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The healing of full-thickness burns treated by using plasmid DNA encoding VEGF-165 activated collagen—chitosan dermal equivalents

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A B S T R A C T

Repair of deep burn by use of the dermal equivalent relies strongly on the angiogenesis and thereby the regeneration of dermis. To enhance the dermal regeneration, in this study plasmid DNA encoding vascular endothelial growth factor-165 (VEGF-165)/N,N,N-trimethyl chitosan chloride (TMC) complexes were loaded into a bilayer porous collagen—chitosan/silicone membrane dermal equivalents (BDEs), which were applied for treatment of full-thickness burn wounds. The DNA released from the collagen—chitosan scaffold could remain its supercoiled structure but its degree was decayed along with the prolongation of incubation time. The released DNA could transfect HEK293 cells in vitro with decayed efficiency too. Human umbilical vein endothelial cells (HUVECs) in vitro cultured in the scaffold loaded with TMC/pDNA-VEGF complexes expressed a significantly higher level of VEGF and showed higher viability than those cultured in the controls, i.e. blank scaffold, and scaffolds loaded with naked pDNA-VEGF and TMC/pDNA-eGFP, respectively. The four different BDEs were then transplanted in porcine full-thickness burn wounds. Results showed that the TMC/pDNA-VEGF group had a significantly higher number of newly-formed and mature blood vessels, and fastest regeneration of the dermis. RT-qPCR and western blotting found that the experimental group also had the highest expression of VEGF, CD31 and α-SMA in both mRNA and protein levels. Furthermore, ultra-thin skin grafting was performed on the regenerated dermis 14 days later, leading to complete repair of the burn wounds with normal histology. Moreover, the tensile strength of the repaired tissue increased along with the time prolongation of post grafting, resulting in a value of approximately 70% of the normal skin at 105 days.

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1. Introduction

Every year in the world millions of patients are disabled and require hospitalization due to the burn injuries, among which more than 300,000 persons die ultimately [1,2]. Early excision and adequate coverage, which prevent fluid loss and the risk of infection, are crucial steps in the treatment of severe burns [3]. So far the split-thickness autografts have been regarded as the golden standard for treating the full-thickness burns. However, the questions of limited donor sites and repeated surgical procedures have urged the fast development of skin substitutes. So far three types of skin substitutes, i.e. epidermal equivalents, dermal equivalents and composite equivalents, have been used in burn treatment [4]. For example, the cultured epidermal grafts are life-saving for patients suffering from extensive full-thickness burns [5,6]. However, they are rather fragile and difficult of handling and the take rate is unpredictable.

It is known that the regeneration of full-thickness skin pertains to dermal regeneration. The need for a proper dermal equivalent, which functions as a regenerating template for ingrowth and deposition of new dermal tissue, has been recognized. For example, Alloderm™ obtained from cadaver skin can serve as a scaffold for the ingrowth of cells and blood vessels and is usually combined with autografting [7]. Integra® developed by Yannas and coworkers is composed of a collagen/chondroitin sulfate porous scaffold and a silicone membrane, and has been widely applied for dermal regeneration [8,9]. The cellular dermal equivalents such as Dermagraft™ (neonatal foreskin fibroblasts cultured on a polylactin mesh) can facilitate healing by stimulating the ingrowth of fibrovascular tissue and reepithelialization too [4]. Also, the composite substitutes including both dermal and epidermal components will undoubtedly simplify burn treatment, such as Apligraf® (type I bovine collagen with cultured human fibroblasts and keratinocytes). Damour et al. developed an in vitro reconstructed skin graft, which...
DNA encoding VEGF can apparently enhance the angiogenesis of angiogenesis, which is a multifactor process controlled by interplay of to be an essential and rate-limiting step in physiological angiogenesis. Signaling by VEGF is thought to accelerate the onset of angiogenesis. Since angiogenesis is the major issue triggering the survival of the BDE, special attention shall be paid to the time dependent vascular number following the immunohistochemical staining. Also, transplantation of ultra-thin skin grafts on the gene-activated BDEs treated burn wounds shall be further performed to demonstrate the possibility of complete healing of the full-thickness burns.

2. Materials and methods

2.1. Materials

DNA (Fish sperm, sodium salt, used as a model to study the physicochemical property of TMC/DNA particles) was purchased from Sigma (Sigma, MO, USA). 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, Zhejiang University, China. Plasmid DNA encoding human vascular endothelial growth factor (pDNA-VEGF) was donated from Dr. Ming Yu, the Fourth Military Medical University, Shanghai, China. The plasmids were amplified in E. coli and purified by a differential precipitation method [34]. The plasmid DNA was lyophilized and stored at −20 °C before use. Collagen type I was isolated from fresh bovine tendon by a trypsin digestion and acetic acid dissolution method [35]. Chitosan (deacetylation degree 90%, Mw 6 k) for the TMC synthesis was purchased from Qingdao Haidebei Co., Ltd (China). Chitosan (deacetylation degree 75–85%, Mw, 10 × 10^5–1.7 × 10^6) for the scaffold fabrication was purchased from Sigma. Silicone membrane was a medical grade product from Shanghai Kinesichem Co., Ltd (China). Human embryonic kidney cells (HEK293) were maintained in Dulbecco’s Modified Eagles Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Sijiqing Co., Ltd, Hangzhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in an atmosphere consisting 5% CO2 and used at an appropriate degree of confluence. Human umbilical vein endothelial cells (HUVECs) were maintained in RPMI 1640 medium supplemented with 20% fetal calf serum (FBS, Sijiqing Co., Ltd, Hangzhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in an atmosphere containing 5% CO2 and used at an appropriate degree of confluence. All other reagents were of analytical grade and used as received. Triple-distilled water was used throughout the study.

2.2. Preparation of TMC and TMC/DNA complexes

N,N-trimethyl chitosan chloride (TMC) was synthesized according to the method reported previously [33]. 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 −OCH3, respectively, according to which the degree of quaternization of 38% was calculated. TMC/DNA complexes were prepared as described previously [30]. 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 [33].

2.3. Fabrication of gene-activated scaffolds/BDEs

The collagen–chitosan scaffolds were prepared according to the procedures described previously [29]. The sterilized PBS containing TMC/DNA complexes (1 mg/ml DNA) was dropped onto the dried collagen–chitosan scaffolds, which was then kept at 4 °C overnight to facilitate the complete incorporation of the TMC/DNA complexes. These DNA loaded scaffolds were carefully washed twice in PBS and then lyophilized to obtain the gene-activated scaffolds. The eventual loading amount of DNA on the collagen–chitosan scaffolds was quantified by fluorometer (LS55, PerkinElmer, UK) with a fluorescent dye Hoechst 33258 as described previously [33], which was about 50 μg DNA per mg scaffold.

For in vivo experiments, the gene-activated scaffolds were covered with silicone membranes, which functioned as a temporary epidermis, to obtain the gene-activated bilayer dermal equivalents (BDEs). Four types of BDEs 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 DNA.

2.4. Characterization of gene-activated scaffolds

The morphology of the TMC/DNA complexes was observed using TEM (JEOL, JEM-200). The cross-sections of the scaffolds with or without the TMC/DNA complexes 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. An in vitro release assay was conducted to evaluate the release kinetics of DNA from the gene-activated scaffolds by immersion in 3 ml of sterile PBS at 37 °C. At
The healing properties of the gene-activated BDEs on full-thickness burns were studied in a porcine model. The miniature pigs were subject to a skin burn injury by a method described previously [37]. Briefly, the Bama miniature pigs weighing about 15 kg were fasted for 12 h, and then anesthetized by intraperitoneal injection of pentobarbital sodium solution (45 mg/kg body wt). Before surgery, the dorsal surface was shaved and sterilized with 5% povidone–iodine (PVP–I). Under anesthesia, 4 dorsal burn wounds with a diameter of each of 30 mm were made on the pigs. The thickness of the skin grafts were determined by a human VEGF ELISA Kit (Ming Rui Biotech Company, Shanghai, China) following the procedures described in the manual. Viability of the seeded HUVECs at day 1, 3, 5 and 7 was studied by MTT assay. Optical density of the solution was recorded using a Multiskan RC microplate reader (Thermo Scientific, Finland). The freshly fabricated DNA complexes were used as the control.

2.5. In vitro VEGF expression and viability of HUVECs

VEGF expression of HUVECs cultivated in vitro in the gene-activated scaffolds was examined by enzyme-linked immunosorbance assay (ELISA). Briefly, the four kinds of scaffolds seeded with HUVECs at a density of 10^5 cells per well were cultured at 37 °C in an atmosphere containing 5% CO2. The culture medium was changed every 2 days. At scheduled time intervals, the scaffolds were washed three times with PBS and then homogenized in the lysis buffer (0.1% tris–HCl, 2 mM EDTA, 0.1% Triton X-100). The lystate (2 ml) was centrifuged at 12,000 rpm at 4 °C for 5 min and the supernatant was added with a concentration of 1 μg DNA per 106 cells. The transfection efficiency was analyzed by a FACSCalibur flow cytometry (FCM, BD Bioscience). The freshly fabricated DNA complexes were used as the control.

2.6. Animal test in a porcine model

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about 200 nm (Fig. 1B). After incorporated into the collagen–chitosan scaffolds, these particles were clearly visible on the pore walls (Fig. 1C). Moreover, the average pore size of the collagen–chitosan scaffold was decreased to about 100 μm.

The incorporated DNA complexes could be released in a sustained manner for at least 2 weeks, with a gradual decrease release rate along with time prolongation (Fig. 2A). No apparent burst release was found. The loaded DNA was released ~50% at the first 5

Fig. 1. SEM images of collagen–chitosan scaffold before (A) and after TMC/DNA complexes loading (C). (B) TEM image of TMC/pDNA complexes.

Fig. 2. (A) Cumulative release of DNA from the scaffolds as a function of time. (B) Electrophoresis of plasmid DNA. Lane1, molecular weight marker; lane 2, pristine plasmid DNA; lane 3–7, plasmid DNA released at day1, 3, 7, 14, and 28, respectively. (C) The percentage of HEK293 cells transfected by the freshly prepared DNA complexes (+ctrl) and DNA complexes released at different time from the scaffolds.
days, and more than 90% at 28 days. The structural integrity of the released DNA was examined using gel electrophoresis after 1, 3, 7, 14 and 28 days of release (Fig. 2B). All the released DNAs from the complexes showed similar double bands as that of the naked DNA. However, the band brightness weakened gradually along with the incubation time. These results confirm that the released DNAs could preserve their intact structure, but the degree is decreased at longer release time. The transfection competence of the released DNA was assessed by in vitro culture with HEK293 cells, showing that the transfection efficiency of the released DNA was lower than that of the freshly prepared DNA complexes (Fig. 2C). Consistent with the DNA integrity results (Fig. 2B), the transfection efficiency was decayed along with the release time, yet was still remained 25% after 28 days (Fig. 2C).

3.2. In vitro VEGF expression and cell viability

Fig. 3A shows the VEGF expression of HUVECs in vitro cultured in four different scaffolds, revealing the amount of the expressed VEGF augmented with the culture time. The VEGF expression of the
TMC/pDNA-VEGF loaded scaffolds was significantly higher than all other groups, resulting in a value of 1.8 and 2.9 folds to the blank one at day 3 and day 7, respectively.

The viability of the HUVECs in all the scaffolds increased monotonously along with the culture time (Fig. 3B). The TMC/pDNA-VEGF complexes loaded scaffolds always showed the highest viability, although no significant difference was found during the first 3 days. At day 5, its cell viability was significantly higher than that of the blank and TMC/pDNA-eGFP scaffolds, and at day 7 significantly higher than all other three groups.

3.3. Gross observations of burn wounds healing

Fig. 4 shows the gross observation of the burn wounds treated with the four different BDEs after transplantations for 7, 14 and 21 days. Along with the prolongation of the implantation, all the wounds became from relatively white at day 7 to reddish at day 21. At each time interval, the burn wounds treated by the TMC/pDNA-VEGF loaded BDEs looked more reddish than those treated by other BDEs. At day 7, the collagen—chitosan scaffolds with white color were clearly observed on the burn wounds treated by the blank and pDNA-VEGF loaded BDEs, but less scaffold remnants were found for the TMC/pDNA-VEGF group (Fig. 4A–D). At day 14, the scaffolds integrated better with the wounds for TMC/pDNA-VEGF group. In contrast there were still some unintegrated scaffolds in the blank, pDNA-VEGF and TMC/pDNA-eGFP groups (Fig. 4E–H). At day 21, the flat surface of the wounds and some degree of reepithelialization were observed for the TMC/pDNA-VEGF group, but there were still some unintegrated scaffolds present in the other three groups (Fig. 4I–L).

3.4. Histology

Histological analyses were performed to assess the regenerated tissues guided by the different BDEs, and the results are present in Fig. 5 and quantitatively summarized in Table S2. 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 BDE group (Fig. 5A), the fibroblasts had infiltrated throughout the scaffold of the TMC/pDNA-VEGF group, in which a large number of blood vessels could be observed too (Fig. 5C). At day 14, the numbers of granulocytes decreased in all the groups, and only a few of macrophages still existed. All the scaffolds were integrated better with the surrounded tissues (Fig. 5E–H). Specifically, in the TMC/pDNA-VEGF group there was no obvious gap between the newly-formed tissues and the implanted scaffold (Fig. 5G). At day 21, the moderate inflammatory responses still existed in the blank and TMC/pDNA-eGFP groups. However, only very few definitive inflammatory cells could be found in the TMC/pDNA-VEGF group and more blood vessels were found (Fig. 5K).

3.5. Immunohistochemistry and immunofluorescence

The newly-formed blood vessels of the burn wounds were characterized by CD31 (a marker of endothelial cells) immunohistochemical staining (Fig. 6), from which the average vessel density is quantified and summarized in Fig. 7. It shows that the number of blood vessels increased along with the implantation time for all the groups. At all time points, the TMC/pDNA-VEGF group had the
highest vessel density, the values of which were 43/\(C_{6}8\), 53/\(C_{6}7\) and 67/\(C_{6}7\) per mm\(^2\) at day 7, 14 and 21, respectively. The vessel density of the pDNA-VEGF group was also higher compared with the other two groups. At all the time intervals, no significant difference was found between the blank and TMC/pDNA-eGFP groups in terms of the vessel density. It has to mention that the blood vessel density in the TMC/pDNA-VEGF group did not increase unlimitedly but remained in a reasonable level. For example, the vessel density was 45/\(C_{6}8\) and 37/\(C_{6}5\) per mm\(^2\) after ultra-thin skin grafting for 14 days and 28 days on the TMC/pDNA-VEGF treated burn wounds, respectively.

As illustrated in Fig. 8, the mature vessels were further characterized with immunofluorescence by co-staining CD31 and \(\alpha\)-SMA (a marker of vascular smooth muscle cells) [40,41]. The number of the mature blood vessels is summarized quantitatively in Fig. 9. Again, the density of the mature blood vessels increased monotonously along with the implantation time for all the groups, but their values were smaller than that of the total blood vessels (Fig. 7). The TMC/pDNA-VEGF group always had the significant higher density of the mature vessels at all the detection time. No significant difference was found among the other three groups. The blood vessels with thicker walls and round shape were present in the TMC/pDNA-VEGF group at day 14 and 21 too, indicating the higher maturity (Fig. 8G and K).

3.6. RT-qPCR assay

To further confirm the results, RT-qPCR was used to quantify the in vivo expression of human VEGF mRNA, which was only found in the samples of pDNA-VEGF and TMC/pDNA-VEGF groups and their values decreased from day 7 to day 21 (Fig. 10A). 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 \(\approx 2\) times at day 7 to \(\approx 8\) times at day 21).

The mRNA expression of pig VEGF, CD31 and \(\alpha\)-SMA, which are known to be associated with the angiogenesis, were also analyzed by RT-qPCR. The VEGF, CD31 and \(\alpha\)-SMA mRNA expression showed...
similar alteration patterns, which increased from day 7 to day 21 for all groups (Fig. 10BeD). However, the TMC/pDNA-VEGF group showed significantly higher mRNA levels than the other three groups for all the genes examined at all time points. The pDNA-VEGF group had higher values than the other two groups too.

3.7. Western blotting analysis

Western blotting analysis of the tissue extracts was conducted to directly detect the VEGF, CD31 and α-SMA contents in the regenerated tissues. As shown in Fig. 11, at day 7 and day 14, the VEGF bands of the TMC/pDNA-VEGF and pDNA-VEGF groups were apparently darker than those of the blank and the TMC/pDNA-eGFP groups, all of which became similar at day 21. In the case of CD31, the bands of the TMC/pDNA-VEGF were darkest compared to the other three groups at day 7 and day 14, but was comparable with that of the pDNA-VEGF at day 21. As to α-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 at all the detection time.

3.8. 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 loaded BDEs and followed by transplantation of ultra-thin skin grafts for different time (Fig. 12B–D). At day 28 after grafting, many inflammatory cells were observed, but no obvious gap was found between the epidermis and the newly-formed dermis (Fig. 12B). At day 56, only fewer inflammatory cells were remained. Moreover, the papillary structure was present, conveying a tighter connection between the epidermis and the newly-formed dermis (Fig. 12C). After 105 days grafting, the skin showed a very similar structure as the normal skin, where the collagen fibers became denser and regularly aligned (Fig. 12D).

Fig. 8. Fluorescence triple staining of sections of burn 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 time, respectively. Endothelial cells (CD31), smooth muscle cells (α-SMA) and cell nuclei were stained with red, green and blue colors, respectively. The co-existence of red and green represents mature vessels of dually positive for CD31 and α-SMA. Bar indicates 100 μm.

Fig. 9. Number of mature blood vessels of burn wounds after treated with blank BDE, and BDEs loaded with pDNA-VEGF, TMC/pDNA-VEGF, and TMC/pDNA-eGFP for different time (n ≥ 6; * denotes statistically significant difference, p < 0.05).
The tensile strength of the healed skin is summarized in Table 1, showing that the value of the burn wounds treated with the TMC/pDNA-VEGF group was increased from 2.26 MPa to 5.67 MPa after the grafting time prolongation from 28 to 105 days. The value at 105 days reached approximately 70% of the normal skin. By contrast, the tensile strength of the control group was significantly lower than that of the experimental group at each time interval.

4. Discussion

Skin is the largest organ of human body and serves a wide range of sensory, mechanical and regulatory functions. In the case of severe burns, damage to the skin is too extensive to allow the natural healing, and thereby grafting is required to cover the wound site. So far autografts, allografts and xenografts have been used to treat burn wounds. However, allografts and xenografts can only provide temporary coverage due to their immunogenic activity, while serious burn patients are frequently limited by lacking sufficient donor skin for autografts [42]. By contrast, skin substitutes offer a potential alternative in the treatment of burn wounds. Actually, it has been well recognized that dermal regeneration is essential to realize the functional and aesthetic outcomes for the healing of full-thickness skin defects. Therefore, the dermal equivalents are helpful to regulate and alter the wound healing process, leading to regeneration of the dermis and thereby the good repair outcome [43,44]. Up to present several kinds of dermal equivalents such as Alloderm™, Integra™ and Dermagraft™ have been reported and applied in burn treatment [4].

However, the major limitation of the dermal equivalents for treatment of the burn wounds is the slow vascularization, which may result in graft necrosis. The sufficient vascularization of the implanted dermal equivalent plays a crucial role in determining the survival of the ultra-thin skin graft. Thus, a faster vascularization rate is highly demanded to improve the take rate of the grafts and then shorten the second surgery time. Angiogenesis, the process of new blood vessel growth, is a basic process of vascularization, and is extensively enhanced by various angiogenic factors such as bFGF, VEGF and PDGF etc [18-22]. Despite of the vast interest in growth factors and their potential for wound healing, clinical trials have, in most cases, been disappointing [7]. By contrast, plasmid DNA encoding functional proteins either injected directly [45] or loaded...
into matrices [46] can avoid the problems of short half-time and low stability of proteins. Thus, combination of the dermal equivalent with gene therapy would be an appealing therapeutic approach to improve the clinical outcomes for burn wounds. It is known that the gene transfection efficiency is greatly dependent on the gene delivery vectors. From the viewpoint of in vivo applications, the ease of synthesis and biodegradability should be taken into account. In this regard, TMC was adopted as the vector, which had shown great success in regeneration of full-thickness incisional skin loss [30].

However, compared to the incisional wounds the burn wounds are much more complicated because of the damage of surrounding cells and blood vessels [13,47]. The implanted BDEs will be much harder to be vascularized. In this study, the physical and biological properties of the gene-activated BDEs were examined. The in vivo angiogenesis process of the gene-activated BDE in Bama miniature pigs was studied in mRNA and protein levels. Comparisons were made with the naked pDNA-VEGF and non-functional TMC/pDNA-eGFP complexes. Finally, ultra-thin skin grafts were performed on the burn wounds treated by the gene-activated BDEs to evaluate the full-thickness skin repair effects.

SEM characterization showed that the average pore size of the collagen—chitosan scaffold decreased from 150 μm to 100 μm after loading of the TMC/DNA complexes (Fig. 1A and C). This phenomenon is well documented previously and is caused by the freezing and lyophilization after the TMC/DNA complexes loading [26,30]. Moreover, the pore size and structure of the scaffold in the dry state may further alter in the culture medium [26]. Nonetheless, this alteration would not bring significant influence on the dermal regeneration as reported previously [30,48] and also demonstrated in this study. Incorporation of the DNA complexes into the collagen—chitosan scaffold was confirmed by the appearance of the particles on the pore walls, which are stabilized by surface adsorption or/and electric interactions. Quantitative analysis found that the DNA loading amount was about 50 μg/mg collagen—chitosan scaffold.

The effective interactions between the DNA complexes and the scaffold are also evidenced by the sustained release of DNA, which could be lasted up to 28 days (Fig. 2A). The results in Fig. 2B confirmed that the released DNA could remain its supercoiled conformation [49], but its degree was decayed along with the time prolongation. This is in good agreement with the steady decrease of the in vitro transfection efficiency (Fig. 3B), illustrating that the supercoiling absent DNA can not take the normal transfection. It has to mention that the porous structure of the scaffold can increase the surface area and ensure the sustained release of DNA complexes, which may maintain an effective local concentration in vivo and then transfect adjacent cells [50]. The incorporation of pDNA-VEGF, no matter with or without the protection of TMC, has shown some positive effects on the VEGF expression. However, the use of TMC can effectively enhance the VEGF expression (Fig. 3A). Moreover, loading of the TMC/pDNA-eGFP had no influence on the VEGF expression, demonstrating that the overexpression of VEGF by the TMC/pDNA-

Table 1

<table>
<thead>
<tr>
<th>Days after grafting</th>
<th>Tensile strength (MPa)</th>
<th>Normala</th>
<th>Controlb</th>
<th>Gene-activated BDEsc</th>
</tr>
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<tr>
<td>28</td>
<td>9.29 ± 1.04</td>
<td>1.59 ± 0.05</td>
<td>2.26 ± 0.11*</td>
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<tr>
<td>56</td>
<td>9.85 ± 0.92</td>
<td>3.22 ± 0.16</td>
<td>4.39 ± 0.72*</td>
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<tr>
<td>105</td>
<td>8.42 ± 0.7</td>
<td>4.35 ± 0.23</td>
<td>5.67 ± 0.23*</td>
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*a Indicates significant difference (p < 0.05) between the control and gene-activated BDE group.

*b Control is the burn wounds treated with petrolatum gauze dressings.

*c The burn wounds were treated with the gene-activated BDEs for 14 days and then transplanted with ultra-thin skin grafts for different time.

Fig. 12. H&E staining of the normal skin tissue (A) and the tissue sections of burn wounds after treated with gene-activated BDEs for 14 days, and then transplanted with ultra-thin skin grafts for 28 days (B), 56 days (C) and 105 days (D). Bar indicates 200 μm.
VEGF scaffold is surely caused by the loaded DNA complexes. As a result of this overexpression, the HUVECs showed higher viability in the TMC/pDNA-VEGF complexes loaded scaffolds (Fig. 3B).

Next, the in vivo importance and functions of the TMC and functional gene loaded BDE on burn wounds repair were evaluated in the same pig (Fig. 51), which can maximally exclude the individual difference. The results showed that the TMC/pDNA-VEGF complexes loaded BDEs had the fastest vascularization rate in all the four groups and thereby the better dermal regeneration (Figs. 6–9). Furthermore, the naked pDNA-VEGF also had some positive effect on angiogenesis, though no significant difference was found compared with the other two controls. As a transmembrane protein expressed early in vascular development, CD31 can be used as a marker to evaluate the newly-formed blood vessels [51]. To form the mature blood vessels, however, the endothelial cells need to be completely surrounded by smooth muscle cells, which can be indicated by a plasma protein, α-SMA. Therefore, the co-existence of endothelial cells and smooth muscle cells represents the mature blood vessels. Figs. 6–9 confirm that the TMC/pDNA-VEGF group had significant higher numbers of both newly-formed blood vessels and mature blood vessels. However, these values are smaller than their counterparts of incisional wounds treated by the same BDE at the same time interval [30], revealing that the burn wounds are harder to be vascularized as a result of severe damage of blood vessels.

The in vivo results confirm the necessary use of the functional DNA and the delivery vector. Compared with the blank BDEs, the TMC/pDNA-eGFP complexes loaded BDEs did not show significant difference in terms of blood vessel number and expression of VEGF, CD31 and α-SMA in mRNA and protein levels, revealing that incorporation of the non-functional DNA does not bring positive effect on the angiogenesis. Moreover, loading of the naked pDNA-VEGF produced a very limited effect on the angiogenesis and dermal regeneration, illustrating the necessary use of the cationic TMC vector. It is likely that under the condensation and protection of TMC, the DNA complexes are more easily endocytosed to realize the more effective transfection, and thereby the production of functional protein [33]. Actually, RT-qPCR and western blotting analyses demonstrated that the TMC/pDNA-VEGF group had the highest mRNA and protein expression of those key factors related to vascular development in vivo, i.e. VEGF, CD31 and α-SMA (Figs. 10 and 11). It is worth mentioning that the increase of pig VEGF mRNA expression in the pDNA-VEGF and TMC/pDNA-VEGF groups may be resulted from the recruitment of endothelial cells which express endogenous growth factor [52], and the positive expression of VEGF in the blank and TMC/pDNA-eGFP groups (Fig. 52) should be caused by cross-reaction between the human VEGF antibody and pig VEGF [53].

It is well documented that the local, microenvironmental dose rather than the total dose of VEGF determines the safety and efficacy in a therapeutic setting [54,55]. Generally, to attain stable blood vessels the exogenous VEGF needs to be supplied over several weeks to allow the endothelial cells enwrapped by pericytes or smooth muscle cells [40,56,57]. Figs. 10 and 11 show that the VEGF in the experimental group can express to a significant higher level at least for 21 days, although its absolute value decreases along with the time prolongation. Besides the blood vessels, the granulocytes, macrophages, endothelial cells, and fibroblasts which take the functions of tissue repair were present and their relative numbers altered along with the tissue formation (Fig. 5). Enhanced formation of granulation tissue and faster integration of the scaffolds with the host tissues (Figs. 4 and 5) were observed for the TMC/pDNA-VEGF group. These results are closely related with the faster angiogenesis, which sufficiently supplies oxygen and nutrients for the repair cells, and thereby facilitates the synthesis of extracellular matrix components.

To evaluate the full-thickness repair results, ultra-thin skin grafting was performed after the burn wounds were treated by the gene-activated BDEs for 14 days, which is much shorter than that of the normal BDE transplantation on the burn wounds (21–28 days), but slightly longer than that of the incisional wounds (~10 days) [30]. Actually, the skin grafts could only survive well on the burn wounds treated by the TMC/pDNA-VEGF group. All the other three groups could not yield satisfactory wound beds to support the grafts (Fig. 53). There are many methods [58,59] to assess the degree of the healing skin, in which tensile strength measurements, reflecting the overall quality of newly-formed tissue, is adopted widely. The data in Table 1 show that the tensile strength of the repaired skin reached about 70% of the normal skin after 105 days grafting, which is in good accordance with its histological analysis (Fig. 12). This is slightly smaller than that (~80%) of the incisional wound with the same treatment after grafting for 112 days. This difference reveals again the severe damage of the skin collagen of the burn wounds [60].

Various artificial dermal equivalents such as Integra® and AlloDerm™ are available commercially and used to treat full-thickness burn wounds. Integra® is an immediate and temporary coverage for full-thickness burned patients, which takes 3–6 weeks to form a vascular neodermis. Thereafter, the silastic layer is removed and an ultra-thin skin graft is transplanted [7]. The longer vascularization time brings the increase of infection risk. Later on, fibrin glue and negative-pressure therapy have been adopted to shorten the period from coverage to its integration following which the skin graft could be applied [61]. AlloDerm™ can be used as a template for the ingrowth of cells and blood vessels in the treatment of full-thickness burn and is usually combined with autografting. The use of AlloDerm™ for burn wounds has shown that it is repopulated by host cells, revascularized and incorporated into the tissue [4]. However, concerns are still remained on the immunogenic response and the risk of virus transfer [42]. In the present study, gene therapy was combined with the BDE to fabricate the gene-activated BDE, which had shown enhanced angiogenesis in the treatment of burn wounds. Consequently, the skin grafting could be applied after the gene-activated BDE treatment for only 14 days. Together with its lower cost and immunogenicity, it is an appealing equivalent for the treatment of full-thickness burn.

5. Conclusion

Herein the TMC/pDNA complexes loaded collagen—chitosan/silicone membrane was used to treat the full-thickness burn, resulting in faster angiogenesis and dermal regeneration. The TMC/pDNA complexes could be released in a sustained manner from collagen—chitosan scaffold up to 28 days. The supercoiled structure of the released DNA could be still remained, but its content was decayed along with the prolongation of the incubation time. A consistent alteration of the transfection efficiency of the released DNA complexes to HEK293 cells was observed in vitro. In vitro culture of HUVECs revealed that the TMC/pDNA-VEGF complexes loaded scaffold had highest VEGF expression and HUVECs viability over a period of 7 days. After the BDEs were transplanted onto the full-thickness burn wounds of miniature pigs, the gene-activated BDE group had a significant higher number of newly-formed and mature blood vessels and fastest regeneration of the dermis. Expression of human VEGF mRNA, and the pig VEGF, CD31 and α-SMA in mRNA and protein levels was also highest in the regenerated tissues by the gene-activated BDEs. 14 days later, a further ultra-thin skin grafting was performed on the regenerated dermis, leading to complete regeneration of the full-thickness skin of the burn wound. The tensile strength of the repaired skin reached ~70% of the normal skin after skin grafting for 105 days.
Acknowledgements
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Appendix

Figures with essential color discrimination. Figs. 4–6, 8 and 12 in this article 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.2010.08.087.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.08.087.

References


