Enhanced angiogenesis of gene-activated dermal equivalent for treatment of full thickness incisional wounds in a porcine model

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A B S T R A C T

Angiogenesis of dermal equivalent is one of the key issues for treatment of full thickness skin defects. To develop a gene-activated bilayer dermal equivalent (BDE), N,N,N-trimethyl chitosan chloride (TMC), a cationic gene delivery vector, was used to form complexes with the plasmid DNA encoding vascular endothelial growth factor-165 (VEGF-165), which was then incorporated into a collagen–chitosan/silicone membrane scaffold. To evaluate the angiogenesis property in vivo, full thickness skin defects were made on the back of pigs, into which the TMC/pDNA-VEGF complexes loaded BDE and other three control BDEs, i.e. the blank BDE, and the BDEs loaded with pDNA-VEGF and TMC/pDNA-eGFP complexes, respectively, were transplanted. Biopsy specimens were harvested at day 7, 10 and 14 after surgery for histology, immunohistochemistry, immunofluorescence, real-time quantitative PCR (RT-qPCR) and western blotting analyses. The results showed that the TMC/pDNA-VEGF group had the strongest VEGF expression in mRNA and protein levels, resulting in the highest densities of newly-formed and mature angiogenesis in the healing skin. At 112 days grafting, the healing skin had a similar structure and ~80% tensile strength of the normal skin.

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1. Introduction

As the largest organ of body, skin loss caused by trauma, burn and chronic diseases has become one of the most serious problems in clinic [1–3]. Millions of patients suffering from skin loss need the treatment of skin substitutes such as wound dressings, allografts and autografts. However, traditional autografts are limited by the timely availability and donor sites. Recent advances in regenerative medicine and tissue engineering have expanded the understanding of wound healing and developed diverse methods for skin repair and regeneration. The skin defects in full thickness cannot repair autologously because of the lack of dermis. Therefore, a proper dermal equivalent, which functions as a regenerating template in wound healing, is highly demanded.

In the past decades, numerous dermal equivalents have been developed and applied for the treatment of full thickness skin defects in hospital. Integra, the first bilayer dermal equivalent (BDE) developed by Yannas and his coworkers [4–9], contains a collagen/chondroitin sulfate porous scaffold and a silicone membrane. The porous scaffold offers an extracellular matrix analog which functions as the artificial dermis template for host cells infiltration, proliferation and differentiation, whereas the silicone membrane serves as a temporary epidermis which play a role in controlling water loss and inhibiting bacterial entry until an ultra-thin skin graft is transplanted. No pre-cell seeding is needed inside such a type of BDEs thus they are easily manufactured, preserved and applied with very low production cost.

In our previous studies [10–14], a BDE composed of a collagen–chitosan porous scaffold and a silicone membrane has been developed. In the preliminary animal test, the BDE has shown positive effects in the healing of full thickness skin defects, whose complete vascularization takes place within 4 weeks. To develop the next generation BDE of higher performance, a faster rate of angiogenesis is highly demanded, which can thereby shorten the time of post-transplantation of ultra-thin skin graft, decrease the risk of infection, and improve the healing effects.

Angiogenesis is one of the major issues in wound repair, which is indispensable for delivery of oxygen and nutrients to cells on the wound sites. So far many approaches have been developed to
enhance angiogenesis of the engineered constructs, among which incorporation of angiogenic factors such as bFGF, VEGF and PDGF etc. is most powerful [15–21]. However, delivery of the growth factors is sophisticated due to their sensitivity and instability, since the half-lives of which are only in the order of minutes in serum. By contrast, loading of functional genes into the scaffolds (the so-called gene-activated matrix, GAM) is an attractive way to locally produce the growth factors, which can then avoid the many problems of growth factor delivery. The plasmid DNA delivered from the GAM can increase the transfection efficiency compared to direct injection, and to a great extent block the release of the DNA into the circulatory system [22]. So far the GAMs have been employed to promote regeneration of several tissues such as bone, cartilage and skin [23–25]. For example, Shea et al. [22] incorporated plasmid DNA encoding platelet derived growth factor (PDGF-B) into a three dimensional poly (lactide-co-glycolide) (PLGA) sponge, implantation of which in a rat dermis showed enhancement of granulation tissue and vascularization. Other types of matrices such as collagen and PVA sponges loaded with genes were also developed and used to treat cutaneous wound, resulting in improvement of flap survival, granulation tissue formation, angiogenesis and reepithelization [26–28].

It is known that the angiogenesis is controlled to a large degree by levels of VEGF [29,30], a 35–45 kDa homodimeric glycoprotein, which can stimulate the proliferation, migration of endothelial cells and the subsequent formation of new capillary tubes. In our previous study, a non-viral gene vector, N,N,N-trimethyl chitosan chloride (TMC) (TMC) was synthesized [31] and applied to condense the plasmid DNA encoding VEGF (pDNA-VEGF). The TMC/pDNA-VEGF complexes were further incorporated into a collagen sponge. Subcutaneous implantation of this GAM in mice demonstrated that the angiogenesis was enhanced significantly [32]. However, their applicability in the BDEs for treatment of full thickness skin loss is not known so far.

Encouraged by all of the previous results, in the present study the BDE of collagen–chitosan porous scaffold and silicone membrane is combined with the gene therapy by loading the TMC/pDNA-VEGF complexes to obtain a gene-activated BDE. Its angiogenesis ability in the treatment of full thickness incisional wounds is assessed in a porcine model. Finally, transplantation of ultra-thin skin grafts on the gene-activated BDEs-treated wounds is further performed to demonstrate the complete repair results of full thickness skin wounds.

2. Materials and methods

2.1. Materials

Plasmid DNA encoding enhanced green fluorescence protein (pDNA-eGFP) was a gift from Dr. Jun Li, State Key Laboratory of Diagnosis and Treatment for Infectious Diseases (China). Plasmid DNA encoding human vascular endothelial growth factor-165 (pDNA-VEGF-165) was donated from Dr. Ming Yu, The Fourth Military Medical University (China). The plasmid DNA was amplified by Escherichia coli growing in Luria–Bertani medium and purified by a differential precipitation method [33]. The plasmid DNA was lyophilized and stored at −20 °C before use. Collagen type I was isolated from fresh bovine tendon as described previously [12]. Chitosan (deacetylation degree 90%, Mw = ~6 k) for TMC synthesis was purchased from Qingdao Haidabei Co., Ltd (China). Chitosan (deacetylation degree 75–85%, Mw: 10 × 10³–1.7 × 10⁴) for the scaffold fabrication was purchased from Sigma. Silicone membrane is a medical grade product from Shanghai Xincheng Co., Ltd (China). All other reagents were of analytical grade and used as received. Triple-distilled water was used throughout the experiments.

2.2. Synthesis of TMC and preparation of TMC/DNA complexes

N,N,N-trimethyl chitosan chloride (TMC) was synthesized according to the method reported previously [31]. The chemical structure of the product was characterized by 1H NMR spectroscopy. The peaks at 3.2 ppm and 2.0 ppm were assigned to −N(CH3)3 and −COCH3, respectively, according to which the degree of quaternization of 38% was found.

TMC and DNA were dissolved in PBS (pH 7.2) to form solutions of 10 mg/ml and 5 mg/ml and then filtered through syringe filters with a pore size of 0.22 μm for sterilization, respectively. To prepare the TMC/DNA complexes, 1.52 ml TMC solution was added into 0.48 ml DNA solution, vortexed violently for 30 s, and incubated for 30 min at 37 °C. The N/P ratio (molar ratio of amine group to phosphate group) used in this study was fixed at 10, since the TMC/DNA complexes with this ratio have the highest transfection efficiency in vitro [31].

2.3. Fabrication of gene-activated dermal equivalent

The collagen–chitosan/silicone membrane bilayer dermal equivalents (BDEs) were prepared according to the procedures described previously [14]. 1 ml sterilized PBS containing TMC/DNA complexes (1 mg/ml DNA) was dropped onto the dried BDE, which was then kept at 4 °C overnight to facilitate the complete incorporation of the TMC/DNA complexes. These DNA loaded-BDEs were carefully washed twice in PBS and then lyophilized to obtain the gene-activated BDEs. The eventual loaded amount of DNA on the BDEs was assessed using Hoechst 33258 dye and fluorometer (LS55, Perkin–Elmer, U.K.) as described previously [31], which was about 500 μg per BDE. Four groups of BDEs with a diameter of 30 mm and thickness of 2 mm were prepared in this study, i.e. the blank BDEs, and the BDEs loaded with naked pDNA-VEGF, TMC/pDNA-VEGF, and TMC/pDNA-eGFP, respectively. The last one was used as a control to study the potential influence of non-functional plasmids.

2.4. Scanning electron microscopy (SEM) observation

The cross-sections of the BDEs were examined by SEM (Hitachi, S-3000N) with an accelerating voltage of 25 kV after the samples were sputter-coated with a thin gold layer.

2.5. Animal test in a porcine model

The healing properties of the gene-activated BDEs on full thickness skin defects were studied in a porcine model. Briefly, Bama miniature pigs weighing about 15 kg were fasting for 12 h, and were then anesthetized by intraperitoneal injection of pentobarbital sodium solution (45 mg/kg body wt). Before surgery, the dorsal surgical area was shaved and sterilized with 5% povidone-iodine (PVP-I), a full thickness incisional wounds with a diameter of 3 cm were made symmetrically by scissors on the back of each pig. In this experiment, a polypropylene (PP) wound isolation chamber was implanted into each wound to inhibit the natural skin shrinkage. Then the four types of sterilized BDEs were applied on the wounds following the surgical process illustrated in Fig. 1. The tissue samples were harvested at 7, 10 and 14 days post-surgery. Totally 36 wounds were created and analyzed in the transplantation step.

To examine the overall healing property of the gene-activated BDEs, ultra-thin skin grafts with a thickness of 0.4 mm was transplanted on the full thickness wounds after treated with TMC/pDNA-VEGF loaded BDE for 10 days. The PP wound isolation chamber was removed before skin grafting. The wounds treated by petroleum gauze were used as control. At 21, 42, 70 and 112 days after grafting the tissue samples were harvested. Totally 24 wounds were studied in this step. All the tissue samples were cut in a full thickness manner from the transplant plantation sites, and then were used for the histological analyses, or frozen in liquid nitrogen for molecular and protein analyses and were maintained at −80 °C until processing.

2.6. Histology

For histological analyses, the harvested samples were fixed in 4% formaldehyde PBS solution at 4 °C, dehydrated with a graded series of ethanol and embedded in paraffin. The sectioned samples with a thickness of 5 μm were then stained by hematoxylin–eosin (H&E), and visualized by an optical microscope.

2.7. Immunohistochemistry and immunofluorescence

To study the angiogenesis behavior during the wound healing, three key factors related to the angiogenesis, i.e. VEGF, CD31, and alpha smooth muscle actin (α-SMA) were detected by immunohistochemistry or immunofluorescence. For immunohistochemical staining, the paraffin sections (5 μm) were deparaffinized and washed three times in PBS for 5 min, blocked with 5% serum for 30 min. Then the slides were exposed to mouse anti-VEGF primary antibody (1:50, Abcam, Cambridge, UK) and rabbit anti-CD31 primary antibody (1:200, Abbiotec, CA, USA) at 4 °C overnight, respectively. After rinsed three times with PBS, the slides were incubated with goat-anti-mouse secondary antibodies or goat-anti-rabbit secondary antibody (1:200, Dako, CA, USA) at 37 °C for 20–30 min, developed with 3,3’-diaminobenzidine tetrahydrochloride (DAB) solution and finally counterstained with hematoxylin. Positive staining was indicated by a brown color observed under an optical microscope. The number of newly-formed blood vessels was counted by CD31-positive staining per area.

Immunofluorescence was performed following the procedures described previously [34]. Briefly, paraffin sections (5 μm) were deparaffinized, washed three times in PBS for 5 min, blocked with 5% serum for 30 min and incubated overnight at 4 °C with rabbit anti-CD31 primary antibody (1:100, Abbiotec, CA, USA) and mouse anti-α-SMA primary antibody (1:50, Abcam, Cambridge, UK). After rinsed three times
with PBS, the slides were incubated with rhodamine-conjugated goat anti-rabbit secondary antibody and FITC-conjugated goat anti-mouse secondary antibody (1:50; Dako, CA, USA) for 30 min. After washed three times in PBS, the cell nuclei were stained by DAPI (Sigma-Aldrich, Buchs, Switzerland) for 10 min at room temperature. Images were acquired with an Olympus IX 81 fluorescence microscope. The number of mature vessels was counted by dually SMA-positive and CD31-positive vascular structures per area.

2.8. Real-time quantitative PCR (RT-qPCR) analysis

Total RNAs of the tissues were extracted using Trizol Reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions [35]. 1 μg of total RNA was used for reverse-transcribed into complementary DNA (cDNA) by M-MLV Reverse Transcriptase CDNA synthesis kit (Promega, WI, USA). Gene-Primers for human VEGF, and pig VEGF, CD31 and α-SMA as well as the calibrator reference gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) were summarized in Table 1. The real-time PCR was performed using iQ SYBR green PCR Master Mix (Bio-Rad, CA, USA). The samples were subjected to the following conditions in a One iCycler iQ5 (Bio-Rad, CA, USA): initial denaturation at 95 °C for 10 min followed by 45 cycles of 94 °C for 5 s, 62 °C for 20 s. Ct (threshold cycle) values were calculated using the iQ5 optical system software (Version 2). The mathematical model previously described in detail [36] was used to determine the expression ratio of genes.

2.9. Western blotting analysis

The frozen tissue samples were completely homogenized in RIPA lysis buffer (150 mm sodium chloride, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mm Tris, pH 8.0) having protease inhibitors. The lysates were then clarified by centrifugation at 12 000 rpm for 15 min at 4 °C, and separated on SDS-PAGE. After being transferred to a PVDF membrane (Millipore, MA, USA), the proteins were incubated overnight with antibodies and detected with ECL (ECL Western Blotting Substrate, Pierce, USA) system following treatment with 5% milk powder in TBS to prevent non-specific reaction. The specific antibodies used for this experiment were mouse anti-VEGF primary antibody (Abcam, Cambridge, UK), rabbit anti-CD31 primary antibody (Abbiotec, CA, USA) and mouse anti-α-SMA primary antibody (Abcam, Cambridge, UK).

2.10. Mechanical test of the healing skin

Tensile strength of the healed full thickness skin was tested via a universal testing machine (Instron, model 5543, High Wycombe, UK). Standardized strip specimens (6 mm × 50 mm) were cut from the middle of each wound and stored in normal saline for a few hours before the mechanical test. The accurate width and thickness of each tissue sample was determined by a slide caliper. The tensile property of the sample was measured at room temperature with a 5 mm/min crosshead speed using 1000 N load cell up to rupture. The tensile strength was

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![Fig. 1. Surgical process of BDE transplantation into the full thickness wounds of a miniature pig. The left-bottom shows the transplanted sites of different BDEs.](image)

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**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Anneal. Tm.</th>
<th>bp</th>
<th>Source b</th>
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<td>E14233</td>
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<tr>
<td></td>
<td>R GAGGCCATCGCTGCACTCA</td>
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<td></td>
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<tr>
<td>Pig VEGF</td>
<td>F GATCGCCGAGACGGTGTAAATGTTCC 62°C 77</td>
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<td></td>
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<td></td>
<td>R CGTTCGTTTAACTCAAGCTGCCTC</td>
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<td>Pig CD31</td>
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<td></td>
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<tr>
<td></td>
<td>R AGACTCCACCTCTGGCTCAG</td>
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<td>Pig α-SMA</td>
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<td></td>
<td>R CCTCGCCACCAAAGTCGTTTCC</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pig GAPDH</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R ETGCTTCTTGGAAGATGTTGAT</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

a indicates sequence of forward (F) and reverse (R) primers. 
b indicates GeneBank accession numbers where applicable.
defined as the ratio of the maximum load at rupture and the cross-section area of the sample. The wound treated with petrolatum gauze dressing was used as a control.

2.1. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed by two-tailed Student's t-tests between two groups or by one-way ANOVA between more groups. All statistical computations were performed using SPSS version 16.0 (SPSS Inc., Chicago, USA). The significant level was set as p < 0.05.

3. Results

3.1. Macroscopic shape and microstructure of gene-activated BDE

As shown in Fig. 2A, the gene-activated BDEs consisted two layers, i.e. the collagen–chitosan scaffold with a foam-like appearance and the silicone membrane. All other control BDEs had the same macroscopic shape. The gene-incorporated collagen–chitosan scaffold had an average pore size of ~100 μm (Fig. 2B) and a porosity of 92% measured by ethanol inhalation [37]. These values were smaller than that of the control collagen–chitosan scaffold (Fig. 2D), which was ~150 μm and ~95%, respectively. All pores were highly interconnected with each other to form the open pore structure regardless of the existence of TMC/DNA complexes. Tiny particles were observed on the slices of the DNA-incorporated scaffold (Fig. 2C), which are indicative to the TMC/DNA complexes by comparison to the control collagen–chitosan scaffold.

3.2. Gross observation of wound healing process

Fig. 3 shows the gross observations of wounds treated with the four types of BDEs after transplantations for 7, 10 and 14 days. After transplantation, all the wounds became from relatively white at day 7 to reddish at day 14. At each time interval, the wounds treated by the TMC/pDNA-VEGF loaded-BDEs looked more reddish than those treated by other BDEs. For example, the collagen–chitosan scaffolds with white color were clearly visualized at day 7, but less scaffold remnants were found for the TMC/pDNA-VEGF loaded-BDEs (Fig. 3A–D). At day 10, all the scaffolds integrated well with the wounds, in particular for the TMC/pDNA-VEGF group, but there were still some unintegrated scaffolds in the blank, pDNA-VEGF and TMC/pDNA-eGFP groups (Fig. 3E–H). At day 14, all the wounds showed ruddy surface, but the wounds treated by the TMC/pDNA-VEGF loaded-BDEs showed the reddest appearance, implying the better vascularization of the implanted scaffolds (Fig. 3I–L).

3.3. Histology

Histological analyses were performed to assess the regenerated tissues guided by the different types of BDEs, and the results are present in Fig. 4 and quantitatively summarized in Table 2. At day 7, there was a typical infiltration of granulocytes in all the groups. Macrophages could also be observed at this stage. The implanted scaffolds could be clearly distinguished from the host tissues. Compared to the fewer infiltrated fibroblasts in the blank BDEs group (Fig. 4A), the fibroblasts had infiltrated throughout the scaffold of the TMC/pDNA-VEGF group (Fig. 4C). At day 10, the numbers of granulocytes decreased in all the groups, and only a few of macrophages still existed. All the scaffolds were integrated well with the surrounding tissues (Fig. 4E–H). Specifically, in the TMC/pDNA-VEGF group there was no obvious gap between the newly-formed tissues and the implanted scaffold, in which a large number of vessels could be observed (Fig. 4G). At day 14, the moderate inflammatory responses still existed in the blank and TMC/pDNA-
eGFP groups. However, only very few definitive inflammatory cells were found in the TMC/pDNA-VEGF group, while the vessels were more apparent and their numbers were increased pronouncedly too (Fig. 4K).

3.4. Immunohistochemistry and immunofluorescence

To examine the existence of VEGF of the BDEs-treated wounds, immunohistochemistry staining was performed and the results are displayed in Fig. 5. Positive staining of VEGF was observed for all the tissue samples regardless of the implantation time, but its level depended greatly on the type of BDEs and the implantation time. At day 7 and day 10, the regenerated tissues of the TMC/pDNA-VEGF group showed more abundant VEGF than those of other groups (Fig. 5A–H). Comparatively, at this stage the samples of the pDNA-VEGF group showed stronger positive staining than the other two groups too. After transplantation for 14 days, all the groups showed strong positive staining (Fig. 5I–L).

The newly-formed vessels of the wounds were characterized by CD31 staining (Fig. 6), from which the average vessel density is quantified and summarized in Fig. 7. The vessel density is defined as the number of staining positive for CD31 per mm². It shows quantitatively that the number of vessels increased monotonously along with the prolongation of the implantation time for all the groups. However, the absolute vessel number is different. At day 7, the vessel density of the TMC/pDNA-VEGF group was found to be the highest (56 ± 7 per mm²), but had no significant difference with that of the pDNA-VEGF group (54 ± 4 per mm²). They are both significantly larger than that of the blank (36 ± 6 per mm²) and TMC/pDNA-eGFP BDEs (37 ± 6 per mm²). At day 10 and day 14, the vessel densities of the TMC/pDNA-VEGF group were increased to 87 ± 10 and 107 ± 7 per mm² and significantly higher than those of other groups of the same time, respectively. At all the time intervals, no significant difference was found between the blank and TMC/pDNA-eGFP groups in terms of the vessel density, implying that incorporation of the non-functional gene has no effect on generation of vessels in the scaffolds. It has to mention that the vessel density in the TMC/pDNA-VEGF group did not increase unlimitedly but remained in a reasonable level after transplantation of the ultra-thin skin graft. For example, the number of vessels was found to be 68 ± 7 and 50 ± 8 per mm² after transplantation of the ultra-thin skin for 14 days and 21 days on the wounds treated by the TMC/pDNA-VEGF loaded-BDEs for 10 days, respectively.

As illustrated in Fig. 8, mature vessels were further characterized with immunofluorescence by co-staining CD31, a marker of endothelial cells, and α-SMA, a marker of smooth muscle cells around the vessels [38, 39]. The number of the mature vessels was summarized quantitatively in Fig. 9. Again, the numbers of the mature vessels increased monotonously along with the implantation time for all the groups, but their absolute values were smaller than that of the total number of vessels shown in Fig. 7. Moreover, the number of mature vessels of the TMC/pDNA-VEGF group was always significantly higher than those of the other groups in all the detection time. At day 7, the pDNA-VEGF group had significantly higher mature vessels than the TMC/pDNA-eGFP group. However, at day 10 and day 14, no significant difference was found among these control groups.

3.5. RT-qPCR analysis

Three key factors related to angiogenesis, i.e. VEGF, CD31 and α-SMA, were further characterized by their gene expression using
RT-qPCR. Firstly, human mRNA for VEGF was only found in the samples of pDNA-VEGF and TMC/pDNA-VEGF groups, whose values increased until 10 days and then decreased significantly at 14 days (Fig. 10A). But the TMC/pDNA-VEGF group always had a higher mRNA expression than the pDNA-VEGF group, and their difference was enlarged along with the time prolongation (from 1.5 times at day 7 to 5 times at day 14). The pig mRNAs for VEGF, CD31 and \( \alpha \)-SMA showed a different alteration pattern, which increased monotonously along with the time prolongation regardless of the groups (Fig. 10B–D). At day 7, the TMC/pDNA-VEGF group showed significantly higher mRNA levels for all the factors than those of the blank control and the TMC/pDNA-eGFP groups, but no significant difference with the pDNA-VEGF group. At day 10 and day 14, the mRNA levels of the TMC/pDNA-VEGF group were significantly higher than those of other groups regardless of the types of genes.

Table 2  
Tissue response to the implanted BDEs.

<table>
<thead>
<tr>
<th>Time</th>
<th>Sample</th>
<th>Scaffolds</th>
<th>Macrophages</th>
<th>Granulocytes</th>
<th>Fibroblasts</th>
<th>Capillaries</th>
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<tr>
<td></td>
<td>pDNA-VEGF</td>
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<tr>
<td></td>
<td>TMC/pDNA-VEGF</td>
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<td>+</td>
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<tr>
<td></td>
<td>TMC/pDNA-eGFP</td>
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<td></td>
<td>pDNA-VEGF</td>
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<td>+</td>
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<td>++</td>
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The scores were made from not present (–) to abundantly present (+++).

* Scaffolds = collagen–chitosan scaffolds.

3.6. Western blotting analysis

Western blotting analysis of the tissue extracts was conducted to detect directly the VEGF, CD31 and \( \alpha \)-SMA contents in the regenerated tissues. As shown in Fig. 11, at day 7 and day 10, the VEGF bands of the TMC/pDNA-VEGF and pDNA-VEGF groups were apparently darker than those of the blank control and the TMC/pDNA-eGFP groups, which became similar at day 14 for all the samples. In the case of CD31 and \( \alpha \)-SMA, the bands of the TMC/pDNA-VEGF and pDNA-VEGF groups were always darker than those of the blank control and the TMC/pDNA-eGFP groups regardless of the investigating time. Qualitatively, the TMC/pDNA-VEGF group showed slight larger band areas than the pDNA-VEGF group at the same implantation time in terms of the CD31 and \( \alpha \)-SMA.

Fig. 4. H&E staining of sections of wounds treated by blank BDE (A, E, I), and BDEs loaded with pDNA-VEGF (B, F, J), TMC/pDNA-VEGF (C, G, K), and TMC/pDNA-eGFP (D, H, L) for different days, respectively. The bar indicates 200 \( \mu \)m. Arrows indicate scaffolds.
Fig. 5. VEGF immunohistochemical staining of sections of wounds treated with blank BDE (A, E, I), and BDEs loaded with pDNA-VEGF (B, F, J), TMC/pDNA-VEGF (C, G, K), and TMC/pDNA-eGFP (D, H, L) for different days, respectively. The bar indicates 100 μm.

Fig. 6. CD31 immunohistochemical staining of sections of wounds treated with blank BDE (A, E, I), and BDEs loaded with pDNA-VEGF (B, F, J), TMC/pDNA-VEGF (C, G, K), and TMC/pDNA-eGFP (D, H, L) for different days, respectively. The bar indicates 100 μm. Arrows indicate vessels.
3.7. Histology and tensile strength of healed full thickness skin

Fig. 12 shows the histological images of the normal skin (Fig. 12A) and the healed full thickness skin treated by TMC/pDNA-VEGF complexes loaded-BDEs and followed by transplantation of ultra-thin skin grafts for different time (Fig. 12B-D). At day 21 after grafting, a few of inflammatory cells were still observed, but no obvious gap was found between the epidermis and the newly-formed dermis (Fig. 12B). At day 70 after grafting, only fewer inflammatory cells but a lot of dense collagen fibers were found (Fig. 12C). The epidermis had formed papillary structure, conveying its well differentiation. After 112 days grafting, the skin showed a very similar structure as the normal skin (Fig. 12D), where the collagen fibers became denser and regularly aligned.

The tensile strength of the healing tissues is presented in Table 3. At day 21, the tensile strength of the regenerated skin was only $1.04 \pm 0.29$ MPa, which was much smaller than that of the normal skin.
4. Discussion

Wound healing is a dynamic process based on tissue growth and regeneration, which involves the interactions between extracellular matrix (ECM), cell growth factors and various types of resident cells. It comprises three overlapping phases: inflammation, tissue formation and remodeling [40]. During the healing of full thickness skin wound, the dermal regeneration is essential to realize the functional and aesthetic outcomes. So far it has been well recognized that artificial dermis equivalents can well regulate and alter the above process, leading to repair of the dermis and thereby facilitating the repair results. These equivalents have been widely used for wounds, burns and orthopaedics [41-45]. Natural polymers such as fibrin [46], hyaluronic acid [47,48] and collagen [49,50] are usually used to formulate into different matrices. In our group [10-14], a BDE composed of a collagen-chitosan porous scaffold and a silicone membrane was designed and examined in vitro and in vivo in terms of their basic properties and biological responses including fibroblast growth and dermal repair. Results show that it can effectively induce the regeneration of dermal layer with satisfactory vascularization degree within 4 weeks in a pig model [14].
One of the key issues for the second transplantation of the ultra-thin skin graft is the sufficient vascularization of the implanted artificial dermis, which is essential for delivery of nutrients and metabolic wastes and thereby the survival of the graft. A faster vascularization rate is therefore highly demanded to shorten the second operation time. It is known that angiogenesis, a basic process of vascularization, is extensively enhanced by various angiogenic factors such as bFGF, VEGF, PDGF etc. Actually, these factors have shown great promise and been applied singly or simultaneously in matrices to improve angiogenesis [15–21]. Among them, VEGF, a homodimeric glycoprotein produced by numerous cell types, is very effective for vasculogenesis, angiogenesis, vascular remodeling, and vessel stabilization [51]. To overcome its short half-time and low stability, an alternative strategy is proposed by incorporation of plasmid DNA encoding VEGF. The simplest way is the use of naked DNA, which is either injected directly [52] or loaded into matrices [53]. However, it has some drawbacks such as enzymatic degradation of the unprotected DNA and low cell transfection efficiencies. Therefore, many polycationic molecules such as PLL [54] and polyethylenimine (PEI) [55] have been previously used as gene delivery vector to condense and protect the DNA. We reported previously that the polycation, TMC could be easily manufactured and had a low cytotoxicity, but showed comparable transfection efficiency in vitro as that of PEI in gene delivery [31]. It was also used to deliver pDNA-VEGF. A collagen sponge loaded with the TMC/pDNA-VEGF complexes showed faster angiogenesis when it was implanted subcutaneously in mice [32].

Based on all these results, in this work the BDE of collagen–chitosan porous scaffold/silicone membrane was combined with the TMC/pDNA-VEGF complexes in order to develop a gene-activated BDE which may have faster angiogenesis. Since many physical and biological properties of the BDE have been well characterized previously, herein we focused mainly on the in vivo angiogenesis process in a pig model and the corresponding alterations of relevant factors such as VEGF, CD31 and α-SMA brought by loading of the TMC/pDNA-VEGF complexes. Comparisons were also made with the naked pDNA-VEGF and non-functional TMC/pDNA-eGFP complexes to assess their influences. Here a pig model was

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**Table 3**

Tensile strength of wounds treated with TMC/pDNA–VEGF loaded BDE.

<table>
<thead>
<tr>
<th>Days after grafting</th>
<th>Tensile strength (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NormalΑ</td>
</tr>
<tr>
<td>21</td>
<td>8.29 ± 2.24</td>
</tr>
<tr>
<td>42</td>
<td>8.82 ± 1.52</td>
</tr>
<tr>
<td>70</td>
<td>11.52 ± 0.8</td>
</tr>
<tr>
<td>112</td>
<td>10.29 ± 0.67</td>
</tr>
</tbody>
</table>

* indicates significant difference (p < 0.05) between control and TMC/pDNA–VEGF group.

Α Normal is the healthy skin tissue.

Β Control is the incisional wounds treated with petrolatum gauze dressings.

Γ The incisional wound was treated with TMC/pDNA–VEGF loaded BDE for 10 days and then ultra-thin autograft was transplanted for different days.
selected, since the pig skin has the largest similarity as that of human among other animals [56], which can maximally mimic the healing process and repairing effects in human.

SEM analysis showed that the pore size of the collagen—chitosan scaffold decreased from 150 μm to 100 μm after loading of the TMC/pDNA complexes. The pore size reduction was also observed previously [25] and is caused by the further freezing and lyophilization after TMC/DNA complexes loading. Here electrostatic interaction between the positively charged TMC/DNA complexes and the scaffold may exist besides the physical adsorption, which may contribute to the alteration of the pore size as well. It has to mention that the pore size and structure in the dry state may further change in the culture medium [25]. Nonetheless, the collagen—chitosan scaffold with a pore size between 100 μm and 150 μm has shown similar property for dermal regeneration [10,57]. Indeed, this slight variation of the physical structures did not have significant influence on the angiogenesis rate as demonstrated in this study by comparison between the blank BDE and the TMC/pDNA-eGFP complexes loaded BDE.

In order to identify the importance and functions of TMC and functional gene in wound repair, here the TMC/pDNA-VEGF group was compared with three control groups. The four types of BDEs were transplanted in the same pig to exclude individual differences [Fig. 4]. Since the BDE (bilayer collagen—chitosan and silicon membrane porous scaffold) has been demonstrated a good template for dermal regeneration, no further change was made for the sake of constructing an effective and useful full thickness skin repair BDE. The in vivo results showed that the BDE having TMC/pDNA-VEGF complexes had a significant faster rate of vascularization than all other control groups, especially at longer transplantation time (~10 days) (Figs. 6–9). Moreover, the naked pDNA-VEGF also showed some effect on angiogenesis, although the difference is not significant compared with the other two controls.

Here immunochemical staining for CD31, a transmembrane protein expressed early in vascular development, was used to evaluate the newly-formed vessels [58]. To attain mature vessels, the endothelial cells need to be surrounded by the smooth muscle cells, which can be indicated by α-SMA. Therefore, the mature vessels can be identified by colocalization of the endothelial cells and the smooth muscle cells. The results of immunohistochemistry and immunofluorescence indicate that the TMC/pDNA-VEGF group cannot only promote the number of newly-formed vessels, but also improve their maturity [59,60]. The TMC/pDNA-VEGF group showed enhanced granulation tissue formation and faster integration (Figs. 3 and 4) as well, which is resulted from the faster angiogenesis, sufficient supply of oxygen and nutrients, and thereby facilitating the infiltration of fibroblasts and collagen synthesis. All these results confirm that the gene therapy aiming at faster angiogenesis has brought great promise for the dermal repair by the BDE.

This positive effect is reasonably attributed to the expression of VEGF by the loaded repair TMC/pDNA-VEGF complexes, since the same loading of pDNA-VEGF or TMC/pDNA-eGFP complexes did not bring significant influence. Actually, the expression of human VEGF mRNA was only detected in the TMC/pDNA-VEGF and the pDNA-VEGF groups, and a significant higher level was found in the TMC/pDNA-VEGF group for up to 14 days (Fig. 10A). It is known that the vector of TMC can condense and protect the DNA, and their complexes are easily endocytosed to realize more effective transfection than that of the naked DNA [31]. There is no detectable human VEGF mRNA in the blank control and the TMC/pDNA-eGFP groups, which is quite reasonable. The inability of TMC/pDNA-eGFP complexes delivery to induce anything beyond a limited inflammatory response confirms that the angiogenesis was dependent on the VEGF gene delivery instead of inflammatory response caused by complexes delivery. Therefore, the combination of TMC and pDNA-VEGF into complexes is indispensable to achieve the faster angiogenesis. It is understood that the positive expression of VEGF in all the groups characterized by immunohistochemistry (Fig. 5) should be caused by cross-reaction between the human VEGF antibody and pig VEGF [61].

It has been well demonstrated that the VEGF has a strong ability to induce angiogenesis. Furthermore, it is recognized that the local, microenvironmental dose rather than the total dose of VEGF determines the safety and efficacy in a therapeutic setting [60,62]. Generally, the exogenous VEGF needs to be supplied over several weeks to allow maturation of the newly-formed vessels into stable vessels by enwrapping of pericytes or smooth muscle cells [38,63,64]. The RT-qPCR results show that the TMC/pDNA-VEGF group had the highest mRNA expression of those key factors related to vascular development in vivo, i.e. VEGF, CD31 and α-SMA (Figs. 10 and 11). Moreover, the increase of pig VEGF mRNA expression in the pDNA-VEGF and TMC/pDNA-VEGF groups is likely resulted from the recruitment of endothelial cells which express endogenous growth factor [65]. Actually, a longer VEGF mRNA expression in the TMC/pDNA-VEGF group was observed, showing a high level at day 10 and lasting by a rapid decrease during post TMC/pDNA-VEGF ~70 days (Data not shown). However, the western blotting analyses showed that the TMC/pDNA-VEGF group only had a moderate increase in the VEGF protein expression. This discrepancy is not well understood yet. Nevertheless, as demonstrated in this work and also many other studies [66], this moderate increase of the VEGF level already generates strong enough effects on vascular development and maturation.

To evaluate the full thickness skin repair results, ultra-thin skin grafting was performed on the wound treated by TMC/pDNA-VEGF loaded BDE for 10 days, which is much shorter than that of the normal BDE transplantation (14–21 days). Actually, the skin grafts transplanted onto all other control groups at 10 days could not survive well. Among many methods [67,68] for assessment of skin healing, the tensile strength is frequently adopted to show the overall quality of the newly-formed tissue. The data in Table 3 show that the tensile strength of the repaired skin reached up to about 80% of the normal skin after 112 days grafting, which is in good accordance with its histological analysis (Fig. 12). The good deposition and alignment of collagen (Fig. 12D) also reveals the well remodeling of the regenerated full thickness skin. Therefore, the enhanced angiogenesis property can not only shorten the grafting time of ultra-thin skin, but also facilitate the overall repair quality of the regenerated skin in a long run.

5. Conclusions

A gene-activated BDE was fabricated by incorporation of the TMC/pDNA-VEGF complexes into the collagen–chitosan/silicone membrane scaffold (BDE). After loading of the TMC/pDNA-VEGF complexes, the porous structure and interconnectivity between pores of the BDE were maintained, but the pore size was decreased from 150 μm to 100 μm as a result of refreeze-drying. In vivo experiment in a full thickness skin wound model showed that the TMC/pDNA-VEGF complexes loaded BDE could accelerate the angiogenesis in which the numbers of newly-formed and matured vessels were all increased. Consequently, enhanced granulation tissue formation and faster integration were also achieved. Control experiments demonstrated that the combination of TMC and pDNA-VEGF into complexes was indispensable to achieve the faster angiogenesis. RT-qPCR and western blotting analysis showed that the TMC/pDNA-VEGF group had the highest mRNA and protein expression of those key factors related to the vascular development
in vivo, i.e. VEGF, CD31 and z-SMA. By using this type of BDE, the time of ultra-thin skin grafting was shortened from 14–21 days to 10 days and the tensile strength of the repaired skin at 112 days reached up to about 80% of the normal skin. Good remodeling of the regenerated full thickness skin was also realized.

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Appendix

Figures with essential color discrimination. Many of the figures in this article have parts that are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.06.013.

References


