in thrombotic events with heparin-coated Palmaz-Schatz stents in normal porcine coronary arteries. Circulation 1996;93:423-30.
41. Burchenal JEB, Marks DS, Mann JT, Schweiger MJ, Rothman MT, Ganz P, et al. Effect of direct thrombin inhibition with Bivalirudin (Hirulog) on restenosis after coronary angioplasty. Am J Cardiol 1998;82:511-5.
42. van de Werf F. More evidence for a beneficial effect of platelet glycoprotein IIb/IIIa-blockade during coronary interventions: latest results from the EPILOG and CAPTURE trials. Circulation 1996;93:637.
43. Aggarwal RK, Ireland DC, Azrin MA, Ezekowitz MD, deBono DP, Gershlick Ah. Antithrombotic potential of polymer-coated stents eluting platelet glycoprotein IIb/IIIa receptor antibody. Circulation 1996;94:3311-7.
44. Sollott SJ, Cheng L, Pauly RR, Jenkins GM, Monticone RE, Kuzuya M, et al. Taxol inhibits neointimal smooth muscle cell accumulation after angioplasty in the rat. J Clin Invest 1995;95:1869-76.
45. Axel DI, Kunert W, Goggelmann C, Oberhoff M, Herdeg C, Kuttner A, et al. Paclitaxel inhibits arterial smooth muscle cell proliferation and migration in vitro and in vivo using local drug delivery. Circulation 1997;96:636-45.

FIBRIN-BASED MATRICES FOR ANGIOGENIC STIMULATION

Andreas H. Zisch, MD
U. Schenk
B. Schweitzer
S. Sakiyama-Elbert
J. C. Schense
J. A. Hubbell
Institute for Biomedical Engineering
Zurich, Switzerland

Our laboratory's research in vascular tissue engineering focuses on two distinct goals: first, in the context of building blood vessels de novo, we aim to engineer biomaterial surfaces that are able to attract and retain endothelial cells organized as two-dimensional endothelial cell sheet under flow conditions, similar to the the native luminal vessel wall. Second, featuring the three-dimensional organization of endothelial cells, the generation of biomaterial matrices, which when grafted to the wounded or ischemic tissue, locally stimulate neovessel formation in the surrounding of the graft and also in the graft itself. In both cases, the material designs aim the vascular grafts to become populated in situ with endothelial cells derived from the body's endogenous sources (ie, from the artery adjacent to the graft, from existing blood vessels, or from circulating endothelial progenitor cells). For that reason, these grafts must have properties specifically attractive for endothelial cells. Through basic research, a better knowledge has been developed about the molecular aspects and requirements for reendothelialization and blood vessel formation. This biological information can be inferred on a biomaterial by incorporating peptide or protein motifs that are necessary for the recruitment and the binding of endothelial cell binding. Polypeptide growth factors, in particular vascular endothelial growth factor (VEGF), have evolved as critical for many aspects of endothelial cell biology, such as the maturation, the proliferation, the assembly, and the survival of endothelial cells. The Hubbell laboratory has recently developed methodology that allows fibrin to be modified by covalent incorporation of bioactive peptides or proteins during the coagulation process. The base matrix fibrin is a natural ingrowth matrix for endothelial cells. To confer the angiogenic features of VEGF upon fibrin, we used this methodology to covalently incorporate VEGF121, an otherwise diffusible VEGF isoform into the fibrin matrix. This VEGF121/fibrin matrix exhibits strong angiogenic responses both in vitro and in vivo as described below. Further, we will discuss a sophisticated and elegant fibrin modification scheme in which growth factors, such as the VEGF165 isoform, can be entrapped and retained within the matrix through their heparin-binding abilities. From these depots, growth factors can be released by controllable diffusion as well as the activity of matrix-degrading enzymes secreted by cells invading the matrix.

The clinical demand for angiogenic materials—modified fibrin as material for clinics

Design of materials that induce angiogenesis in vivo is important for many areas of tissue regeneration and wound healing. One of the more relevant and therefore well studied needs is in treatment of chronic wounds, such as diabetic ulcers, pressure ulcers, or venous static ulcers that are caused by deficient vascular supply. Scores of trials with ulcer patients have been conducted over the past years using growth factor proteins or cytokines as therapeutics, with many discouraging results. One likely explanation for the failure of many therapies is the current modes of growth factor delivery in the context of creams and sprays, which are often inappropriate for providing sustained delivery of active factors. Without enbedment, however, the soluble protein factors are cleared very rapidly from the target site, or never reach the target in sufficient quantities, or reach it only at extreme dose. For angiogenic stimulation with VEGF, this dilemma of delivering VEGF protein active is now attacked in parallel by tissue engineering and somatic gene therapy approaches. These include first, novel, "smarter" biomaterials that present the polypeptide growth factors properly and

therefore work with much smaller doses of active factors; second, the in situ expression of the gene after intramuscular injection or adenovirus mediated delivery of the DNA; and third, representing a combination of point one and two, the use of so called gene activated matrices where the gene is added as plasmid into a biomatrix that can be invaded by cells that pick up the DNA and subsequently express the gene product. Indeed, the results with VEGF gene therapy are encouraging for the treatment of myocardial and peripheral ischemia.[1-5] As vehicle for the delivery of the protein itself, modified fibrin represents an excellent candidate material. Fibrin is nontoxic and as "fibrin glue," accepted for clinical application. It can be readily formed directly at the site of therapeutical need from a mix of the precursor solutions containing fibrinogen, thrombin and factor XIIIa. Through novel methodology developed in the Hubbell laboratory, exogenously added peptides or proteins, modified by the N-terminal addition of the transglutamination substrate sequence NQEQVSPL, can be covalently attached to the fibrin network through the action of factor XIIIa during the coagulation process.[6] We have used this scheme to incorporate growth factor into the fibrin gel or to confer growth factor release characteristics onto fibrin. Polypeptide growth factors are not freely diffusible when present in tissues but associated with extracellular matrix components, such as heparan sulfate proteoglycans. The interaction with matrix components is critical to stabilize the factors' active conformation. Degradation of the matrix components by cellular enzymes such as heparinases or proteases then leads to the release of the growth factors in soluble form. We exploit the heparin-binding activity of many polypeptide growth factors to add a heparin-based release system to fibrin to mimic the growth factors' natural presentation through heparan sulfate proteoglycans in the extracellular matrix. A synthetic heparin-binding peptide derived from antithrombin III is covalently incorporated into fibrin during the coagulation process to accomplish this. When heparin is added simultaneously into the mixture before coagulation, a matrix rich in heparin, in character similar to extracellular matrix, is created. Any growth factor entrapped within this matrix would then interact with the incorporated heparin molecules and be retained within the matrix.[7,8] The rate of diffusion of the growth factor out of the gel can be readily controlled by the chosen concentrations of heparin-binding peptide, heparin, and growth factor in the fibrin gel. The heparin-binding VEGF165 isoform, which now became available in our laboratory, will be tested in this scheme.

Fibrin loaded with VEGF121 promotes angiogenesis

VEGF is present endogenously in several isoforms, differing in molecular weight and their ability to bind heparin.[9] The isoform VEGF121 is a non-heparin-binding VEGF variant. Its mitogenic activity for endothelial cells appears to be somewhat weaker compared with the heparin-binding variant VEGF165, and therefore the VEGF165 isoform has been favored for use in previous therapeutic studies. However, the different activities of VEGF variants might also reflect their differing abilities to bind to matrix components that stabilize their active conformation. VEGF121 has been succesfully used in adenoviral gene therapy for treatment of coronary artery disease.[4] In our studies, VEGF121 was covalently incorporated into fibrin. By that, its release was dependent on the stability of fibrin itself, as only degradation of fibrin should result in VEGF release. Incorporation was accomplished by generating through recombinant DNA methodology a novel molecule TG/VEGF121, containing the factor XIIIa substrate sequence NQEQVSPL from α2 plasmin inhibitor added to the aminoterminus. The protein was expressed and purified in *E coli.* In soluble form, this modified TG/VEGF retained its mitogenic activity for endothelial cells that compared the published activity of unmodified VEGF121. The covalent incorporation of TG/VEGF into fibrin was analyzed using radiolabeled TG/VEGF. One to 120 μg/mL gel was added to the precursor solutions, and over this range the amount of incorporated VEGF was linearly dependent on the amount of added growth factor, with a degree of incorporation of approximately 50%. Hence, because of the incomplete incorporation, the resulting matrix contained a combination of both free and matrix-bound VEGF and thus represented a matrix that combined both chemotactic and haptotactic features for endothelial cells. Angiogenic stimulation by the VEGF121/fibrin matrices was assayed in both three-dimensional endothelial cell cultures and the chick chorioallantoic assay. Human umbilical vein endothelial cells or pulmonary aortic endothelial cells were cultured either directly in TG/VEGF fibrin gels that were formed by the addition of 1 μg of TG/VEGF per milliliter gel, or in plain fibrin gels that were formed adjacent to TG/VEGF loaded fibrin gels. Control cultures were seeded in fibrin only. Endothelial cells grown directly in TG/VEGF loaded fibrin or in unmodified fibrin in proximity to TG/VEGF loaded fibrin were viable over 2 weeks and very prominently exhibited all classical hallmarks

of angiogenesis, such as capillary networks and cord formation. Notably, these characteristics were absent in cells cultured in isolated, unmodified fibrin. Using the chick chorioallantoic membrane (CAM) assay, we confirmed the strong angiogenic stimulation mediated by TG/VEGF loaded fibrin in vivo. Fibrin gels of 100 μL, loaded with 1 to 10 μg of TG/VEGF, were directly formed on the chorioallantoic membrane and microscopically analyzed after 3 days in culture. VEGF-loaded fibrin gels were strongly vascularized, whereas control gels made of fibrin only remained basically vessel free.

Prospects for angiogenic therapy

It is likely, that somatic gene therapy and engineered biomaterials will compete friendly for better angiogenic performance, which is good news for hundreds, thousands, or millions of patients suffering from tissue ischemia or impaired wound healing worldwide. A certain beauty shared by both approaches, intramuscular gene delivery with "naked" cDNA, as well as protein delivery through modified fibrin, is the fact that formulation respective material generation is relatively simple, which may facilitate their approval as clinical tools. Yet a big demand remains for technical solutions that allow the administration of these therapeutics inside the body with minimal invasion. But fibrin gels can be readily formed in the wound bed of chronic skin ulcers. One of our laboratory's immediate goals is to test if fibrin-based delivery systems with various growth factors, including VEGF, improve wound healing in skin. We hope that one of these growth factors will ride the fibrin horse for therapy in man.

This work was supported by the Swiss National Fond Project 38, grant NF 4038-52235.

REFERENCES

1. Baumgartner I, Pieczek A, Manor O, et al. Constitutive expression of phVEGF165 after intramuscular gene transfger promotes collateral vessel development in patients with critical limb ischemia. Circulation 1998;97:1114-23.
2. Losordo DW, Vale PR, Symes JF, et al. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF as sole therapy for myocardial ischemia. Circulation 1998;98:2800-4.
3. Losordo DW, Val PR, Isner JM. Gene therapy for myocardial angiogenesis. Am Heart J 1999;138:132-41.
4. Rosengart TK, Lee LY, Patel SR, et al. Angiogenesis gene therapy: phase I assesment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. Circulation 1999;100:468-74.
5. Symes JF, Losordo DW, Vale PR et al. Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease. Ann Thorac Surg 1999;68:830-6.
6. Schense JC, Hubbell JA. Cross-linking exogenous bifunctional peptides into fibrin gels with factor XIII. Bioconjug Chem 1999;10:75-81.
7. Sakiyama S, Schense JC, Hubbell JA. Incorporation of heparin-binding peptides into fibrin gel enhances neurite extension-an example of designer matrices in tissue engineering. FASEB J. In press.
8. Sakiyama-Elbert S, Schense JC, Hubbell JA. Development of fibrin derivatives for controlled release of heparin binding growth factors. J Controlled Release. In press.
9. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997;18:4-25.