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Enhancement of capillary and cellular ingrowth in ePTFE implants with a proangiogenic recombinant construct derived from fibronectin

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Abstract

Based on our discoveries of a unique, synergistic interplay between vascular endothelial growth factor (VEGF) and specific domains of the matrix protein fibronectin (FN), we used recombinant technology to create a new protein construct derived from the cell-binding and VEGF-binding domains of FN. We wished to test the hypothesis that this prototype recombinant FN (rFN) protein would enhance cellular and capillary ingrowth *in vivo* into expanded polytetrafluoroethylene (ePTFE) implants. ePTFE disks of high porosity (60 micron internodal distance) were embedded with fibrin gel and heparin, with/without mixtures of VEGF and rFN and were implanted subcutaneously in rats. Control implants embedded with fibrin glue and heparin alone showed an average of 8.5% ($\pm 0.51\%$ standard error mean (SEM)) cellular ingrowth. The addition of either VEGF or rFN caused a modest but significant increase in cellular ingrowth ($12.7 \pm 1\%$ and $11.8 \pm 0.98\%$, respectively, $p < 0.004$). However, the combination of rFN/VEGF/heparin dramatically increased cellular ingrowth ($27.6 \pm 1.62\%$, $p < 0.001$), compared with all other treatments. Quantification of capillary ingrowth yielded the same pattern. These results suggest that the incorporation of such biological modulators into cardiovascular implants could offer new strategies for the design of a readymade small diameter prosthetic graft with enhanced capacity for neovascularization and endothelialization.

Keywords

expanded polytetrafluoroethylene; fibronectin; neovascularization; vascular endothelial growth factor; prostheses and implants; rats

INTRODUCTION

A major challenge to the effectiveness of biomaterial implants is the induction of a natural regenerative and healing response by the host. This is especially an obstacle for prosthetic replacements of small diameter blood vessels in humans. When no autogenous replacement is available, expanded polytetrafluoroethylene (ePTFE) is a favored substitute for blood vessels of 6-mm diameter and smaller. However, the ingrowth of cells and capillaries never fully penetrates the interstices of ePTFE grafts implanted in humans. Luminal endothelialization is rare, apart from the pannus ingrowth of endothelium from the ends of the arteries. For many years, techniques to induce spontaneous endothelialization of

prosthetic grafts have been extensively studied in a variety of animal models and conditions. The species, the age of the animal, and the type of surrounding tissue all affect the ease with which a prosthetic graft may endothelialize.¹ Still, the human species remains most resistant of all to endothelialization of prosthetic arterial grafts, regardless of the tissue location.

One strategy for inducing more natural tissue and capillary ingrowth is to embed pro-angiogenic molecules that will modulate the host's healing response within the implant. Vascular endothelial growth factor (VEGF) has been used by many investigators, with some improvements in graft healing and endothelialization. Our research has focused on identifying and refining key biomolecules that can encourage capillary and tissue ingrowth into ePTFE implants. In recent work, we have discovered that there is a unique and dedicated interplay between VEGF and specific domains of the matrix protein fibronectin (FN).²⁻⁴ Unlike other matrix proteins, such as vitronectin or collagen, FN actively binds VEGF to its heparin-II domain (type III repeats 12–14). This FN-VEGF complex positively modulates neocapillary growth and enhances the biological effects of VEGF on endothelial cells. Furthermore, it seems that the entire FN molecule is not required—only key functional domains are necessary. Using recombinant technology, truncated constructs of FN (hereafter denoted rFNIII_{9-10/12-14}) that link the cell-binding domain (type III repeats 9–10, the $\alpha 5\beta 1$ integrin ligand) with the VEGF-binding domain (type III repeats 12–14) were developed and expressed. Extensive *in vitro* experiments revealed that this truncated FN construct (rFN) forms a complex with VEGF that significantly enhances VEGF's proangiogenic effects, especially in the presence of heparin.⁴ This ternary complex of rFN/VEGF/heparin was more potent than VEGF alone or VEGF plus native FN. Furthermore, FN fragments containing these linked domains were most effective in directing endothelial precursor cells to differentiate into the endothelial line.³

We wished to test the hypothesis that this pro-angiogenic construct would increase the cellularity and capillarity of the interstices of ePTFE implants. Accordingly, ePTFE disks of high porosity (60 micron internodal distance) were embedded with mixtures of VEGF and heparin, with/without rFNIII_{9-10/12-14}, were implanted in rats, and then analyzed for the number of cells and capillaries within the implant.

MATERIALS AND METHODS

Recombinant human VEGF-165 was obtained from Genentech, South San Francisco, CA. A well-characterized unfractionated porcine mucosal heparin was purchased from Celsus, Cincinnati, OH. It has an $M_r \sim 15,000$, an anti-Xa activity of 181 units/mg, and a single batch was used for all experiments. Vascular ePTFE tube grafts measuring 6 mm in diameter, 0.6-mm wall thickness, and a porosity of 60 micron internodal distance were obtained from Atrium Medical, Hudson, NH. They were steam sterilized for 4 min before use.

Recombinant FN expression

Recombinant human FN construct encompassing type III repeats 9–10/12–14 was expressed in the *Pichia pastoris* expression system (Invitrogen). DNA construction and cloning into expression vector pPICZ α -B was performed as previously described.⁴ Expression of proteins was performed as described by the manufacturer. Briefly, the rFNIII_{9-10/12-14} vector was electroporated into the KM71H yeast strain. After selection with zeocin (1 mg/mL) on YPD plates, selected colonies were cultured in BMGY medium at 30°C until cultures reached an OD₆₀₀ of 5. Cells were pelleted and resuspended in BMMY medium and cultured for 4 days. Fresh methanol (final concentration 1%) was added daily. After 4 days, supernatants were precipitated with ammonium sulfate (70%) overnight at 4°C. Pellets were resuspended in 10 mM Tris-HCl, pH 7.2. The desired recombinant protein was purified by

FPLC affinity and S-sepharose ion-exchange chromatography (GE Healthcare BioSciences). Purity was greater than 98% as determined by silver nitrate staining of SDS-PAGE.

Implant preparation

The interstices of graft segments were prepared with fibrin glue containing various concentrations of experimental biological substances to be tested, using two methods of impregnation. In the first phase of the experiments, the ePTFE tube grafts were slit longitudinally into a flat configuration. The material was mounted in a 96-well dot blot manifold (Schleck & Schueller), and the liquid fibrin glue mixtures were applied under vacuum through the ePTFE until the material was saturated through and the fibrin had polymerized. In the second phase experiments, the ePTFE tube grafts were degassed in 100% ethanol for 15 min, then rinsed multiple times with phosphate-buffered saline. Clamping one end of the tube graft, the fibrin glue mixture was introduced into the lumen under pressure, until the interstices were saturated and the fibrin gelled. In both methods, the excess surface fibrin was removed by either gentle wiping or the method described by Zarge et al.⁵ for the tube grafts. Disks were cut to 4-mm diameter and then immediately implanted. All materials and procedures were performed under sterile conditions. To ensure that the interstices of the graft were filled with fibrin material, we added India Ink (Speedball) at a 1:500 dilution to the fibrin gel mix and treated a test piece of ePTFE with the mix. It was then fixed, paraffin-embedded, sectioned, and stained with H & E.

The fibrin glue was prepared with 5 mg/mL plasminogen-depleted fibrinogen (Calbiochem) to which was added 0.05 units/mL thrombin (Enzyme Research Laboratories) just before impregnation of the ePTFE. This stoichiometry of fibrinogen and thrombin was empirically developed to allow sufficient time for the liquid protein mixture to penetrate the interstices of the graft material before gelation.

Animal implant model

All animal procedures were performed following protocols approved by the VA Puget Sound Health Care System's Animal Care and Use Committee, and in compliance with the National Institutes of Health "Guidelines for the Care and Use of Laboratory Animals." Animal housing, care, and surgeries were conducted in facilities approved by the American Association for Accreditation of Laboratory Animal Care. Long Evans rats (Charles River) approximately 6-month old and weighing an average of 600 g were used for the implantation model. Under an isoflurane general anesthetic, four ePTFE disks of 4-mm diameter were implanted via surgical incisions on the dorsum of the rat approximately 1/2 inch on either side of the spine behind the scapulae. After surgery, each rat received one dose of buprenorphine for pain medication. The rats tolerated the implants well and showed no signs of distress or infection throughout the duration of the experiment.

The first phase of experiments consisted of pilot studies to determine the duration and location of implantation. The performance of control implants (containing fibrin glue alone) was compared with experimental implants containing the complete mixture of rFN, VEGF, and heparin (100 µg/mL VEGF; 25 µg/mL rFNIII_{9-10/12-14}, and 25 µg/mL heparin). These optimal ratios were chosen based on our empirical studies of their synergistic effects on endothelial cells *in vitro*.⁴ Pairs of these implants were placed in subcutaneous or intramuscular locations in 4 rats and explanted at 2 or 4 weeks.

In the second phase of experiments, the implants were placed subcutaneously in 8 rats and analyzed at 2 weeks. The purpose was to measure the independent and combined effects of embedding VEGF, rFNIII_{9-10/12-14}, and heparin within the ePTFE material. Disks were impregnated with fibrin glue containing each of four treatments: (A) heparin (control); (B)

heparin + VEGF; (C) heparin + rFNIII_{9-10/12-14}; and (D) heparin + VEGF + rFNIII_{9-10/12-14}. The biological additives were all used in the same concentrations as in the first phase, and a set of four matching disks representing ePTFE treatments A–D were implanted in each rat. Explantations were performed at 2 weeks. The rats were euthanized by sedation with isoflurane and then a lethal dose of pentobarbital. Within 20 min of sacrifice, the disks were retrieved and rinsed in 20 mL of ice-cold PBS before fixing in 10% neutral buffered formalin (Fisher Scientific).

Histology, cytochemistry, and quantitative analysis

Explant samples were immediately fixed for at least 24 h, then embedded in paraffin and cut in 5 micron sections. Dewaxed sections were stained with hematoxylin and eosin. The lectin *Griffonia simplicifolia* (biotinylated GSL-1, Vector Laboratories) was used to visualize and quantify endothelial cells. This lectin recognizes carbohydrate epitopes on endothelium, with mild cross-reactivity with cells from the monocyte/macrophage line.^{6,7} Endothelial structures were selected by their positive staining and characteristic vascular profiles. Staining with mouse anti-smooth muscle cell actin (Dako) was performed to confirm the identity of mature microvessel structures. Inflammatory monocyte/macrophage cells were identified by their typical morphologies on H&E staining and by specific staining with a mouse anti-rat CD68 antibody (AbD Serotec). The Vector Elite ABC Reagent kit was used to develop the lectin and antibody stains, using DAB (Vector Laboratories) as the substrate. Hematoxylin (Vector Laboratories) was used as the counter stain.

The total number of cells within the interstices of the ePTFE was quantified from DAPI fluorescent stained sections (Vector Laboratories), using the digital image analysis tools of Adobe Photoshop CS3. From high resolution 10× photomicrographs of sections, the total cross-sectional area of the interstitial space within the ePTFE grafts and the area occupied by DAPI positive nuclei of cells within the graft interstices were measured. Results were expressed as the percentage of vacant interstitial graft space that was occupied by cells, or “percent cellular ingrowth.” For each experimental and control explant, at least 5 complete cross sections (~0.6 mm by 3–4 mm) of ePTFE were quantified.

To quantify the cellularity of the border regions of the implant, areas of 300 × 300 microns were randomly selected beginning at the edge of the implant, and the cells counted as above.

Capillary ingrowth was measured from 40× magnifications of sections stained with GSL-1. Capillaries were counted as positive when they stained positively with GSL-1 and bore the typical cross-sectional or longitudinal morphology of capillaries. Some macrophages also stained positively with GSL-1, but these were easily distinguished from capillaries by their characteristic morphology and nuclei, and their positive staining for CD68. Measurements were made from four to six independent, complete sections from each explant, and calibrated with a micrometer standard. The capillary index was thus expressed as the number of capillaries per square millimeter of explant. The results of different treatments were compared by the Mann-Whitney rank sum test, using Sigmastat software.

RESULTS

The completeness of fibrin saturation of the interstices of the ePTFE was determined by microscopic examination of hematoxylin stained grafts that were embedded with fibrin but not implanted—and was found to be adequate. Furthermore, dry runs of graft preparation, in which the fibrin glue preparation was spiked with India ink, demonstrated penetration across the full thickness of the ePTFE (Fig. 1).

In the pilot study, eight pairs of disks were implanted in subcutaneous or intramuscular locations in four rats, explanted at different intervals, and analyzed for total cellular ingrowth by quantitative DAPI staining. The results showed that there were no significant differences between subcutaneous or intramuscular implantation sites and that cellular ingrowth peaked at 2 weeks. They also showed that the complete synergistic mixture of rFN/VEGF/heparin had a significantly positive effect on tissue ingrowth.

Figure 2 illustrates the results of these implants. Addition of the rFN/VEGF/heparin mixture to the fibrin glue dramatically increased cellular ingrowth within the ePTFE. This was true for both subcutaneous and intramuscular implants, at both 2 and 4 weeks. Second, the degree of cellular ingrowth at 4 weeks was not significantly different than that at 2 weeks. Finally, the intramuscular space did not appear to be significantly less conducive to cellular ingrowth, compared with the subcutaneous space, although there was a trend suggesting that the treated disks implanted in the intramuscular space at 4 weeks had slightly less ingrowth. At explantation, we also observed that the disks did not remain as consistently embedded in the intramuscular space.

A more definitive series of implantations were then conducted to distinguish the relative values of each experimental bioactive additive in promoting cellular and capillary ingrowth. Based on the pilot study, a 2-week duration, and subcutaneous location were chosen for all implants. Eight rats underwent implantation of four disks each, containing four different combinations of additives: (A) fibrin gel and heparin alone; (B) fibrin gel, heparin, VEGF; (C) fibrin gel, heparin, rFN; and (D) fibrin gel, heparin, VEGF and rFN.

Lymphocytes and small inflammatory cells were the predominant cell type within the interstices, with rarer macrophages and multinucleated giant cells at the entry points of internodal spaces (Fig. 3). Capillaries could be seen penetrating from the host tissue into the internodal spaces, and the abundance of capillaries paralleled the density of cellular infiltrate. Figure 3 contains illustrative photomicrographs. There was variability in the cellularity of the border regions of the tissue-implant interface, although this did not correlate with the experimental treatment group. The most significant qualitative difference observed between the treatments was the extent to which cells had migrated from the border of the implant into its center. Some implants showed full, transmural penetration of cells, whereas others displayed a progressive tapering of cell density, with a void of cells at the center. Figure 4 shows representative sections of the four different implant treatments, stained with DAPI, with and without polarizing light to enhance the ePTFE. These images illustrate the progressive increase in cellularity filling the empty spaces between the ePTFE nodes. In many sections treated with rFN/VEGF/heparin, the majority of the interstitial space was fully occupied with cells. These images also illustrate a general finding that the cellular density at the borders of the implant did not correlate well with the degree of central penetration.

Quantitative analysis of cellular density revealed marked differences according to the experimental treatment (Fig. 5). The inclusion of either VEGF (11% median ingrowth) or rFN (8.25% median ingrowth) caused a modest but significant increase in cellular ingrowth compared to the controls (fibrin gel plus heparin, $p < 0.004$). The ternary mixture of rFN/VEGF/heparin dramatically increased cellular ingrowth of the interstitial space of ePTFE grafts (27% median ingrowth, $p < 0.001$ compared with all other treatments). Quantitative analysis of the peri-implant cellularity showed no differences in cell density. The cell density for treatments A–D were $34.2\% \pm 12.6$, $25.1\% \pm 6.5$, $31.8\% \pm 5.9$, and $35.1\% \pm 6.8$, respectively.

The capillary index of the ePTFE explants closely paralleled the results of cellular ingrowth. Figure 6 shows that the control samples (A) containing only fibrin gel and heparin had significantly fewer capillaries than the samples embedded with VEGF/rFN/heparin (D). As expected, the capillarity of the B and C treatments was intermediate (data not shown).

DISCUSSION

These studies of ePTFE implants in rats confirm the potency of an engineered FN construct to significantly enhance cellular and capillary ingrowth. Previous studies confirmed the concept that FN and VEGF shared a unique biological synergism. This synergism absolutely required the VEGF-binding domain of FN (III₁₂₋₁₄), and the cell-binding domain (III₉₋₁₀). In a variety of *in vitro* studies of human umbilical vein endothelial and endothelial precursor cells, we showed that a recombinant protein construct derived from these two domains linked together was necessary and sufficient to significantly augment the biological actions of VEGF. The rFNIII_{9-10/12-14} construct dramatically enhanced VEGFR-2 and Erk phosphorylation.⁴ This truncated construct was more potent than native FN, suggesting either that the conformation of the construct enhances the functionality of these domains, or that native FN contains other regions that interfere with its pro-VEGF effects. We have observed this previously with ePTFE grafts implanted in the dog.⁸

In preliminary subcutaneous and intramuscular implants, the primary determinant of cellular ingrowth was found to be the presence or absence of the ternary mixture of rFN/VEGF/heparin, rather than the anatomic location of the implant. More detailed studies examining the independent roles of VEGF and the rFN construct again showed that maximal ingrowth and capillarity was achieved with the ternary complex. This could be due to enhanced VEGF signaling as shown by our previous studies or that VEGF bound to rFNIII_{9-10/12-14} is retained in the local environment much longer. Studies by Ehrbar et al.^{9,10} have shown increased vascularization when VEGF is bound and released on demand. The binary combinations of VEGF plus heparin and rFN plus heparin also significantly enhanced ingrowth compared with controls but less so. It was of interest to observe that both VEGF and the rFN construct were equally pro-angiogenic in the absence of the other. One explanation is that in a healing bioprosthesis, there may be limiting concentrations of local, endogenously produced VEGF and cryptic fragments of FN. Increasing the local concentrations of either may enhance angiogenesis to some extent.

Local inflammation is a key stimulant for neocapillary formation, and however when engineering new biological environments for healing, it is important to avoid the unintended consequences of excessive inflammation. Williams et al.¹¹ observed a more intense inflammatory response of macrophages and giant cells in ePTFE pretreated with FN, without further advancement of capillary ingrowth. We did not observe more inflammation in the presence of the rFN construct, perhaps because this construct lacks most of the adhesive domains of plasma FN, save for the cell-binding and the VEGF-binding regions.

To test the efficacy of our modulations of the biological milieu of the prosthetic implant, we specifically chose materials and models that are relevant to the human situation—that is, the ePTFE porosity and the animal implant model and location. By virtue of its manufacture, the internodal distance (IND) and fibril density of ePTFE are the dominant parameters of internal structure. Studies suggest that fibril morphology does not appear to be as important in cellular ingrowth as the IND, which is the primary determinant of porosity.^{12,13} The optimal IND for capillary ingrowth appears to be around 60 microns.^{14,15} The upper limit of effective porosity in the human is not known, but 100 micron implants are filled with more activated macrophages, and fewer capillaries.¹⁴ In baboon grafts, 90 micron IND forms an intima but it later sloughs off.¹⁶ The anatomic location of the implant is another important

factor. We chose a subcutaneous implant site that was likely to be more resistant to capillary and cellular ingrowth. Other anatomic locations are more conducive to neovascularization, such as the adipose tissue of the epididymal fat pad in the rat,¹⁷ the retropleural space in the dog, or retroperitoneum in the baboon.^{1,18,19}

This study examined one dominant factor controlling capillary and tissue ingrowth into bioprostheses: the biological milieu present within the scaffold. Recent reviews have summarized the biologically active molecules that have been used to mimic the natural biological environment necessary for directing cell differentiation and growth.^{20–23} The ideal choice for coating the interstices of a graft could be a mixture of the very proteins that are naturally laid down by endothelial cells. Williams and coworkers^{11,24} have confirmed this concept, by depositing a natural angiogenic matrix on ePTFE grafts using live cells. However, the cells are removed before implantation. Shimada et al.²⁵ demonstrated improved healing of small caliber ePTFE grafts using covalently bonded FN. The perspective of this study was to determine whether a mixture of bioactive molecules could be manufactured and embedded preoperatively, thus avoiding the impracticality of “growing up” each individual graft prosthesis over days or weeks. In this study, we tested the ability of a pro-angiogenic construct derived from FN to synergize with VEGF in promoting capillary ingrowth.

To reduce the potential of sampling errors, quantitative image analysis tools were used to accurately measure an entire histological section of the ePTFE implant. Because of the polarizing nature of the ePTFE, an accurate estimate of one of the most clinically relevant questions for bioimplants was obtained, namely how much of the potential “empty space” within the graft becomes occupied with living tissue and capillaries. The fluorescent marker DAPI stains the nuclear material of cells, not the cytoplasm, so obviously the parameter “percent cellular ingrowth” will never reach 100%. However, because the measurements are applied consistently for all implants, the comparative value of this measure is strong. Correlations of H&E sections adjacent to DAPI sections suggest that when the area of nuclei measured by DAPI approaches 30–40% of the available empty space, the majority of the interstitial space between ePTFE nodes is occupied by cells.

This study is a clear demonstration that a rationally engineered construct of FN can enhance capillary and cellular ingrowth into porous ePTFE *in vivo*. It does, however, have limitations. We primarily assessed the roles of VEGF and a recombinant FN, but a more complex mixture of growth factors and pro-angiogenic modulators may ultimately be optimal. Although capillary networks are a dynamic phenomenon, and very long term implant studies may be helpful, we did not observe significant fall-off in neovascularization and cellularity at 4 weeks. In addition, these implants were not true blood vessel prostheses that possess an inside and outside. A more complex model of vascular grafting will be needed to determine whether this enhanced cellular and capillary ingrowth will translate into a more rapid or complete endothelialization of the lumen of an ePTFE vascular graft. The role of fluid dynamics within the graft construct will also need to be examined, because bends in a graft construct can lead to less than optimal healing.²⁶ Measurements of fibrous capsule formation and its effects on graft healing and endothelialization will also be required. We are currently pursuing studies to examine both the durability and long-term efficacy of these biological modulators in such models. We are hopeful that approaches such as these may bring us closer to the goal of a ready-made, off the shelf, small diameter vascular prosthesis that possesses improved capacities for healing and endothelialization.

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References

1. Sterpetti AV, Hunter WJ, Schultz RD, Farina C. Healing of high-porosity polytetrafluoroethylene arterial grafts is influenced by the nature of the surrounding tissue. *Surgery*. 1992; 111:677–682. [PubMed: 1595064]
2. Wijelath ES, Murray J, Rahman S, Patel Y, Ishida A, Strand K, Aziz S, Cardona C, Hammond WP, Savidge GF, Rafii S, Sobel M. Novel vascular endothelial growth factor binding domains of fibronectin enhance vascular endothelial growth factor biological activity. *Circ Res*. 2002; 91:25–31. [PubMed: 12114318]
3. Wijelath ES, Rahman S, Murray J, Patel Y, Savidge G, Sobel M. Fibronectin promotes VEGF-induced CD34 cell differentiation into endothelial cells. *J Vasc Surg*. 2004; 39:655–660. [PubMed: 14981463]
4. Wijelath ES, Rahman S, Namekata M, Murray J, Nishimura T, Mostafavi-Pour Z, Patel Y, Suda Y, Humphries MJ, Sobel M. Heparin-II domain of fibronectin is a vascular endothelial growth factor-binding domain: Enhancement of VEGF biological activity by a singular growth factor/matrix protein synergism. *Circ Res*. 2006; 99:853–860. [PubMed: 17008606]
5. Zarge JI, Gosselin C, Huang P, Vorp DA, Severyn DA, Greisler HP. Platelet deposition on ePTFE grafts coated with fibrin glue with or without FGF-1 and heparin. *J Surg Res*. 1997; 67:4–8. [PubMed: 9070173]
6. Hansen-Smith F, Banker K, Morris L, Joswiak G. Alternative histochemical markers for skeletal muscle capillaries: A statistical comparison among three muscles. *Microvasc Res*. 1992; 44:112–116. [PubMed: 1386405]
7. Hansen-Smith FM, Watson L, Joswiak GR. Postnatal changes in capillary density of rat sternomastoid muscle. *Am J Physiol*. 1989; 257:H344–H347. [PubMed: 2750947]
8. Murray-Wijelath J, Lyman DJ, Wijelath ES. Vascular graft healing. III. FTIR analysis of ePTFE graft samples from implanted bigrafts. *J Biomed Mater Res B Appl Biomater*. 2004; 70:223–232. [PubMed: 15264304]
9. Ehrbar M, Djonov VG, Schnell C, Tschanz SA, Martiny-Baron G, Schenk U, Wood J, Burri PH, Hubbell JA, Zisch AH. Cell-demanded liberation of VEGF121 from fibrin implants induces local and controlled blood vessel growth. *Circ Res*. 2004; 94:1124–1132. [PubMed: 15044320]
10. Ehrbar M, Zeisberger SM, Raeber GP, Hubbell JA, Schnell C, Zisch AH. The role of actively released fibrin-conjugated VEGF for VEGF receptor 2 gene activation and the enhancement of angiogenesis. *Biomaterials*. 2008; 29:1720–1729. [PubMed: 18155761]
11. Williams SK, Kleinert LB, Hagen KM, Clapper DL. Covalent modification of porous implants using extracellular matrix proteins to accelerate neovascularization. *J Biomed Mater Res A*. 2006; 78:59–65. [PubMed: 16602088]
12. Hirabayashi K, Saitoh E, Ijima H, Takenawa T, Kodama M, Hori M. Influence of fibril length upon ePTFE graft healing and host modification of the implant. *J Biomed Mater Res*. 1992; 26:1433–1447. [PubMed: 1447228]
13. Miura H, Nishibe T, Yasuda K, Shimada T, Hazama K, Katoh H, Watanabe S, Okuda Y, Kumada T. The influence of node-fibril morphology on healing of high-porosity expanded polytetrafluoroethylene grafts. *Eur Surg Res*. 2002; 34:224–231. [PubMed: 12077509]
14. Davids, L.; Dower, T.; Zilla, P. The lack of healing in conventional vascular grafts. In: Zilla, P.; Greisler, HP., editors. *Tissue Engineering of Prosthetic Vascular Grafts*. Austin: R.G.Landes; 1999. p. 3–44.

15. Salzmann DL, Kleinert LB, Berman SS, Williams SK. The effects of porosity on endothelialization of ePTFE implanted in subcutaneous and adipose tissue. *J Biomed Mater Res.* 1997; 34:463–476. [PubMed: 9054530]
16. Golden MA, Hanson SR, Kirkman TR, Schneider PA, Clowes AW. Healing of polytetrafluoroethylene arterial grafts is influenced by graft porosity. *J Vasc Surg.* 1990; 11:838–844. [PubMed: 2359196]
17. Williams SK, Berman SS, Kleinert LB. Differential healing and neovascularization of ePTFE implants in subcutaneous versus adipose tissue. *J Biomed Mater Res.* 1997; 35:473–481. [PubMed: 9189825]
18. Hayashida N, Han MT, Wu MH, Shi Q, Wechezak AR, Sauvage LR. Differential effect of the retropleural and retroperitoneal environments on healing of the inner wall of porous fabric prostheses in the thoracic and abdominal aorta of the same dog. *Ann Vasc Surg.* 1995; 9:369–377. [PubMed: 8527338]
19. Hazama K, Nishibe T, Shimada T, Miura H, Watanabe S, Kondoh S, Takahashi T, Okuda Y, Kato H. Effects of omental wrap on performance of small-caliber high-porosity expanded polytetrafluoroethylene grafts. *J Surg Res.* 1999; 81:174–180. [PubMed: 9927537]
20. Alsberg E, Anderson KW, Albeiruti A, Rowley JA, Mooney DJ. Engineering growing tissues. *Proc Natl Acad Sci USA.* 2002; 99:12025–12030. [PubMed: 12218178]
21. Sakiyama-Elbert SE, Hubbell JA. Functional biomaterials: Design of novel biomaterials. *Annu Rev Mater Res.* 2001; 31:183–201.
22. Stock UA, Vacanti JP. Tissue engineering: Current state and prospects. *Annu Rev Med.* 2001; 52:443–451. [PubMed: 11160788]
23. Xue L, Greisler HP. Biomaterials in the development and future of vascular grafts. *J Vasc Surg.* 2003; 37:472–480. [PubMed: 12563226]
24. Kidd KR, Nagle RB, Williams SK. Angiogenesis and neovascularization associated with extracellular matrix-modified porous implants. *J Biomed Mater Res.* 2002; 59:366–377. [PubMed: 11745574]
25. Shimada T, Nishibe T, Miura H, Hazama K, Kato H, Kudo F, Murashita T, Okuda Y. Improved healing of small-caliber, long-fibril expanded polytetrafluoroethylene vascular grafts by covalent bonding of fibronectin. *Surg Today.* 2004; 34:1025–1030. [PubMed: 15580386]
26. Lyman DJ, Stewart SF, Murray-Wijelath J, Wijelath E. Role of fluid dynamics on the healing of an in vivo tissue engineered vascular graft. *J Biomed Mater Res B Appl Biomater.* 2006; 77:389–400. [PubMed: 16278848]

FIGURE 1.

Saturation of interstices of ePTFE with fibrin. H&E stained cross section demonstrating the completeness of the fibrin saturation of the ePTFE using fibrin spiked with India Ink. Arrows denote deposition of the India Ink. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 2.

Quantitative analysis of timecourse and location of implants. Means $\pm$ SEM of eight pairs of implants placed in the subcutaneous (SC) and intramuscular (IM) locations, explanted at 2 weeks (left) and 4 weeks (right). The experimental implants (expmntl) were embedded with rFN/VEGF/heparin, and the controls (ctrl) with fibrin glue alone. The experimental implants showed significantly greater cellular ingrowth at all time points and locations compared with their respective controls ($p < 0.001$, rank sum test). There were no significant differences in cellular ingrowth at 2 versus 4 weeks for the same treatments.

FIGURE 3.

Cellularity, capillaries, and cell phenotypes in explants. (A) H&E staining shows progressively sparser ingrowth from the border of the implant (40×). (B) Comparable section stained for endothelial cells with GSL-1 lectin (40×). (C) Smooth muscle cell actin staining of mature capillaries (10×). Arrows indicate capillaries penetrating the interstices (A–C). (D) Anti-CD68 antibody staining of macrophages/giant cells found predominantly at the implant borders (10×). Arrows indicate macrophages. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 4.

Ingrowth into treated ePTFE Implants. Representative 2-week explants stained with DAPI to mark nuclei, photographed at 10× under fluorescent and polarizing light (upper) and fluorescent light alone (lower). Cellular ingrowth was quantified as the % of empty interstitial space occupied by DAPI positive cells. Treatment codes and quantitative results are in Figure 5. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 5.

Quantitative analysis of cellular ingrowth of implants. Boxes indicate the median and 25/75 percentile values and whiskers the 5/95 percentiles. Treatments B and C were significantly different than the control (A) ($p = 0.004$), and D was significantly greater than all other treatments ($p < 0.001$), all by the rank sum test.

FIGURE 6.

Capillary indices of explants. The number of capillaries/mm² was measured from GSL-1 stained sections of control (A) explants, and those treated with rFn/VEGF/heparin (D). The differences between control and treatment were highly significant ($p < 0.001$), rank sum test.