×100 %.

**Histology examination.** At 2 and 4-week post implantation, the animals were sacrificed and the scaffolds were recovered postoperatively. The fixed samples were processed for routine HE and Masson’s staining for histological analysis. The number of capillary vessels was marked and counted by two independent examiners blinded from at least 3 randomly selected fields of HE results under a magnification of 200 and analyzed using Pro 5.0 software (IBM, New York, USA).

**Statistical analysis.** Data were presented as means ± SD. Statistical comparisons were performed using ANOVA (T-test), and differences at \( P < 0.05 \) were considered statistically significant.
RESULTS AND DISCUSSION

Pore characteristics. The images of scaffolds in Fig 1 demonstrated that the bSF scaffolds had a continuous and interconnected network porous structure with average pore diameters in the range of 200～300 μm and porosities in the range of 85～91% (Fig 1, a). Moreover, the bFGF were immobilized on scaffolds and had no influence on internal pore structure of SF scaffolds (Fig 1, b).

Cells viability. The bioactivity of bFGF released from SF scaffolds was measured using culturing L929 cells in vitro. The SEM images showed that bSF scaffolds supported the adhesion and growth of L929 cells after 5-day incubation (Fig. 2, a). With the culturing time increased, the growth of cells were greatly improved on the bSF scaffolds significantly, suggesting that the SF scaffolds stabilized the bFGF bioactivity and the releasing of bFGF from SF scaffolds promoted the cell growth and proliferation (Fig. 2, b). Those results demonstrated that bSF scaffolds was beneficial to stabilize the bFGF bioactivity and enhance cells growth and proliferation, which was potential to be applied in wound sites.

Graft viability. Fig. 3 shows the representative images of wound healing with SF scaffolds and bSF scaffolds. There was no infection and scab in both SF scaffolds (a-0) and bSF scaffolds (b-0) after treatment. After 2-week operation, a higher wound recovery rate in bSF scaffolds compared with SF scaffolds. Most of the scab was falling off from the wound in bSF scaffolds group, and the wounds were mostly filled with hair growth (b-2). At week 4 after operation, morphological results showed that the regenerated skin of bSF scaffolds group were covered with epidermis, the defect disappeared and the wound areas were closed, which was similar to normal skin (b-4). The wound healing images were quantified to show the graft viability of each experimental group after 2 and 4 weeks treatment (Fig. 3 c). As shown in Fig. 3 c, the graft viability of bSF scaffolds groups were significantly higher than that of SF scaffolds groups (p<0.05) during the skin wound healing. The evaluation clearly indicated the faster wound healing rate of bSF scaffolds groups was closely related to the sustained release of bFGF.

Figure 1. Morphologies of bSF scaffolds (a) and bFGF on bSF scaffolds (b).

Figure 2. Cells growth (a) and viability (b) on bSF scaffolds (P < 0.05).
Histology examination. HE staining in Fig. 4 appeared that host tissue in-growth into the pore space of the scaffolds was observed in all SF scaffolds, but the extent of blood capillary in-growth into the scaffolds was different after 2-week implantation. The capillary in-growth of SF scaffolds was low (a-2), while that were high in bSF scaffolds (b-2). And more new vessels and granulation tissue were detected in bSF scaffold than that in the SF scaffold (b-4), which indicated that local sustained delivery of bFGF from SF scaffolds was effective to induce angiogenesis in the inner and outer part of the implanted scaffolds. At the fourth week, the number of mature capillary vessels in bSF scaffolds were significantly increased (Fig. 4, c). It clearly demonstrated that sustained release of bFGF in SF scaffolds induced the formation of large and mature blood vessels, as judged by the massive layer of mural cells surrounding endothelial cells, and improved tissue recovery after implantation. In addition, the bFGF should induce strong proliferation on numerous inflammatory cells, high metabolic rate and low oxygen content in regenerated tissues, which was supposed to promote vasculogenesis. From this perspective, bSF scaffolds can be utilized to accelerate wound healing at the damaged tissue site where blood supply is insufficient and regenerate various tissues with built-in angiogenic activity.

Fig. 5 shows the deposition of collagen by Masson’s staining in the different SF scaffolds at different time points. The collagen production was clearly observed in all SF scaffolds at week 2 after treatment, and more collagen fibers were observed in the bSF group (b-2). With the prolongation of wound healing, the deposition of collagen increased and the distribution of collagen was more uniform. The alignment of collagen fibers in bSF scaffolds was lined up in order, which was similar to the normal dermal tissue after 4 week operation (b-4). The alignment of the collagen exhibited the similar development tendency in SF scaffolds (a-4), but the collagen production was slower than that in bSF scaffolds. Masson’s staining results showed that the collagen fibers were uniform and well oriented in all SF scaffolds, especially in bSF scaffolds, owing to the gradual release of bFGF from SF scaffolds enhanced collagen deposition and promoted the arrangement of collagen fibers to similar to the normal tissues. These findings were corroborated with HE staining.
CONCLUSIONS

The bsF scaffolds were prepared by lyophilization using EDC as crosslinking agent for covalent immobilization of bFGF on SF. The bFGF were immobilized on SF scaffolds, and maintained bioactivity to improving the growth and proliferation of cells greatly in vitro. The bsF scaffolds revealed significantly higher wound recovery rate compared with SF scaffolds. Higher blood vessel density and collagen extents were achieved in bsF scaffolds after 4 wk treatment in vivo, indicating the gradual release of bFGF from SF scaffolds promoted the reconstruction of wound skin. This study presents an alternative method for immobilization of SF via covalenting bFGF, and provides a potential use of the SF scaffolds for the regeneration of wounded tissue.

ACKNOWLEDGMENTS

This work was supported by the National Nature Science Foundation of China (51403146, 51302177) and Priority Academic Program Development of the Jiangsu Higher Education Institutions.

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