Fibrin Gel-Immobilized VEGF and bFGF Efficiently Stimulate Angiogenesis in the AV Loop Model

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The modulation of angiogenic processes in matrices is of great interest in tissue engineering. We assessed the angiogenic effects of fibrin-immobilized VEGF and bFGF in an arteriovenous loop (AVL) model in 22 AVLs created between the femoral artery and vein in rats. The loops were placed in isolation chambers and were embedded in 500 µL fibrin gel (FG) (group A) or in 500 µL FG loaded with 0.1 ng/µL VEGF and 0.1 ng/µL bFGF (group B). After two and four weeks specimens were explanted and investigated using histological, morphometrical, and ultramorphological (scanning electron microscope (SEM) of vascular corrosion replicas) techniques. In both groups, the AVL induced formation of densely vascularized connective tissue with differentiated and functional vessels inside the fibrin matrix. VEGF and bFGF induced significantly higher absolute and relative vascular density and a faster resorption of the fibrin matrix. SEM analysis in both groups revealed characteristics of an immature vascular bed, with a higher vascular density in group B. VEGF and bFGF efficiently stimulated sprouting of blood vessels in the AVL model. The implantation of vascular carriers into given growth factor-loaded matrix volumes may eventually allow efficient generation of axially vascularized, tissue-engineered composites.

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INTRODUCTION

There is no doubt that cells transplanted into the body require sufficient supplies of oxygen and nutrients for survival. Suboptimal initial vascularization often limits the survival of cells in the center of large cell-containing constructs (1,2). Numerous studies investigating the importance of vascularization for cell survival have been conducted since the beginning of the 20th century (3,4). Studies proved that no given cell can survive if located further than 200-500 microns from a capillary (1,5). Basically, there are two different patterns of vascularization. Vascularization from the periphery toward the center, which was defined as "extrinsic vascularisation" by Cassell and coworkers (6), and vascularization from a vascular axis radiating from the center toward the periphery, which represents an outgrowth from within, called "intrinsic vascularization." Angiogenic factors may be used to shorten the time period between implantation and vascularization of matrices (7,8). The most extensively tested angioinductive growth factors in animal models are basic fibrobast growth factor (bFGF) and vascular endothelial growth factor (VEGF). bFGF possesses a distinct angiogenic potential in addition to other biological properties (9). VEGF and bFGF show considerable cooperative activity on angiogensis in vivo (10). VEGF is an endothelial cell-specific mitogen with proven angioinductive effects (11,12). Five different human VEGF isoforms are known. These consist of monomers of 121, 145, 165, 189, and 206 amino acids that are derived from a single gene by alternative splicing (13). VEGF has a relatively short biological half-life owing to rapid biodegradation, which constitutes a major limitation for delivery of a recombinant protein (14). Fibrin sealants have already been tested as a means to deliver growth factors in animal models of wound healing and revascularization (15-18). bFGF is released relatively slowly from the fibrin clot because of covalent binding of bFGF to fibrin(ogen) (19). VEGF121 binds relatively weakly to fibrinogen, with almost half of the amount being released from the fibrin clot during the first day. Delivery of a combination of growth factors (e.g., VEGF and bFGF) is more effective than delivery of single growth factors and results in increased neovascularization with formation of more mature vessels (20).

In 1979 Erol and Spira reported that vascular sprouting from an implanted arteriovenous loop (AVL) created by artery and vein grafts in a rat model leads to

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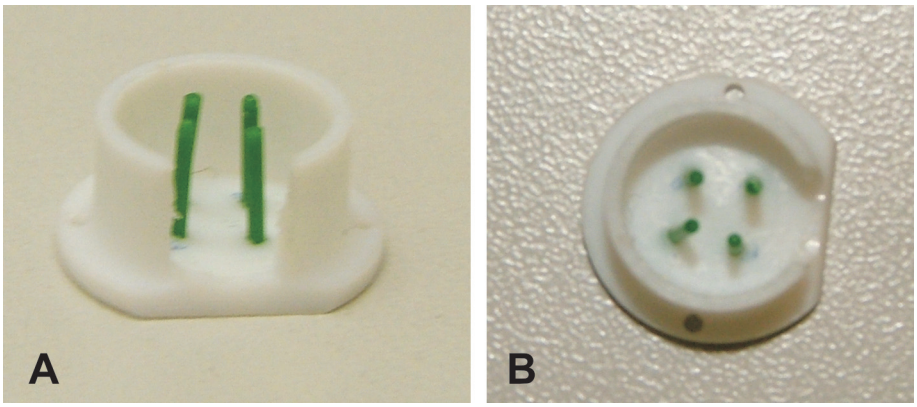

Figure 1. The cylindrical chamber with rounded lid is made of heat-resistant medical-grade Teflon (inner diameter 10 mm, height 6 mm) (A). In the center of the chamber, four tubes consisting of medical plastic are placed to avoid a dislocation of the loop (B).

formation of a new capillary bed (21). Morrison et al. refined this model and implanted the AVL into polycarbonate isolation chambers filled with different types of matrices (6,22). This concept allows vascularization and subsequent transfer of biomaterials independent of local conditions at the recipient site. Our group recently vascularized a custom-made processed bovine cancellous bone matrix by means of an AVL (23). In the same model, vascularization by means of an *AVL* increased survival of transplanted osteoblasts (24).

The aim of this study was to quantitatively evaluate the angiogenic effects of VEGF and bFGF immobilized in a fibrin-based drug delivery system in the AVL model.

MATERIALS AND METHODS

Experimental Design

We divided 22 syngenic male Lewis rats into two identical groups (A and B). In animals from group A an AVL was constructed between the left femoral artery and vein using a contralateral vein graft. The loop was placed in a custom-made Teflon isolation chamber and was embedded in 500 µL fibrin gel (10 mg/mL fibrinogen, 2 IU/mL thrombin). In group B the fibrin component was loaded with 0.1 ng/µL VEGF and 0.1 ng/µL bFGF. Four matrices with patent AVLs per group and time point were explanted after two and four weeks and subjected to histologic and morphometric analysis. Two animals of group A and B were used for corrosion cast analysis two weeks after implantation of the AVL. Two animals with thrombosed loops were excluded from the study.

Isolation Chamber and Composition of the Fibrin Matrix

The cylindrical chamber (inner diameter 10 mm, height 6 mm) with a rounded lid was made of heat-resistant medical-grade Teflon (P. Greil, Department of Materials Science, Glass and Ceramics, University of Erlangen). In the center of the chamber, four tubes consisting of medical plastic were placed to avoid dislocation of the loop (Figure 1). In this study, the matrix was comprised of a clinically approved fibrin gel (Tissucol®, Baxter, Unterschleißheim, Germany) with a fibrinogen concentration of 10 mg/mL, a thrombin concentration of 2 IU/mL, and an aprotinin concentration of 1500 KIE/mL to delay fibrinolysis. In group B the same matrix was loaded with 0.1 ng/µL VEGF and 0.1 ng/µL bFGF before implantation. Recombinant human VEGF121 (R&D Systems, Minneapolis, MN, USA) and recombinant human bFGF (Roche Diagnostics, Indianapolis, IN, USA) were used.

Animals

Syngenic male Lewis rats (Charles River Laboratories, Sulzfeld, Germany) were used in this study. German regulations for the care and use of laboratory animals were observed at all times. All experiments were approved by the animal care committee of the University of Erlangen and the Government of Mittelfranken, Germany. The animals were housed in the veterinary care facility of the University of Erlangen Medical Center and submitted to a 12-hour dark-light cycle with free access to standard chow (Altromin, Hamburg, Germany) and water.

Surgical Procedures

All operations were performed by the same microsurgeon using an operative microscope (Karl Zeiss, Jena, Germany) on rats under general anesthesia with isoflurane (Baxter, Unterschleißheim, Germany). In both groups the left femoral vessels were exposed by a longitudinal skin incision from the groin to the knee. The artery and vein were dissected from the pelvic artery in the groin to the bifurcation of the femoral artery in the knee. A 20-mm vein graft was harvested from the right femoral vessels. This vein graft was interposed between the recipient left femoral vein and artery by microsurgical techniques using 11-0 sutures (Ethicon, Norderstedt, Germany) (Figure 2). The chamber was filled with 250 µL fibrin gel and the *AVL* was passed around the four plastic tubes. Then the chamber was filled with the second half of the fibrin gel-matrix to a total volume of 500 µL (Figure 2), the lid was closed, and the chamber was fixed in the groin using Prolene 3-0 (Ethicon, Norderstedt, Germany) sutures. Hemostasis was assured and the wound was closed using Vicryl 5-0 (Ethicon, Norderstedt, Germany). Postoperatively all animals received 0.2 mL Benzylpenicillin-Benzathin (Tardomycel Comp®, Bayer, Leverkusen, Germany), buprenorphin (0.3 mg/kg rat weight, Temgesic®, Essex Chemie AG, Luzern, Switzerland), and Heparine (80 IU/kg Liquemin, Ratiopharm, Germany).

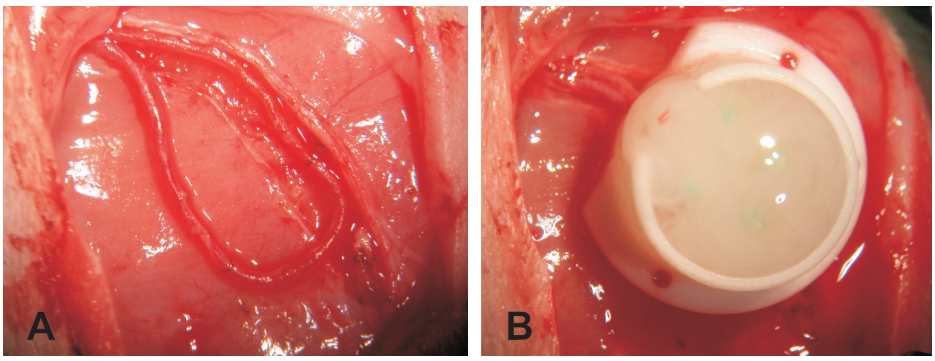

Figure 2 A. An arteriovenous loop is constructed between the left femoral artery and vein using a contralateral vein graft. B: The loop is placed in a custom-made Teflon isolation chamber and is embedded in 500 µL fibrin gel.

India Ink Injection and Explantation of the Matrices

As described previously (23), the animals were perfused with India ink prior to explantation of the matrices. The abdomen was opened via a midline incision and the aorta and inferior vena cava were exposed. After cannulation of the distal descending aorta using a 24-gauge catheter the rats were flushed with 100 mL heparin solution in Ringer (100 IE/mL) until the returning fluid from the severed inferior vena cava was clear. Then, 30 mL of India ink solution [50% v/v India ink (Rohrer&Klingner, Leipzig, Germany) in 5% gelatin (Roth, Karlsruhe, Germany) and 4% mannitol (Neolab, Heidelberg, Germany)] at 37 °C was injected into the aorta. The rat was then placed in -20 °C for approximately 30 minutes; specimens were then explanted *in toto* and fixed in 3.5% formalin solution for histological processing.

Histological and Statistical Analyses

After fixation in 3.5% formalin solution, the constructs were dehydrated in graded ethanol and embedded in paraffin. 3 µm cross sections were obtained from two standardized planes (1 mm proximal and 1 mm distal of the central plane) using a Leica microtome (Leica Microsystems, Bensheim, Germany) as described previously (23). All planes were oriented rectangular to the longitudinal axis of the AVL. Cross sections were stained using hematoxylin eosin. The images were evaluated by two independent and blinded observers. All images of each cross section were generated with a Leica Microscope and Digital Camera under 25x magnification. Afterward the individual images of each cross section were set together (Photoshop, Adobe, San Jose, CA, USA). The composed images were rendered bimodal (standardized threshold) (WinQ, Leica Microsystems, Bensheim, Germany). First the construct size (i.e. cross-section area) was measured, and then the total number of vessels was assessed by counting of all India ink-filled (positive) vessels in the whole cross section. Blood vessel density was consecutively evaluated for each group and each time (blood vessel density = total number of blood vessels/cross section area). India ink-filled (positive) vessels were counted in two high power (x100) fields that were close to artery and vein. The mean values of blood vessel counts per field and blood vessel diameter were calculated separately for artery and vein. Results are given as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, USA). Two-tailed unpaired Student t-test was applied for statistical analysis. The critical level of statistical significance chosen was $P < 0.05$.

CD31 Immunohistology

For CD31 immunostaining specimens were frozen in Tissue Tek (Sakura Finetek, Zoeterwoude, The Netherlands) at -80°C and cut into 5-µm sections. Sections were rehydrated in TBS and incubated with pepsin for 15 s for antigen retrieval, followed by two washes in TBS. Sections were then incubated overnight at 4 °C with the primary antibody [Mouse Anti Rat CD31 (Serotec MCA13346A, Düsseldorf, Germany)]. The secondary antibody [Polyclonal Rabbit Anti Mouse Ig (Dako Cytomation Z0456, Glostrup, Denmark)] and the APAAP-Mouse detection antibody (dilution 1:50, Dako Cytomation D0651) were each diluted in normal goat serum and cycled three times for 20 min. Between the incubations sections were washed twice in TBS. All incubation steps took place in a humidified environment. A Fuchsin/chromogen detecting kit (Dako Cytomation K 0624) was applied according to the manufacturer´s instructions. For counterstaining, Gill´s hematoxylin was used.

Corrosion Cast Technique

After cannulation of the aorta, the vascular system of the rat was rinsed with 200 mL of heparinized Ringer solution (100 IU/mL). The system was then rinsed with 10 mL half-strength Karnowsky solution (0.25% glutaraldehyde, 0.25% paraformaldehyde and 0.1 M Na-Cacodylate buffer adjusted to a pH of 7.2). The caudal vascular system was perfused with 20 mL of a methylmethacrylate resin in a mixture of 4:1 prepolymerized oligomere:methylmethacrylate monomer (Sigma Aldrich Chemie, Schnelldorf, Germany) and benzoyl oxide as a catalyst (Mercox, Ladd Research Industries, Burlington, VT, USA), by use of a method described by Lametschwandtner et al. (25). The rat was left in a warm water bath (50°C) for at least six hours. The construct was then left for 12 hours in 7.5% NaOH at 60°C. The cycle was repeated three times. Final drying was performed by lyophilization.

RESULTS

Surgery and Animals

All 22 animals tolerated the surgical procedure well. Two animals (group A, four weeks, group B, two weeks) were excluded from the study because of thrombosis of the pedicle vessels. There were no major postoperative complications such as infection, hematoma, or wound dehiscence. No extrusion of the implants occurred over the observation period.

Histology

In both groups, the AVL induced the formation of densely vascularized connective tissue with differentiated and functional vessels inside of the fibrin matrix. Vessel patency and continuity with the AVL were visualized using India ink perfusion at all times. The construct size decreased over time in group A (fibrin matrix) and B (fibrin matrix with growth factors). Constructs in the growth factor group B were substantially smaller than constructs in group A at both time points. After two weeks, the constructs of group A showed a low degree of vascularization, mainly in proximity to the vascular axis (Figure 3 A and E). The specimens of group B displayed a higher degree of vascularization of the fibrin matrix at this time (Figure 3 B and F). After four weeks, the use of growth factors also induced a higher vascular density in group B (Figure 3 D and H) compared with group A (Figure 3 C and G).

In both groups a foreign body reaction was not observed at any time point. The amount of connective tissue increased by the use of growth factors as it grew from the vessels of the vascular axis toward the peripheral regions. The newly formed tissue within the matrix was composed of inflammatory cells, fibroblasts, blood vessels, and vascular sprouts in both groups. Between two and four weeks, the vascularized tissue continued to grow centripetally toward the periphery of the vascular axis. Histologically there were no obvious differences between the venous and arterial branches of the AVLs in both groups with regard to the number of vessels, vessel diameter, and amount of newly formed tissue. Vascularization of implanted fibrin gel could be confirmed by immunohistochemical staining of the endothelial cell-associated marker CD31 (Figure 4).

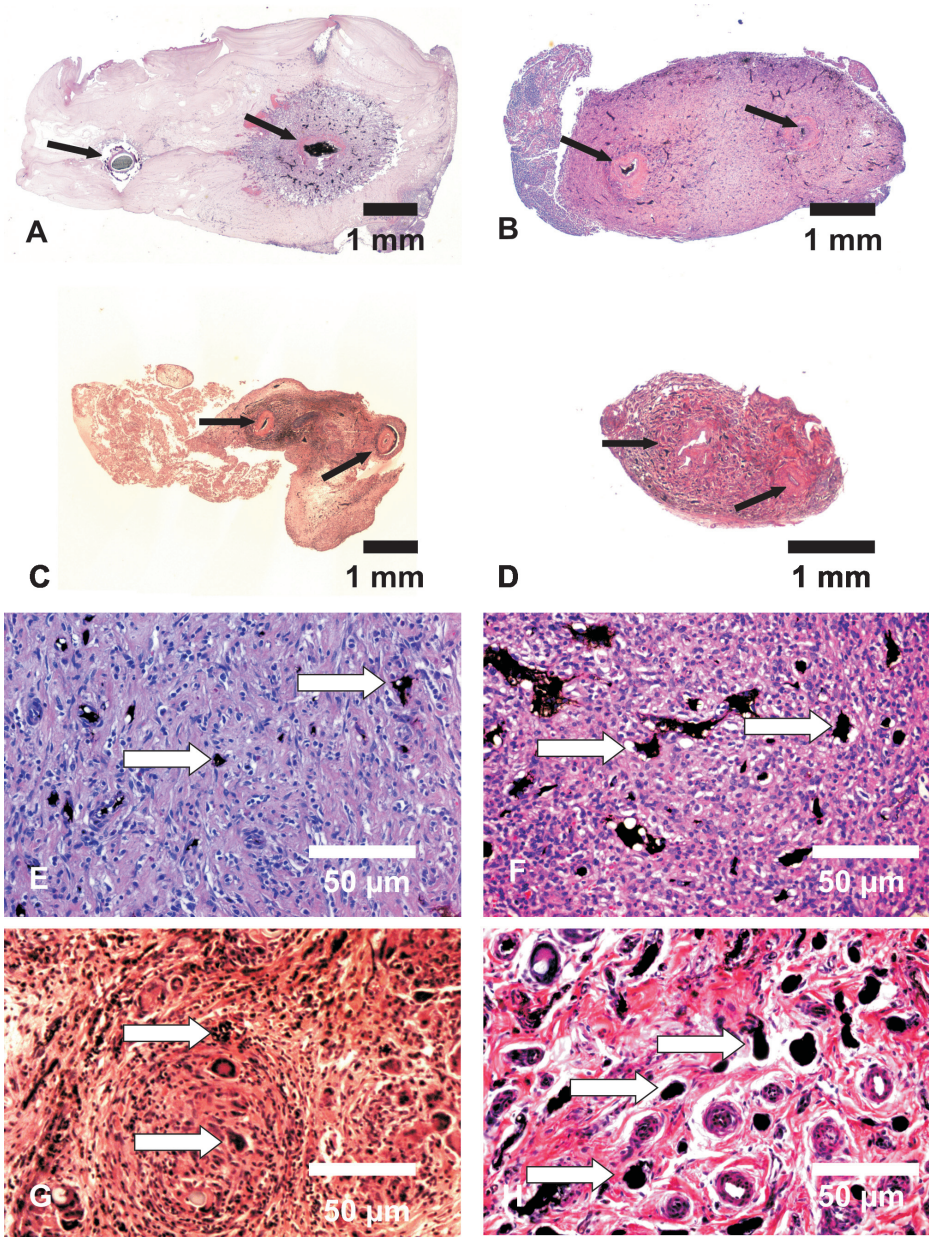

Figure 3. Hematoxylin-eosin staining of matrices explanted after two (A, B, E, F) and four (C, D, G, H) weeks. After two weeks vascular density was low in group A (without growth factors) (A, E) and considerably increased in group B (treated with growth factors) (B, F). After four weeks construct size was decreased in both groups (compare A, B with C, D). Compared to group A (C, G), however, the number of India ink-positive vessels was higher in the growth factor group B (D, H). Arrows indicate India Ink-perfused, patent parts of the AVL. Scale bars A-D = 1 mm, scale bars E-H = 50 µm.

Scanning Electron Microscopy

In all specimens of the 2-week groups the neocapillary network displayed

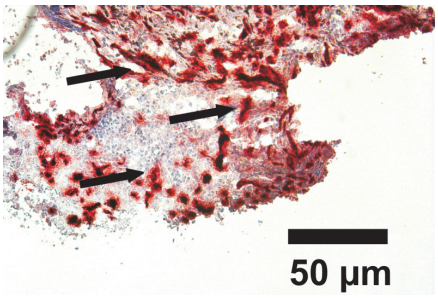

Figure 4. CD31 Immunohistology of the specimens two weeks after implantation (group A). Functional vessels are stained red after two weeks. Specimens were injected with India ink injected before explantation. Scale bar = 50 µm.

characteristics of an immature vascular bed: uniformity of caliber with diameters ranging between 10-15 µm, absence of loop formation, absence of a distinct vascular hierarchy, and vivid angiogenesis in terms of sprout formation (Figure 5). In the treatment group B the density of the neocapillaries was higher, but this effect was not quantitatively assessed in the microvascular replicas. However, this phenomenon could be explained by an enhanced initial sprouting from the lumen of the vascular axis as a result of an initially increased VEGF concentration.

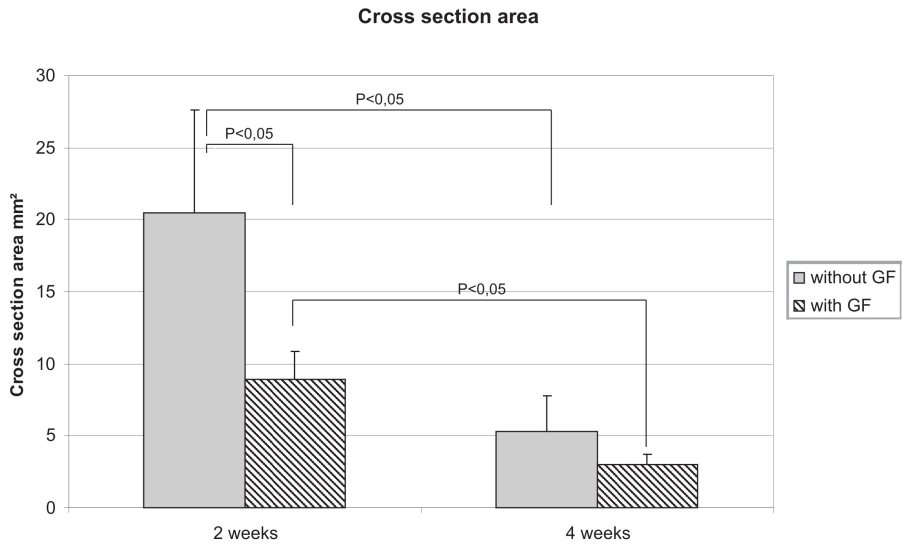

Figure 6. Morphometric analysis of construct size. There is a significantly decreased construct size in the growth factor (GF) group (group B) compared to group A (without growth factors) ($p<0.05$) after two weeks. Moreover, the construct size is considerably decreased after four weeks in group B compared to group A. In both groups, cross section area of the implants decreased between week two and four ($P > 0.05$).

Morphometric Analysis

Morphometric analysis of the constructs demonstrated a significant decrease of construct size after application of growth factors at two weeks (group A, 20.48 ± 7.14 mm^2; group B, 8.89 ± 1.97 mm^2; $P < 0.05$). The same trend was observed in the four-week implants; however, this difference was statistically insignificant. In both groups matrix volume significantly decreased over time ($P < 0.05$) (Figure 6).

The use of growth factors also induced higher absolute and relative vascular density in the implants. After two weeks a significantly higher vascular density was observed in specimens from group B compared to group A (group A, 36.52 ± 15.49 vessels per mm^2; group B, 133.45 ± 38.66 vessels per mm^2,; $P < 0.05$). A similar trend was observed in the four-week groups, with 200.75 ± 200.51 (group B) versus 38.89 ± 20.14 (group A); however, this difference was not statistically significant (Figure 7).

The absolute number of vessels per construct was also significantly higher in group B, with 287 ± 66 compared to 175 ± 78 in group A after two weeks ($P < 0.05$). The same trend was observed at four weeks (group B, 162 ± 171; group A, 46 ± 29); however, this effect was not statistically significant. The results are graphically displayed in Figure 8.

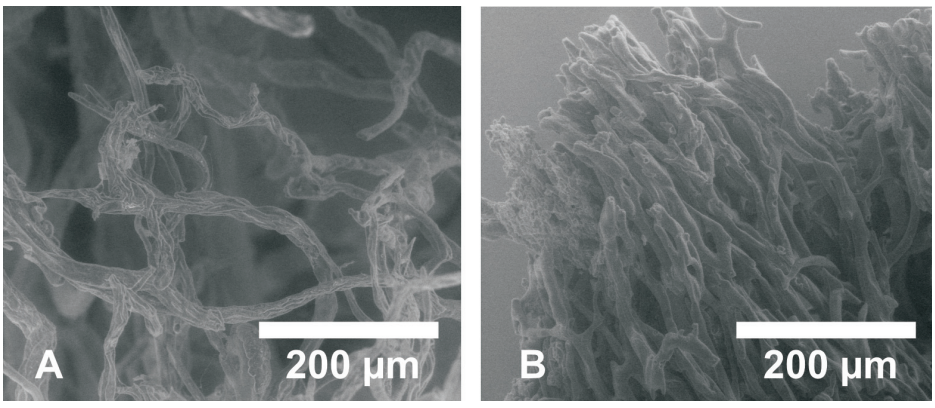

Figure 5. Scanning electron microscopy analysis of vascular corrosion casts of the specimens two weeks after implantation. In all specimens the neocapillary network displayed characteristics of an immature vascular bed, such as absence of a distinct vascular hierarchy and vivid angiogenesis in terms of sprout formation. In comparison to group A (A) the density of the neocapillaries was higher in the treatment group B (B). (magnification x 500).

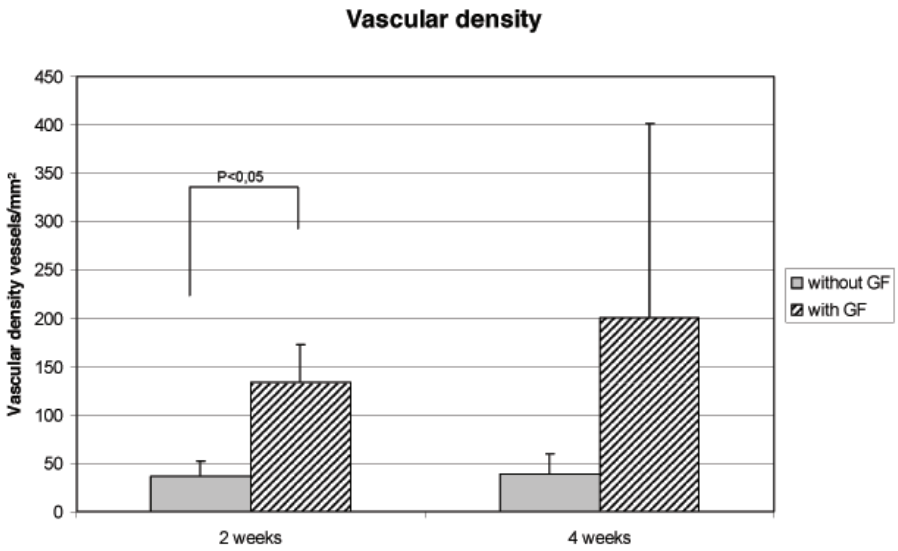

Figure 7. Morphometric analysis of vascular density. In the growth factor (GF) group (group B), a significantly higher vascular density compared to group A (without growth factors) ($p<0.05$) is observed at two weeks. Similar results, without reaching statistical significance are seen in the four week groups ($P > 0.05$).

DISCUSSION

The three main components of tissue-engineered constructs are vascularization, extracellular matrix, and cells. Sufficient vascularization remains the fundamental limitation for successful implantation of tissue-engineered constructs to any recipient site (26). It is well known that cells cannot survive when located further than 200-500 microns away from a capillary (1,5). To permit reliable microsurgical transplantation a vascularized tissue-engineered construct should have a dominant artery and vein. The axial type of vascularization allows vascularization and subsequent transfer of biomaterials independent of local conditions at the recipient site (27). The use of angiogenic factors may shorten the period between implantation and vascularization of matrices (7,8).

The most extensively tested growth factors in animal models are bFGF and VEGF. We used VEGF121 because it is not proteolytically degraded by plasmin when treated in solution (28). It is also known that bFGF acts synergistically with VEGF, additionally stimulating angiogenesis (10). In this study we were able to demonstrate the feasibility of using VEGF and bFGF immobilized in a fibrin-based drug delivery system in the AVL model in the rat. This model was first described by Erol and Spira 1979 (21) and enhanced by Morrison et al., who inserted the AVL into polycarbonate isolation chambers (6,22). Lokmic et al showed that the AVL without any matrix is able to produce a highly vascularized construct (29). Cassell et al demonstrated that Matrigel enables growth of new vessels to a higher extent than fibrin or poly lactic-co-glycolic acid (6). In the same study fibrin matrices reduced the formation of vascularized connective tissue compared to controls without matrix after four weeks. Fibrin also did not integrate with the generating vascularized tissue. Also, because the features of the fibrin matrix compared to fibrin-free controls have been described in detail previously, in this study we focused on fibrin as a drug release system in the AVL to show the angiogenic effects of fibrin-immobilized VEGF and bFGF in the AVL model.

Here we demonstrated that fibrin gel-immobilized VEGF and bFGF significantly enhanced the angiogenic response at two weeks, as evidenced by increased vascular density. The same trend was also observed after four weeks without reaching statistical significance. The absolute number of vessels per construct was significantly higher in group B after two weeks compared to group A without growth factors. Functionality of the newly grown vascular network was confirmed by positive CD31 staining in

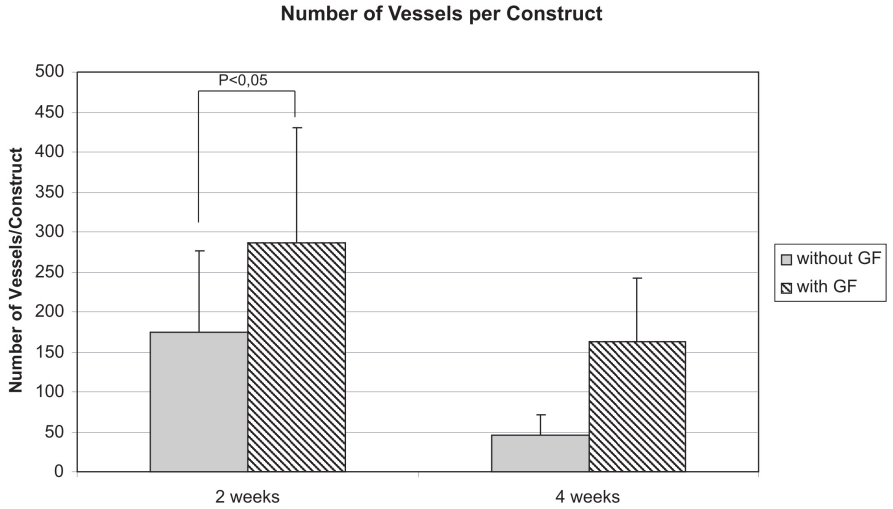

Figure 8. Morphometric analysis of absolute number of blood vessels. The absolute number of vessels per construct was significantly higher in group B compared to group A after two weeks ($P > 0.05$). The same trend was observed at four weeks without reaching statistical significance ($P > 0.05$).

combination with India ink perfusion experiments.

In this experiment we were unable to detect any histological difference between the venous and arterial branch of the AVL in both groups with regard to number of vessels, vessel diameter, and amount of newly formed tissue. Assessment of vascularization patterns, however, requires valid morphological techniques such as corrosion casting. After microdissection to expose the vascular axis, SEM of corrosion casts is a more accurate method to quantify luminal sprouting than histology or macroscopically evaluated casts (30). Recently we observed a higher density of vascular sprouts in the venous proportion of the AVL in a morphometric study of vascular corrosion casts (30).

It is known that bFGF is released in a relatively slow manner from the fibrin clot owing to tight binding of bFGF to fibrinogen (19). VEGF has a relatively short biological half-life, which constitutes a major limitation for its delivery as a recombinant protein. VEGF121 interacts very weakly with the fibrin clot, with almost half of the initial amount being released from the clot during the first day. On the other hand, with the use of growth factors the fraction of fibrovascular tissue increased as it progressed from the vessels of the vascular axis toward the peripheral regions. Two weeks following application of growth factors the fibrin matrix was replaced up to nearly 90% by connective vascularized tissue. Presumably, fibrinolysis in combination with a relatively fast release of growth factors from the fibrin-based drug delivery system led to an angiogenic enhancement in the first two weeks after implantation.

Scanning electron microscopy demonstrated a neocapillary network with characteristics of an immature vascular bed in all specimens of the two week group, with a higher vascular density in the growth factor group B. This phenomenon could be explained by enhanced initial sprouting from the lumen of the vascular axis as a result of an initially increased VEGF concentration. VEGF is known to induce vascular permeability, resulting in leakage of proteins and other molecules from blood vessels (31). Investigations on the relationship between angiogenesis and vascular permeability have led to the proposition that increased vascular permeability is a necessary and sufficient requirement for angiogenesis. Many angiogenic factors such as bFGF are not known to induce vascular permeability, and vascular leakage is not necessarily followed by angiogenesis.

The kinetics of angiogenesis is greatly influenced by differences in concentrations of fibrinogen in the matrix. Recently, we were able to demonstrate that for a fibrinogen concentration of 33 mg/mL the onset of neovascularization was delayed until day 12 to 14 after construction of the loop (30). Therefore we used a lower fibrinogen concentration in this study (10 mg/mL), whereby more rapid vascular growth was accompanied by marked fibrinolysis with disintegration of the matrix. The use of growth factors in group B led to a considerable decrease of construct size after two and four weeks compared to group A (without growth factors). The increased vascular density in group B may have enhanced matrix degradation and remodeling by cell-associated enzymatic activity during cell migration. The degradation of three-dimensional fibrin gel structures is reversely proportional to the supplemented aprotinin concentrations (32). Aprotinin acts as an inhibitor of human trypsin, plasmin, and plasma and tissue kallikrein by forming reversible enzyme-inhibitor complexes. It determines fibrinolysis by inhibiting plasmin. The observed fibrinolysis occurred despite added aprotinin.

To perform future evaluations at other time points, e.g., four weeks, modifications of the fibrin gel matrix will be necessary to further delay the degradation process. The fibrin gel might then serve as a carrier of modulatory substances and angiogenic growth factors in combination with different types of matrices. Eventually the implantation of AVLs into "smart" growth factor-enhanced matrices will facilitate upscaling of bioartificial devices for larger-volume applications (33,34). To the best of our knowledge, the study presented here is the first to demonstrate that the fibrin gel-immobilized angioinductive growth factors bFGF and VEGF121 efficiently stimulate sprouting of blood vessels in the AVL model.

CONCLUSION

Fibrin gel-immobilized angioinductive growth factors efficiently stimulate sprouting of blood vessels in the AVL model. The application of angioinductive factors in combination with the implantation of vascular carriers into given matrix volumes may eventually allow the generation of axially vascularized tissue-engineered composites for application in reconstructive surgery.

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