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Biomimetic fibroblast-loaded artificial dermis with "sandwich" structure and designed gradient pore sizes promotes wound healing by favoring granulation tissue formation and wound re-epithelialization

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ABSTRACT

The structure of dermal scaffolds greatly affects the engineered tissue's functions and the activities of seeded cells. Current strategies of dermal scaffold design tend to yield a homogeneous architecture with a uniform pore size. However, the structures of the human dermis are not homogeneous in terms of either interstitial spaces or architecture at different dermal depths. In the present study, a biomimetic fibroblasts-loaded artificial dermis composed of three-layer scaffolds with different pore sizes was prepared. The three-layer scaffolds, which look similar to a sandwich, mimic the natural structures of the human dermis, which has comparatively larger pores in the outer layers and smaller pores in the middle layer. The fibroblasts-loaded artificial dermis were shown to favor wound healing by promoting granulation tissue formation and wound re-epithelialization, as determined by a histological study and Western blotting. Our data indicated that the biomimetic fibroblasts-loaded artificial dermis with "Sandwich" structure and designed gradient pore sizes may hold promise as tissue-engineered dermis.

Statement of Significance

Pore size effect on wound healing had been extensively studied. However, it is still not well understood whether dermal scaffolds with a uniform pore size are better than that with varied pore sizes, which are similar to human dermis as determined by our previous work. In our study, we demonstrated that the "sandwich" collagen scaffolds mimicking the natural structures of the human dermis significantly promoted wound healing compared with the "Homogeneous" scaffolds with a uniform pore size. These results may be helpful in the design of dermal scaffolds.

1. Introduction

The skin is the largest human organ and accounts for approximately 15% of the total body weight. Extensive skin defects can cause a life-threatening water-electrolyte imbalance and microbial invasion as well as severe contracture due to scarring. Autologous skin grafting is currently the most widely used treatment for full-thickness skin injuries. However, skin autografts have many limitations, including donor site morbidity and limited donor site availability in the case of extensive skin defects. Tissue-engineered skin is believed to provide significant advantages over traditional treatment methods [1]. Porous collagen dermal scaffolds are widely used as tissue-engineered skin because porous structures can provide a microenvironment for cell proliferation to form new tissues [2] and because collagen plays a key role in wound healing [3,4].

The pore size of dermal scaffolds has been reported to greatly affect cell adhesion, migration and proliferation [5–7]. For example, fibroblasts were reported to preferentially adhere to pores with a diameter of 38–150 μm in poly-L-lactic acid (PLLA) scaffolds and to pores measuring greater than 90 μm in porous silicon nitride scaffolds [8,9]. The situation is different for endothelial cells, which cannot cover pores measuring greater than 80 μm in porous silicon nitride scaffolds [8]. Our previous study showed that

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the pore size of human acellular dermal matrix varied widely (131.2 ± 96.8 μm). Moreover, dermal fibers were less frequently found in the dermal regions close to the epidermis and hypodermis than in the middle dermal region, indicating that the interstitial spaces are comparatively larger in the outer dermal regions than between the two regions [10].

The literature indicates that there is an "optimal" pore size for each specific scaffold material and cell type. Unfortunately, no scaffold has been designed according to the natural gradient pore sizes of the human dermis. Instead, the most popular way to design the pores of dermal scaffolds is to try to create a homogeneous architecture and a uniform pore size [5,6]. Whether scaffold design using natural gradient pore sizes can improve wound healing is thus unclear.

Based on the above analysis, we attempted to study the effects of dermal scaffolds with different pore sizes on both the activities of seeded cells and wound healing.

2. Materials and methods

2.1. Materials and animals

Sprague–Dawley (S–D) rats (230 to 320 g, male) and BALB/c mice (15–20 g, male) were purchased from the Experimental Animal Department of the Third Military Medical University and were adaptively bred for two weeks before the experiments. The animals were raised individually under standardized conditions (room temperature: 25 °C; circadian rhythm: 12 h; relative humidity: 50%) with free access to water and autoclaved standard rodent chow. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Third Military Medical University.

Collagen type I was isolated from fresh tail tendons from S-D rats, as described by Ma et al. [11]. Briefly, the rats were sacrificed using high concentrations of carbon dioxide. The tail tendons were acquired and cut into pieces as thinly as possible and then incubated in 0.5 M acetic acid (HAc) at 4 °C for 48 h with tissue trituration to agitate the swollen tendon pieces. After centrifugation, the supernatant was acquired and precipitated using 5 wt% NaCl solution. The precipitate was dialyzed with double-distilled water for 24 h and then re-dissolved in 0.5 M HAc.

The collagen concentration in the extracted solution was determined based on the hydroxyproline (Hyp) content, which was measured using a Hydroxyproline Detection Kit (A030-2, Nanjing Jiancheng Bioengineering Institute, http://www.njjcbio.com/). As Hyp accounts for 13.4% of collagen, the collagen concentration was calculated using the following formula:

$$\text{Collagen concentration (wt\%)} = \text{Hyp concentration (wt\%)}/13.4\%.$$

The collagen solution was then diluted using different amounts of 0.5 M HAc to obtain a series of collagen solutions with three different concentrations (0.26%, 0.19% and 0.13%).

2.2. Preparation of collagen scaffolds with different pore sizes

To evaluate the effect of pore size on the functions of engineered dermis and the proliferation of seeded cells, we prepared a series of scaffolds with different pore sizes, which depended on the concentration of collagen solution used.

The collagen solutions with three different concentrations (0.26%, 0.19% and 0.13%) were transferred into separate wells in 12-well plates using a pipette. The collagen solutions were frozen at −80 °C for 4 h and then freeze-dried for 48 h in a Super Modulyo freeze dryer (Thermo, USA). The freeze-dried scaffolds were placed in 25% aqueous glutaraldehyde solution in a closed box for 6 h at 37 °C for cross-linking. To block the unreacted aldehyde groups, the constructs were immersed in 0.1 M aqueous glycine solution for 48 h and then washed with deionized water five times [5].

2.3. Determination of scaffold pore sizes and microstructures

The mean pore size of the porous collagen scaffolds was observed using hematoxylin–eosin (H&E) staining. Briefly, scaffolds were fixed in paraformaldehyde at 4% (v/v) for 24 h. After dehydration and paraffin infiltration, the embedded specimens were cross-sectioned at a thickness of 6 μm using a Leica microtome. Sections of the scaffolds were then stained with H&E. Five images were acquired for each scaffold, and three pores in each image were randomly chosen for pore size calculation by two independent pathologists using Image-Pro Plus 6.0 (IPP 6.0) software (Media Cybernetics, USA) [12].

The microstructures of the porous collagen scaffolds were observed using a scanning electron microscope (SEM; Hitachi, S-3400N, Japan). The scaffolds were dried, cross-sectioned, sputter-coated with Au for 60 s and observed under high-vacuum conditions at an accelerating voltage of 15.0 kV.

The gross appearance, H&E staining and SEM images of the porous collagen scaffolds are shown in Fig. 1. The mean pore size of the scaffolds made from 0.26%, 0.19% and 0.13% collagen solutions were 87.7 μm, 120.4 μm and 166.9 μm, respectively (Fig. 2A). The prepared porous collagen scaffolds with different pore sizes were used in the following experiments.

2.4. Measurement of scaffold biodegradation in vitro

The *in vitro* biodegradation of the porous collagen scaffolds was measured based on the release of Hyp, which is an indicator of collagen degradation, under the condition of collagenase digestion [11]. Scaffolds with different pore sizes were immersed in 100 mg/ml (28 units) collagenase (type I, Sigma) solution at 37 °C for 4, 13, 28 or 48 h. The collagenase digestion was terminated in an ice bath after the desired time interval. The Hyp content released from each scaffold was measured using a Hydroxyproline Detection Kit (A030-2, Nanjing Jiancheng Bioengineering Institute, http://www.njjcbio.com/).

2.5. Culture and seeding of mouse fibroblasts

Fibroblasts were isolated from neonatal BALB/c mice, as described by Cheng et al. [13]. Briefly, skin tissue was harvested from the mice and washed with PBS three times and then immersed in 0.5 mg/ml Dispase II (Sigma, USA) at 4 °C overnight. The epidermis and dermis were then separated, and the dermal tissue was cut into pieces, followed by digestion in 2 ml 0.25 mg/ml trypsin (Boster, China) at 37 °C for 10 min. Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) was added to stop the digestion. After centrifugation (1000 rpm, 10 min), the suspension was collected and incubated in DMEM containing 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 incubator.

The porous collagen scaffolds were punched into 4 mm-diameter discs that were immersed in 75% alcohol for 30 min for sterilization and then washed with PBS five times. After that, the scaffolds were immersed in cell culture medium for 1 h, followed by placement into separate wells of a 96-well plate. Third-passage fibroblast suspension (3.7×10^4/ml, 50 μl) was loaded onto one side of the scaffolds and incubated for approximately 30 min to allow the cells to adhere to the scaffolds. The scaffolds were then turned over, and another 50 μl fibroblast suspension was loaded onto the other side of the scaffolds. The scaffolds with loaded cells were then incubated at 37 °C in a 5% CO_2 incubator.