

VEGF¹⁶⁵ and bFGF protein-based therapy in a slow release system to improve angiogenesis in a bioartificial dermal substitute in vitro and in vivo

I. Wilcke · J. A. Lohmeyer · S. Liu · A. Condurache ·
S. Krüger · P. Mailänder · H. G. Machens

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Abstract

Background Angiogenesis can be enhanced by several growth factors, like vascular endothelial growth factor-165 (VEGF¹⁶⁵) and basic fibroblast growth factor (bFGF). Delayed release of such growth factors could be provided by incorporation of growth factors in fibrin matrices. In this study, we present a slow release system for VEGF¹⁶⁵ and bFGF in fibrin sealant.

Materials and methods In vitro: Pieces of IntegraTM matrix of 15 mm in diameter were prepared. IntegraTM matrices were divided into four groups (A=control; B=fibrin sealant; C=fibrin sealant+growth factors; D=growth factors). In

vivo: The bioartificial dermal templates were transplanted into a full-skin defect of the back of nu–nu mice. Four different groups included each six matrices at 2 and 4 weeks.

Results In vitro: In groups C and D, continuous release of VEGF¹⁶⁵ and bFGF was eminent. The incorporation of growth factors into fibrin sealant evoked a prolonged growth factor release ($p<0.05$). In vivo: A significantly higher amount of vessels was quantified in groups C and D compared to groups A and B ($p<0.001$).

Conclusions A model of slow protein release by combining VEGF¹⁶⁵ and bFGF with fibrin sealant was produced. This

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1. Wilcke I, Egana T, Lohmeyer JA, Liu S, Thome D, Mailänder P, Machens H-G: VEGF165 and bFGF protein based therapy in a slow release system to improve angiogenesis in a bioartificial dermal substitute in vitro and in vivo. European Conference of Scientists and Plastic Surgeons, 21–23.09.2006, London (oral presentation)
2. Wilcke I, Egana T, Lohmeyer JA, Liu S, Thome D, Mailänder P, Machens H-G: Steuerung der gezielten Freisetzung von bFGF/VEGF165 in einer kollagenen Matrix durch Konstruktion eines ‘Slow release’ Systems in vitro. DGPRÄC 29.09–02.10.2006; Aachen; Plast. Chir. 6 (Suppl. 1):77–78 (2006; poster presentation)
3. Wilcke I, Egana T, Lohmeyer JA, Liu S, Thome D, Condurache A, Mailänder P, Machens H-G: Steuerung der gezielten Freisetzung von bFGF/VEGF165 in einer kollagenen Matrix durch Konstruktion eines ‘Slow release’ Systems in vitro. 28. Jahrestagung der Deutschsprachigen Arbeitsgemeinschaft für Mikrochirurgie der peripheren Nerven und Gefäße (DAM), 16.–18.11.2006, Lübeck-Travemünde (oral presentation).

I. Wilcke · J. A. Lohmeyer · S. Liu · P. Mailänder · H. G. Machens
Department of Plastic Surgery, Burn Unit, University Hospital
of Schleswig-Holstein, Campus Lübeck, Lübeck, Germany

I. Wilcke
Department of Plastic Surgery, University Medical Center
Groningen, University of Groningen, Groningen, The Netherlands

S. Liu
Institute of Burns, Wuhan Hospital No. 3,
Wuhan University, Wuhan,
People’s Republic of China

S. Liu
Department of Hand Surgery, Union Hospital Affiliated to Tongji
Medical College of Huazhong University of Science
and Technology, Wuhan, People’s Republic of China

A. Condurache
Institute for Signal Processing,
University of Lübeck, Lübeck, Germany

S. Krüger
Institute for Pathology, University Hospital of Schleswig-Holstein,
Campus Lübeck, Lübeck, Germany

I. Wilcke (✉)
Plastische, Hand- und Wiederherstellungschirurgie,
Intensivstation für Schwerbrandverletzte,
Universitätsklinikum Schleswig-Holstein, Campus Lübeck,
Ratzeburger Allee 160,
23538 Lübeck, Germany
e-mail: i.wilcke@web.de

model resulted in a prolonged bioavailability of growth factors *in vivo* for functional purposes. Fibrin and collagen can release growth factors *in vivo* and induce significant and faster neovascularisation in bioartificial dermal templates.

Keywords Angiogenesis · Protein-based therapy · VEGF¹⁶⁵ and bFGF · Slow release system · Matrix vascularisation

Introduction

In angiogenesis, vascular cells proliferate and form new capillary structures [1]. Ischaemia causes the release of endogenous biochemical agents, which include growth factors to stimulate angiogenesis [2–4]. All these processes are important in reconstructive strategies using dermal regeneration templates.

In contrast to full thickness skin, bioartificial skin is lacking an original vascular network. This fact has been identified as the main reason for clinical failures due to matrix infection and loss of structural components [5]. The problem can be targeted by creating vascular matrix structures *in vitro* or inducing angiogenesis *in vivo*. Angiogenesis can be enhanced by several growth factors as vascular endothelial growth factor-165 (VEGF¹⁶⁵) and basic fibroblast growth factor (bFGF). The disadvantage of protein-based therapy is quick protein degradation *in vivo*, while a prolonged effect is needed to induce stable capillary structures. A delayed release of proteins could be provided by incorporation of growth factors or other substances in slowly degrading fibrin matrices.

In this work, we present a slow release system for VEGF¹⁶⁵ and bFGF in fibrin sealant. After studying substance release *in vitro*, we used the assay in an *in vivo* model to study whether delayed protein release could induce therapeutic effects.

Materials and methods

Integra™ dermal regeneration template

Integra™ dermal regeneration template (Integra Life Sciences, Plainsboro, NJ) is a bilayered membrane system consisting of a silicone top layer that temporarily replaces the epidermal part of the skin. This layer is meant to retend moisture loss and close the wound bed. A second layer is made of collagen type I and chondroitin-6-sulphate, representing the permanent dermal part [6, 7] (Fig. 1a,b). After harvesting the tissue, the silicone layer was removed to perform quantification of the newly formed blood vessels in the target tissue.

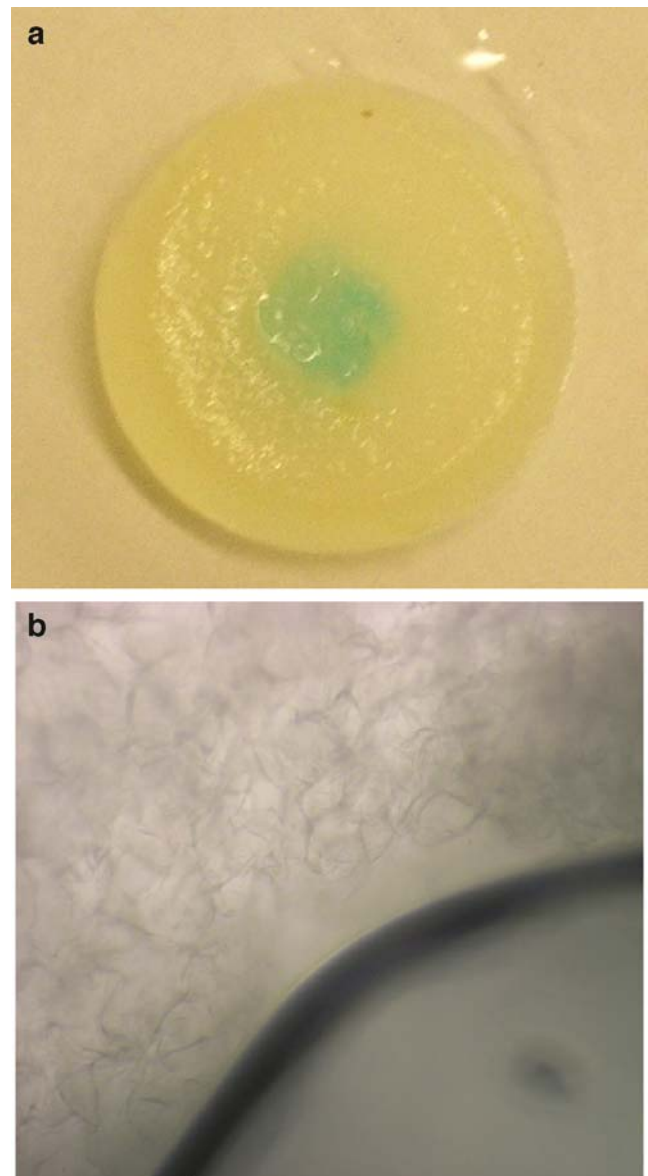


Fig. 1 General view of a prepared Integra™ matrix of 15 mm in diameter (a); microscopical view of the cross-linked collagen and glycosaminoglycan structure with fibrin sealant visible in the *right lower corner* (b)

The growth factors VEGF¹⁶⁵ and bFGF

Recombinant human VEGF¹⁶⁵ was purchased from R&D Systems (Minneapolis, MN, USA). Its source is a DNA sequence encoding the 165 amino acid residue variant of human VEGF that was expressed in Sf 21 insect cells using a baculovirus expression system. The recombinant disulfide-linked homodimeric human VEGF is glycosylated and migrates as a 19–21 kDa protein in sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions [8].

Recombinant human bFGF was also purchased from R&D Systems. Its source is a DNA sequence corresponding

to the mature human bFGF that was expressed in *Escherichia coli*. The 157 amino acid residue recombinant protein has a molecular mass of 17.4 kDa [9].

Fibrin sealant

Fibrin sealant (Tissucol™, Baxter AG, Wien) consists of two human plasma-derived components: (a) a highly concentrated fibrinogen complex composed primarily of fibrinogen and fibronectin along with catalytic amounts of factor XIII and plasminogen and (b) a high potency thrombin. Fibrin sealant clots of varying composition were prepared by mixing diluted solutions of these components. The growth factors were injected into the fibrinogen complex solution before clot formation.

Preparation of fibrin sealant with VEGF¹⁶⁵ and bFGF into a matrix

To determine the release of growth factors, pieces of Integra™ matrix of 15 mm in diameter were prepared (Fig. 1a,b).

Integra™ matrices were divided into four groups A–D (Table 1). In group A, the Integra™ matrices were stored in 1,000- μ l phosphate-buffered saline (PBS) solution (control). The matrices of group B were impregnated with fibrin sealant solution (once [12 μ l], Ersta [12 μ l]) Tisseel™ VH (Baxter AG, Vienna, Austria) mixed with PBS solution (12 μ l). In group C, a combination of fibrin sealant, VEGF¹⁶⁵ and bFGF was used [(growth factors [VEGF¹⁶⁵ (1 μ g) +bFGF (0.5 μ g)]+fibrin sealant (once [12 μ l], Ersta [12 μ l]). Group D incorporated only growth factors (VEGF¹⁶⁵ [1 μ g] +bFGF [0.5 μ g 1 μ g])+288- μ l PBS solution; see Table 1 for general overview]. All matrices were stored in 1,000- μ l PBS solution and kept in a 24-well plate.

Measurements of growth factor release

In the in vitro experiment, three medium samples of each group were taken at 13 definite time points. One thousand microlitres of PBS were harvested and replaced by 1,000- μ l PBS solution every 24 h for 13 days. The collected PBS samples were kept at -80°C and analysed later by using bFGF- and VEGF¹⁶⁵-specific enzyme-linked immunosorbent assay (ELISA) kits (Quantikine immunoassay kit nos.

DFB 50 and DVE 00, R&D Systems) according to the manufacturer's instructions. Measurements were performed once for VEGF¹⁶⁵ and for bFGF. The ELISA samples were read, and the absorbance was measured in a microplate ELISA reader (Sunrise™ absorbance Reader, Tecan Trading AG, Switzerland) at 450 nm.

Animal model

All procedures were performed with approval of the Institutional Animal Care and Use Committee as well as the University Ethical Committee. nu–nu/mice (weight 18–20 g) served for all experiments using Integra™ matrix as bioartificial dermal templates. All animals were anaesthetised with ketamine (Ketamin 10%®, Pfizer; Karlsruhe, Germany, 0.0108 μ g per gram of bodyweight) and xylazine (Rompun®, Bayer; Leverkusen, Germany, 0.0024 μ g per gram of bodyweight) via intraperitoneal injection. A full skin defect 15 mm in diameter was created at anatomically identical sites on the right and left side of the back in each mouse. Twelve animals were operated (three per group) with two matrices each; all matrices were processed as described

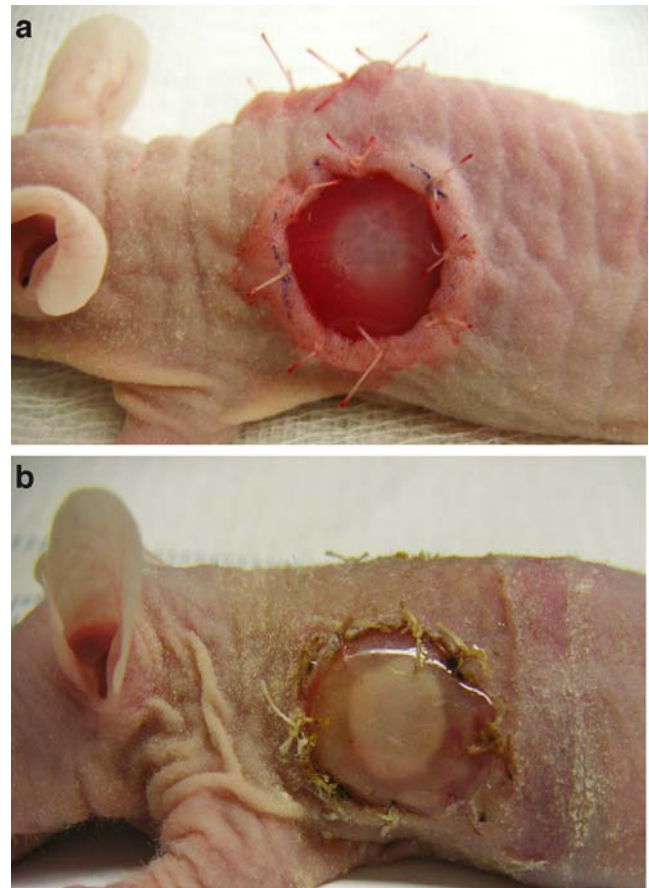


Fig. 2 Matrix transplantation into a full skin defect (a), matrix after 4 weeks in vivo (b)

Table 1 Overview of all groups in vitro and in vivo

Group A	Group B	Group C	Group D
PBS	PBS+FS	GF+FS	GF

FS Fibrin sealant; GF growth factor

and divided into four groups A–D: (A) Control (500 μ l PBS), (B) PBS solution (12 μ l)+fibrin sealant (once [12 μ l], Ersta [12 μ l]), (C) growth factors (VEGF¹⁶⁵ [1 μ g]+bFGF [0.5 μ g])+fibrin sealant ([once [12 μ l], Ersta [12 μ l]), (D) growth factors (VEGF¹⁶⁵ [1 μ g]+bFGF [0.5 μ g])+288 μ l PBS solution. Immediately after preparation, matrices were transplanted into the full skin defects of nu/nu mice (Fig. 2a). After 2 weeks (experiment 1), the animals were killed and the target tissue analysed. In a second set of in vivo experiments (experiment 2), 12 animals (3 animals per group) were used and evenly divided into four groups as described above. Animals were killed 4 weeks after matrix transplantation (Fig. 2b).

Clinical evaluation

All matrix areas were monitored daily for autocannibalism or matrix loss due to infection or other reasons. Photodocumentation of matrix areas was performed in a standard fashion before animal scarification.

Blood vessel quantification

To quantify blood vessel growth into the matrices, the target tissue was harvested and placed upside down on a petri dish. The silicon layer and the sutures were removed, and the inside of the target tissue was turned up. The dish containing the tissue was put on a transilluminator (Fig. 3a), and a picture in tagged image file format (TIFF) quality was taken and segmented (Fig. 3b,c). The amount of newly formed blood vessels in the target tissue was quantified by a VesSeg programme as described before [10]. Briefly, in this programme, vessels are recognised as longitudinal structures. A black and white image is created in which vessels are indicated as white pixels (Fig. 3c). The total area of white pixels reflects the area of blood vessels that allows calculation of the area of blood vessels in the target area (matrix) and in the control area (surrounding tissue). A ratio in between both was formed.

Histology

Cross sections of four matrices of each group from both experiments were analysed. Immunohistochemical cluster of differentiation-31 (CD31) and haematoxylin/eosin staining was performed. Immunohistochemical CD31 staining was done using a standard three-step immunoperoxidase technique and diaminobenzidine as chromogen. Briefly,

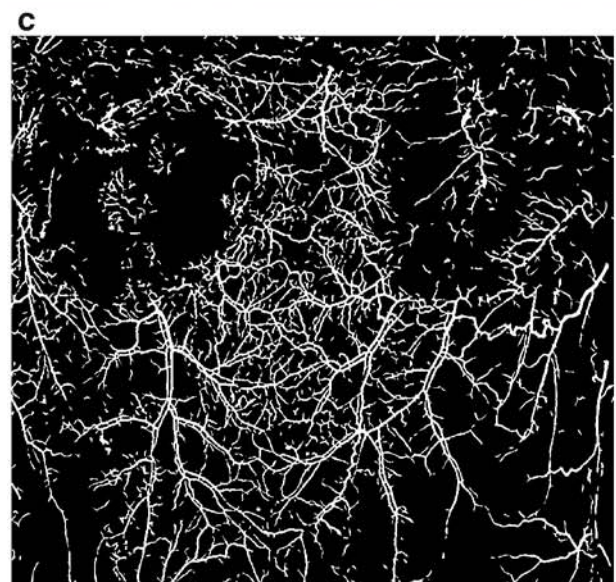
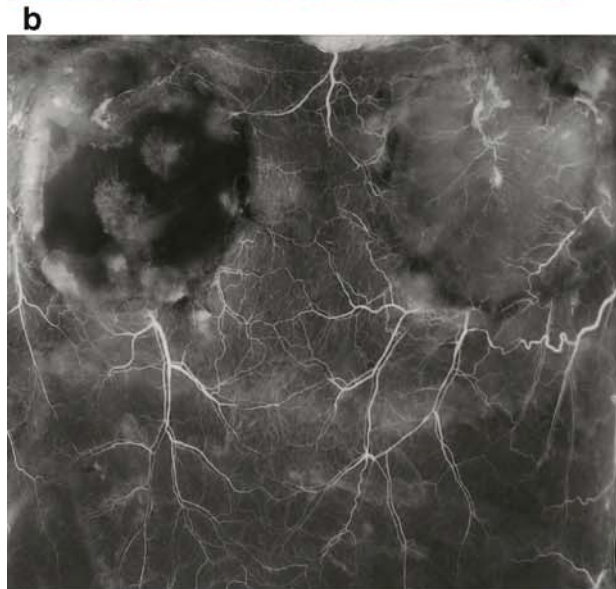


Fig. 3 TIFF picture taken of the target tissue put on a transilluminator (a), picture inverted by the VesSeg programme (b) and segmented by the VesSeg programme (c)

5 μm thick paraffin sections were pre-treated for 45 min in a decloaker (in PBS buffer, pH 9.5) and then incubated for 25 min with the primary polyclonal goat antibody platelet endothelial cell adhesion molecule-1 (dilution 1:60; clone M-20; Santa Cruz, CA, USA), followed by incubation with the secondary antibody (biotin-SP-conjugated mouse anti-goat immunoglobulin G; Dianova, Hamburg, Germany; dilution 1:50). Next, the slides were incubated for 15 min with horse-raddish peroxidase (Dako, Glostrup, Denmark). After another incubation for 10 min with DAB (Dako), the slides were counterstained with Meyer's haematoxilin (Merck, Darmstadt, Germany).

Statistical analysis

All data obtained from in vitro experiments (protein concentrations ng/ml) and in vivo experiments (newly formed blood vessels in percentage) were normally distributed and evaluated for statistical significance by the Student's *t*-test. The level of significance in the in vitro experiments was $p < 0.05$, in the in vivo experiments ($p < 0.05$ and $p < 0.001$).

Results

Release of growth factors in vitro

At the first measurement of the growth factor concentration after 24 h, 9.7 and 6.6% of the initially administered amount of VEGF¹⁶⁵ were detected in the medium in groups C and D, respectively (97.21 ± 13.45 and 66.37 ± 10.25 ng/ml). The corresponding values for bFGF were 1.7 and 0.19% (8.53 ± 0.25 and 0.96 ± 0.21 ng/ml). In groups A and B, we detected no proteins.

In groups C and D, we detected a continuous release of VEGF¹⁶⁵ and bFGF over 13 days, but amounts rapidly declined (Fig. 4a,b). After 48 h, VEGF¹⁶⁵ concentration already diminished to 48.71 ± 8.12 and 31.59 ± 4.51 ng/ml in groups C and D with corresponding values for bFGF of 0.98 ± 0.23 and 0.33 ± 0.07 ng/ml. VEGF¹⁶⁵ almost levelled zero after 9 days in group C and 4 days in group D. bFGF concentration dropped below 0.05 ng/ml after 10 days in group C and 6 days in group D.

However, over 12 days, there was still a significant higher release of bFGF in the fibrin sealant containing group C than in group D ($p < 0.05$). VEGF¹⁶⁵ release was significantly increased over 8 days at last ($p < 0.05$).

Results in vivo

Newly formed blood vessels in the dermal template were specified as the percentage of those of the surrounding tissue (Fig. 5).

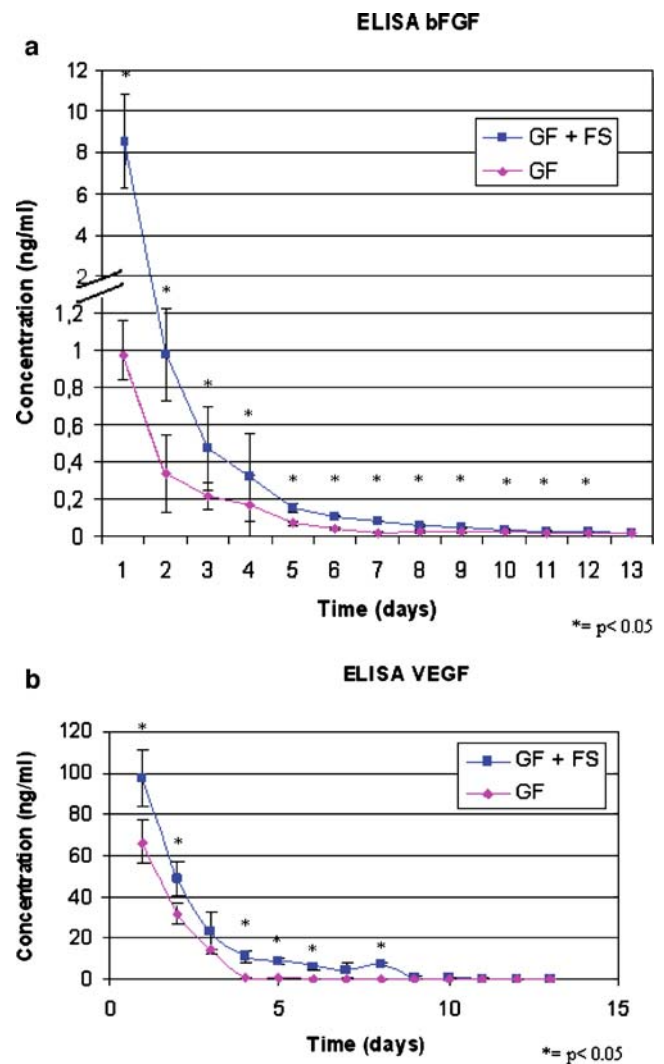


Fig. 4 Release of bFGF (a) and VEGF¹⁶⁵ (b) in vitro. In groups C and D, we detected a continuous release of VEGF¹⁶⁵ and bFGF. At days 1 to 12, there was a significant difference in protein release between groups C and D ($p < 0.05$) for bFGF (a). At days 1, 2, 4, 5, 6 and 8, a significant difference in protein release was obtained between groups C and D ($p < 0.05$) for VEGF¹⁶⁵. bFGF release was less compared to VEGF¹⁶⁵ in groups C and D. FS Fibrin sealant; GF growth factor

Group A was compared to group D, group B was compared to group C and group C was compared to group D (2 and 4 weeks).

After 2 and 4 weeks, group A obtained 17.5 ± 1.3 and $21.9 \pm 1.0\%$ newly formed blood vessels and group B 22.8 ± 1.9 and $27.0 \pm 2.9\%$. Corresponding values for group C were 55.7 ± 2.9 and $80.0 \pm 4.0\%$ and for group D 82.6 ± 4.0 and $62.3 \pm 4.0\%$, respectively.

At both time points, the application of VEGF¹⁶⁵ and bFGF (groups C and D) leads to a significant higher vascularisation in the dermal template when compared to groups A and B ($p < 0.001$). Differences between the sole use of PBS or fibrin sealant (groups A and B) were

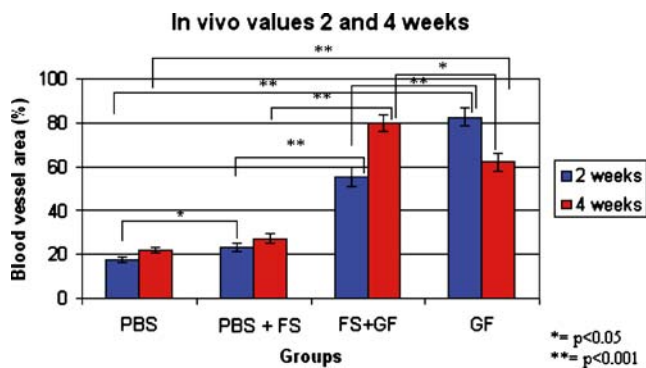


Fig. 5 Vascolarisation of the matrix in vivo after 2 and 4 weeks. After 2 weeks, significantly more blood vessels could be seen in groups C and D, compared to groups A and B ($p < 0.001$). Significantly more blood vessels appeared in group D, compared to group C ($p < 0.001$). After 4 weeks, significantly more blood vessels were detected in groups C and D, compared to groups A and B (0.001). Significantly more blood vessels were evident in group C, compared to group D ($p < 0.05$). *FS* Fibrin sealant; *GF* growth factor

significant in favour of group B after 2 weeks ($p < 0.05$), but not after 4 weeks ($p = 0.1$). Whereas vascularisation prevailed in group D after 2 weeks ($p < 0.05$), more blood vessels could be detected after 4 weeks in the growth factor and fibrin sealant combining group C ($p < 0.001$).

Pictures of the different groups after harvesting the target tissue at 2 and 4 weeks are shown in Fig. 6a,b. The deep-red colorations of the templates were evoked by haemoglobin (bleeding during harvesting). They were not interpreted by the VesSeg programme as blood vessels. The programme selectively recognises only longitudinal structures, but no corpuscular bodies as blood vessels.

Histology

Four matrices of each group from both experiments were analysed. Paraffin sections (5 μm) were stained with haematoxylin and eosin (Fig. 7a) and CD31 (Fig. 7b). Well-defined blood vessels were found in all groups, but it seemed as if more blood vessels were found in the groups treated with growth factors (as well after 2 and after 4 weeks). However, a quantification of the newly formed blood vessels was not performed, because histology is representing only a small part of the target tissue and varies within each sample. Representative pictures are shown in Fig. 7a and b.

Discussion

This article emphasises on the use of biodegradable carriers as vehicles for local and controlled delivery of angiogenic proteins, such as VEGF¹⁶⁵ and bFGF.

A number of specific factors are known to stimulate or inhibit angiogenesis, including vascular growth factors,

inflammatory cytokines, adhesion molecules and nitric oxide. Importantly, regulation of these factors is critical for efficient neovascularisation. Different biological activities are required in the different phases of angiogenesis from initiation to maturation [11]. Several growth factor families and cytokines that are capable to induce physiological blood vessel formation have been identified. In our study, we can confirm previous findings that demonstrate that angiogenesis can be enhanced by several growth factors like VEGF¹⁶⁵ and bFGF [12].

Growth factors are bioactive proteins that play important roles in regulating growth and differentiation of specific cell types and act as cell–cell signalling messengers in the complex process of wound repair [13, 14]. Several of these growth factors, including VEGF and bFGF, are involved in regulating the formation of new blood vessels through angiogenesis [15].

VEGF possesses various biological activities. It enhances microvascular permeability, exerts mitogenic effects on endothelial cells, stimulates the proliferation and migration of endothelial cells, induces the expression of interstitial collagenase and promotes macrophage migration. VEGF is a key mediator of angiogenesis, as it is a potent mitogen for endothelial cells and induces endothelial cell migration and sprouting by upregulation of several endothelial integrin receptors [16]. However, VEGF alone is known to induce instable vessel structures (leaky vessels) [17]. Previous in vivo studies have shown that VEGF¹⁶⁵ can induce temporary angiogenesis within several days [18].

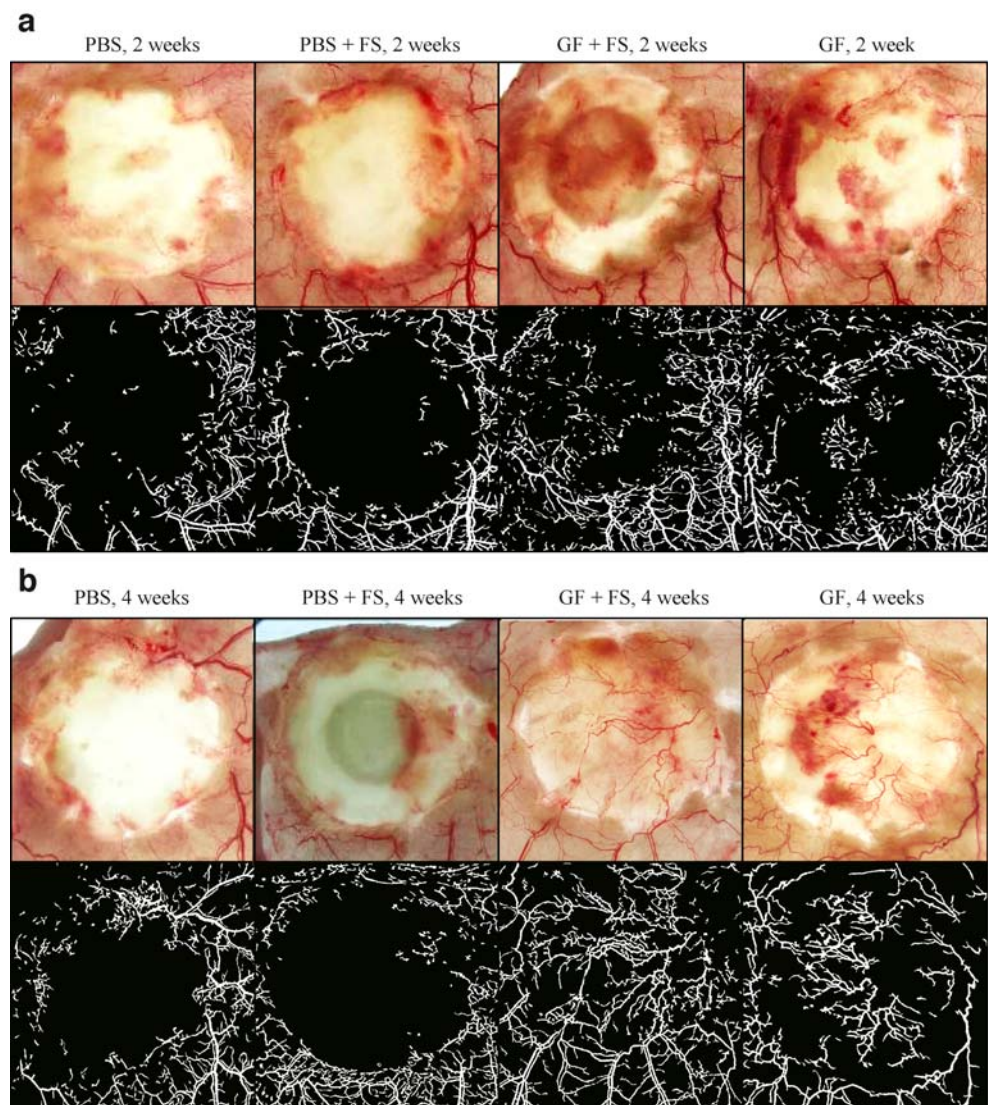
bFGF, another well-studied angiogenic factor, elicits diverse biological effect on numerous cell types, including endothelial cells, and has been used in clinical trials to induce angiogenesis. bFGF has been incorporated into various polymers, such as gelatine, collagen, chitosan and polylactic acid. bFGF reversibly binds to heparin-like molecules and heparin sulphate proteoglycans [19]. This can be exploited to increase the FGF-binding capacity to polymeric delivery vehicles, resulting in a more gradual and sustained release of FGF and an enhancement of angiogenesis [19].

The development of new therapeutic approaches that aim to help the body to exert its natural mechanisms for vascularised tissue growth (therapeutic angiogenesis) has become one of the most active areas of tissue engineering.

One of these areas represents the development of biodegradable carriers. They may facilitate restoration of structure and function of damaged or dysfunctional tissues. Such biodegradable carriers are used in cell-based therapies. They are also used in acellular therapies where materials induce ingrowth and differentiation of cells from healthy residual tissues in situ [20].

In general, the use of acellular biodegradable carriers to support matrix vascularisation is beneficial because it can be used in many different ways, e.g. as matrices for cell

Fig. 6 Pictures of the different groups after harvesting the target tissue at 2 and 4 weeks. The deep-red colorations of the templates are evoked by haemoglobin (bleeding during harvesting). They are not interpreted by the VesSeg programme as blood vessels. The programme selectively recognises longitudinal structures, but no corpuscular bodies as blood vessels



infiltration to induce regeneration and remodelling *in vivo* or as carriers for transplanted cells, which are grafted into tissue defects. But working with cell-based therapies often means difficulties, e.g. purification of the cells or pathogen transmission. For clinical application, protein- or matrix-based therapies comprise the advantage of not including cellular components. This allows the use under the German Act on Medical Devices (Medizinproduktegesetz). This way, an extensive survey that is necessary for advanced cellular therapies according to the new German Tissue Law (Gewebegesetz) can be avoided.

A promising attempt to improve angiogenic performance is presented by biomaterials that allow sequential delivery of growth factors. Biodegradable carriers can serve as a slow release system for angiogenetic proteins.

The use of fibrin-based biomaterials in wound healing therapies is based on the fact that physiologic fibrin clots are an important provisional matrix in normal tissue repair.

The ability of fibrin clots to stimulate and support the growth of new blood vessels is well documented and plays an important role in re-establishing the blood supply in injured areas. A fibrin network is critical for effective wound healing, and it is biodegradable through routine tissue fibrinolysis [15]. As fibrin is lysed slowly, it can serve as a vehicle to deliver various agents that may act to help healing wounds and to promote new vessel growth or to store and slowly release antibiotics and other therapeutic agents. Additionally, fibrin acts as a natural reservoir for the binding and release of certain growth factors as bFGF and VEGF¹⁶⁵ [1].

Controlling the concentration, local duration and spatial distribution of these factors is a key to their utility and efficiency. To address this challenge, controlled delivery systems that incorporate growth factors into polymeric biomaterials have been developed to prolong the tissue exposure time and to maintain growth factor stability [16].

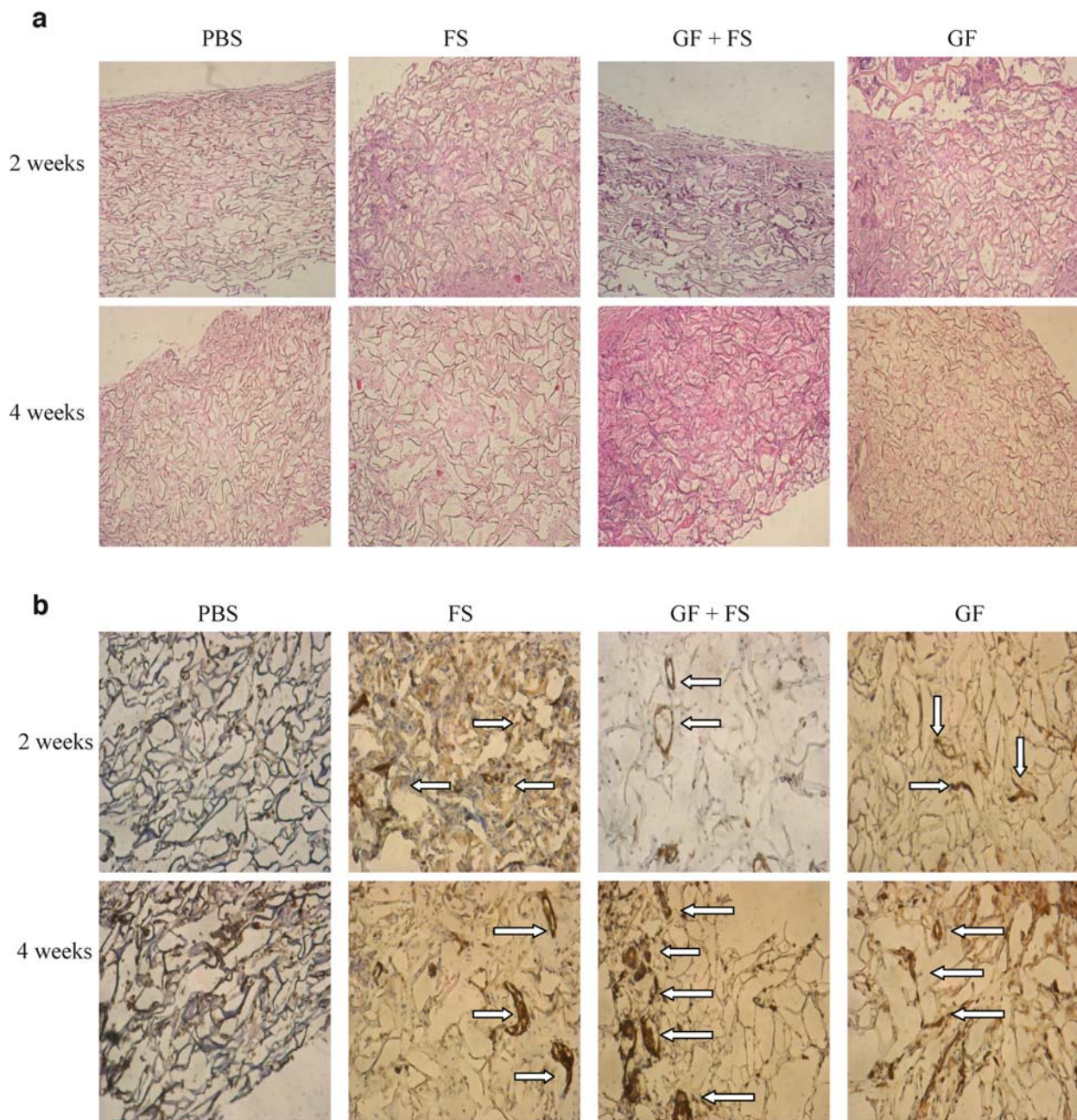


Fig. 7 Representative pictures of cross sections of the matrix are shown. Staining with haematoxylin and eosin; $\times 100$ (a) and CD 31; $\times 200$ (b). Well-defined blood vessels were found in all groups

Attempts have been made to apply growth factors directly or to encode DNA for such factors [21, 22], but it is unknown whether these factors remain at the target site long enough to be effective. A prolonged effect of protein based therapy is needed to induce stable capillary structures. Synergistic effects of bFGF and VEGF to induce mature angiogenesis *in vitro* and *in vivo* have been described by several authors [23, 24]. Some found evidence that a combined therapy for these factors will decrease vessel leakage by reduction in vessel fenestration [25]. This

data is also supported by Kondoh et al. [26], who found a significant increase in smooth muscle cell-positive density in an ischaemic rabbit hind limb model.

Stable angiogenesis can be induced by combined expression of VEGF¹⁶⁵ and bFGF [27]. The combination of VEGF and bFGF has been reported to have potent synergistic effects on neovascular formation in experimental conditions both *in vivo* and *in vitro* [28].

In our *in vitro* experiment, we observed that proteins can be incorporated into the collagen structure of the Integra™

matrix. We were able to produce a model of slow protein release by combining VEGF¹⁶⁵ and bFGF with fibrin sealant. The incorporation of the growth factors in fibrin sealant resulted in a prolonged growth factor release, which is represented by a significantly higher concentration of growth factors over several days in group C compared to group D ($p < 0.05$; Fig. 4a,b).

The matrices initially contained 1 μg of VEGF¹⁶⁵ and 0.5 μg bFGF in groups C and D. Only small amounts of the applied proteins were detected in the medium, especially in the case of bFGF. A fast complete protein diffusion into the medium should have caused a primary peak in the growth factor concentration. But the amount of protein that degrades under physiologic conditions at 37°C before the medium is taken for the first measurement after 24 h would not appear in the values.

As protein measurements were performed only every 24 h, a continuous degradation of both proteins may therefore be responsible for the overall reduced concentration at the time of medium harvesting and processing. Values may only represent the protein release of a markedly shorter time period than the last 24 h. To conclusively answer this question, it would have been necessary to measure total amount of protein at the end of experiment within the matrix. This, however, was not performed.

Another explanation for the low detectable growth factor concentrations could be an interaction of the proteins with collagen or the fibrin sealant altering the function and detectability of the growth factors. At the 24-h time point, 9.7 and 6.6% of the initially administered amount of VEGF¹⁶⁵ were detected in the medium in groups C and D, respectively. The corresponding values for bFGF were 1.7 and 0.19%. bFGF apparently underwent faster degradation, or it showed a stronger, maybe irreversible, binding to fibrin or collagen. To support this assumption, detection of VEGF¹⁶⁵ and bFGF by immunohistochemistry in the dermal templates would be desirable. But detection could not be performed due to strong collateral staining of the collagen matrix and due to cross reactions with rodent VEGF¹⁶⁵ and bFGF *in vivo*.

However, the higher levels of growth factors in the fibrin sealant containing group C compared to group D correspond to the *in vivo* findings of a better long lasting vascularisation in group C after 4 weeks.

In our *in vivo* experiments, we observed that the administration of VEGF¹⁶⁵ and bFGF with or without incorporation in a fibrin sealant lead to a significant and faster neovascularisation of the bioartificial dermal collagen templates (Figs. 5, 6a,b). The incorporation in fibrin sealant initially lead to a minor angiogenetic effect compared to the free protein injection into the collagen. This effect was represented by a higher blood vessel concentration in group D (growth factor). However, after 4 weeks, this ratio

reversed, resulting in a higher blood vessel density in the fibrin sealant and growth factor containing group C (growth factor+fibrin sealant).

This change is probably caused by the stronger retention of growth factors in the slow release system formed by fibrin sealant. A fast flow of the proteins into the surrounding tissue in group D probably resulted in an early stage boost of neovascularisation. But an increased, stable and long lasting vascularisation could only be evoked by the continuous growth factor supply in group C. As mentioned before, bFGF was retained for a longer time in the matrix than VEGF¹⁶⁵. This could contribute to the following biological effect: VEGF is responsible for ‘vessel sprouting’ and the ingrowth of the vessels into the matrix. The part of bFGF left in the matrix is responsible for the stabilisation of the blood vessels [16]. This could be another reason why more blood vessels were detected in group C after 4 weeks.

Due to our developed new segmentation technique, a computer programme called VesSeg, designed by Condurache and Aach [10], we were able to quantify vascularisation of the whole matrix area in groups A to D (Figs. 3a–c and 6a,b). The programme only recognises longitudinal structures as blood vessels. After adjustment of the electronic photograph of the tissue, vessel quantification is nearly completely automated. We were the first to apply this technique for evaluation of the vessel density. The segmentation of the neovascularisation of the target tissue is more representative than the microvessel density shown by the histochemistry. Histology is representing only a small part of the target tissue, while the segmentation technique is showing the entirety. Therefore, we chose the latter for quantification.

Despite of the knowledge regarding the use of dermal regeneration templates in skin defects to induce vascularisation, issues remain in knowing how to give a stable structure to the new developing blood vessels. In other yet unpublished experiments, we could retrieve a stable formation of newly formed blood vessels over more than 6 months in a cell-based bioartificial dermal template after cell transfection with DNA sequences encoding VEGF¹⁶⁵ and bFGF.

Blood vessel development after administration of growth factors combined with fibrin sealant seems to be more effective in producing a patent and stable vasculature than administration of growth factors alone. Our findings indicate that VEGF¹⁶⁵ and bFGF were retained more strongly by the fibrin clots, and thus delivered more slowly. It is the first time this model has been applied for supporting neovascularisation in an artificial dermal substitute. This kind of slow release system may be also promising in the use of other proteins in dermal regeneration templates, for example chemokines, such as stromal cell-derived factor-1 or hypoxia-inducible factor-1 α .

In our experiments, we were able to create a slow release system for VEGF¹⁶⁵ and bFGF by protein incorporation into a slowly degrading fibrin clot placed in a bioartificial dermal regeneration template. The prolonged protein release proved to be beneficial for dermal regeneration. This approach is a promising system for a wide scope of functions in further research in tissue engineering.

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