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Systems for therapeutic angiogenesis in tissue engineering

Abstract The goals in tissue engineering include the replacement of damaged, injured, or missing body tissues with biologically compatible substitutes. To overcome initial tissue-mass loss, improved vascularization of the regenerated tissue is essential. Two pathways of tissue neovascularization are known: vasculogenesis, the in situ assembly of capillaries from undifferentiated endothelial cells (EC), and angiogenesis, the sprouting of capillaries from preexisting blood vessels. Recent advances in our understanding of the process of blood-vessel growth have provided significant tools for the neovascularization of bioengineered tissues. Several growth factors serve as stimuli for EC proliferation and migration as well as the formation of new blood vessels. They convey their effects via specific receptors expressed on the surface of EC. Vascular epithelial growth factor (VEGF) is a major regulator of neovascularization. VEGF plays a major role in the early development of blood-cell progenitors. Basic fibroblast growth factor (bFGF) was identified as the first angiogenic factor. It is a potent inducer of EC proliferation and blood-vessel growth in vitro and in vivo. VEGF and bFGF have been injected into undervascularized ischemic tissues, resulting in new blood-vessel formation and tissue perfusion. Gene-therapy approaches using VEGF cDNA injection into ischemic tissues have augmented the formation of collateral vessels. Angiogenic factors such as VEGF and bFGF have also been incorporated into bioengineered tissues and have facilitated blood-vessel growth. Other approaches such as prevascularization of the matrix prior to cell seeding and incorporation of EC into the bioengineered tissues have produced encouraging results. This article reviews the process of blood-vessel growth and tissue vascularization, placing emphasis on strategies that can be employed for efficient vascularization of engineered tissues in vitro and in vivo.

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The goal in tissue engineering is the replacement of damaged, injured, or missing body tissues with biologically compatible substitutes, which often contain cells that have been cultured in vitro. Significant progress has been made in the cultivation of large amounts of cells in vitro and in the design and use of support materials to deliver the cells in vivo [5, 59]. However, these are usually "single-cell-type" cultures, and attempts to reconstruct tissues with more than one cell type have usually encountered problems. Another critical obstacle in tissue engineering is the ability to maintain large masses of cells alive following their transfer from in-vitro culture conditions into the host [61]. For achievement of the goals of engineering large complex tissues and, possibly, internal organs, vascularization of the regenerating cells is essential. A piece of tissue with a volume exceeding a few cubic millimeters cannot survive by diffusion of nutrients but requires the growth of blood capillaries for the supply of essential nutrients and oxygen [61]. Observations made by Folkman and colleagues [28, 30] over the last two decades have indicated that growing tumors, usually consisting of a "single cell type", do not grow beyond a few millimeters unless they grow their own blood vessels. For their expansion, tumors can direct the ingrowth of capillaries from adjacent blood vessels. This research has resulted in a better understanding of how new capillaries are formed, a process known as angiogenesis [41, 71]. An alternative mode of neovascularization is the formation of new vessels from endothelial cells (EC) and their progenitors (angioblasts). This process, called vasculogenesis, normally takes place during embryogenesis in developing organs [19, 72]. Specific molecules, namely, angiogenic factors, have been identified that can control

and guide blood-vessel growth via vasculogenesis and angiogenesis [35].

This article reviews the process of blood-vessel growth and tissue vascularization, placing emphasis on strategies employed for the engineering of tissues in vitro and in vivo. A number of angiogenic molecules are described, as are models designed for the study of angiogenesis.

Neovascularization: the formation of new blood vessels

The formation of new blood vessels and capillaries comprises two different processes: vasculogenesis, the in situ assembly of capillaries from undifferentiated EC, and angiogenesis, the sprouting of capillaries from preexisting blood vessels. The formation of the first capillaries takes place mostly during the early stages of embryogenesis [34, 96].

Vasculogenesis can be divided into five consecutive steps [19]: (1) EC are generated from precursor cells, called angioblasts, in the bone marrow; (2) EC form the vessel primordia and aggregates that establish cell-to-cell contact but have no lumen; (3) a nascent endothelial tube is formed, composed of polarized EC; (4) a primary vascular network is formed from an array of nascent endothelial tubes; and (5) pericytes and vascular smooth-muscle cells are recruited.

Angiogenesis is a morphogenic process that describes the formation of new blood capillaries from EC of preexisting vessels. Six basic steps are involved in angiogenesis [33]: (1) vasodilatation of the parental vessel, reducing the contact between adjacent EC; (2) degradation of the basement membrane of a parental vessel by the secretion and activation of a wide range of proteolytic enzymes; (3) EC migration and proliferation, resulting in the formation of a leading edge of the new capillary; (4) generation of the capillary lumen and formation of a tube-like structure; (5) basement membrane synthesis; and (6) recruitment of pericytes and vascular smooth-muscle cells.

Angiogenesis plays a major role in wound healing and collateral formation and in the female reproductive system. In wound healing and tissue repair, neovascularization is required for the transport of cells and nutrients into the wound [38]. The new blood capillaries emerge from neighboring vessels during tissue remodeling. Collateral vessel formation is essential throughout the human body. Myocardial collateral vessels that sprout from preexisting capillaries are important in protecting the heart from ischemic damage. In myocardial infarction, new vessels penetrate the necrotic area and the surrounding ischemic heart tissues [73]. In the female reproduction cycle, neovascularization occurs physiologically in the uterus and the ovary every 28 days. Placental tissues represent a major site of active angiogenesis [12, 83].

Besides the important physiologic role of angiogenesis, many serious diseases are associated with excessive capillary growth. In chronic inflammatory diseases such as rheumatoid arthritis, new vessels invade the joint surfaces and degrade the cartilage by proteolysis [9]. Intraocular neovascularization is usually associated with diabetic retinopathy and retinopathy of prematurity [54]. The new blood vessels are leaky and rupture easily, which may result in blindness. However, most importantly, angiogenesis is associated with cancer because it is a prerequisite for tumor growth and metastasis [28]. Tumors usually do not grow beyond a volume of a few cubic millimeters unless they become vascularized. Metastasis is dependent on the tumor vasculature that delivers tumor cells into the circulation.

Angiogenic EC and angiogenic factors

In the adult, large-vessel EC are usually in a quiescent stage due to cell-cell contact and inhibition of proliferation. During conversion to the angiogenic stage, EC capture new properties that enable them to neovascularize the tissue [41]. Conversion of EC to the angiogenic phenotype is stimulated under special conditions, such as low oxygen levels (hypoxia), and is accompanied by (a) changes in cell shape, which facilitate migration; (b) enhanced secretion of proteolytic enzymes, which degrade the basement membrane and the extracellular matrix; and (c) increased sensitivity to angiogenic factors, induced by modulation of receptor expression [27]. When the new vessels are in place and the vascular network matures the neovascular EC resume their quiescent phenotype [16]. Thus, when exposed to angiogenic stimulation the macrovascular EC can go through their developmental program, proceeding from the formation of endothelial tubes to branching and, finally, to the establishment of a vascular network.

Several growth factors serve as stimuli for EC conversion to the angiogenic phenotype [9]. EC possess specific cell-surface receptors that bind these factors and convey the signals into the cell to the nucleus [40]. Tumor biology research in the last decade has yielded a large list of angiogenic molecules. It is important that we determine which cells express these molecules, whether the molecules affect relevant vascular structures directly or indirectly via other cells, and how specific these molecules are in inducing their effects on neovascular EC morphogenesis. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are two examples of well-characterized and important angiogenic molecules.

Vascular endothelial growth factor

VEGF is a major regulator of neovascularization under physiologic and pathologic conditions [23, 25]. VEGF is a potent angiogenic factor in a variety of in vivo models. It promotes new capillary growth in the chick chorioallantoic membrane (CAM), in the cornea, and in the

skin. VEGF is a specific mitogen for EC and it stimulates EC to migrate and to form tubes in vitro. A series of VEGF-receptor gene "knockout" studies has revealed the importance of VEGF protein in vasculogenesis and angiogenesis [36, 78]. VEGF plays a major role in early embryonic development, and embryos lacking even one copy of the VEGF gene do not develop beyond the early embryonic stages [14, 24]. When initially discovered, VEGF was also purified as a vascular permeability factor (VPF), which is consistent with its strong angiogenic activity in vivo [21, 51]. VEGF is produced as five homodimeric isoforms that differ in their expression levels and in their localization. $VEGF_{121}$ and $VEGF_{165}$ are the most abundant isoforms. $VEGF_{121}$ is completely soluble and does not bind the heparan sulfate proteoglycans (HSPG) on the cell surface. $VEGF_{165}$ has moderate affinity for heparin but is mostly soluble. The other isoforms, $VEGF_{145}$, $VEGF_{189}$, and $VEGF_{206}$, have a high degree of affinity for heparin and are almost completely sequestered on the cell surface [44, 67]. VEGF isoforms have similar activities, although $VEGF_{121}$ is 50- to 100-fold less potent than $VEGF_{165}$ in an EC proliferation assay [53]. This greater mitogenic activity may be explained by the interaction of $VEGF_{165}$, but not of $VEGF_{121}$, with the neuropilin-1 receptor [84–86] as discussed below. A recent study described the generation of mice expressing only the $VEGF_{121}$ isoform [15]. These mice had a 50% embryonic lethality, and those that survived died within 6 days due to improper development of the myocardium and insufficient cardiac vascularization.

VEGF is widely expressed in different tissues by a variety of cell types. The expression of VEGF is upregulated in tissues undergoing vascularization during embryogenesis and in the female reproductive cycle [12, 83]. High levels of VEGF are found in various types of tumors, but not in normal tissue, consistent with its role in tumor angiogenesis [20, 56, 69, 82]. High levels of VEGF are found in the vitreous of patients suffering from retinopathy [54]. The synthesis of VEGF is greatly enhanced under hypoxia [81]. It has been found that hypoxia-derived elements stabilize VEGF mRNA and increase VEGF mRNA transcription [57, 58, 81]. Besides the different isoforms of VEGF, other VEGF-homologous proteins with angiogenic activity have been isolated. These include placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and the orf virus VEGF (VEGF-E) [93].

The VEGF family members share a family of VEGF receptors: VEGF-R1 (Flt-1) [18, 80], VEGF-R2 (KDR/Flk-1) [92], VEGF-R3 (Flt-4) [26], and neuropilin-1 (NRP-1) [86, 88]. VEGF-R1 to VEGF-R3 are composed of an extracellular portion that binds VEGF and an intracellular portion with a catalytic domain that triggers a cytoplamic signaling cascade following the association of VEGF with the receptor [93]. The fourth member, NRP-1, lacks the intracellular catalytic unit and probably acts as a coreceptor for VEGF-R2 [86]. NRP-1 is an isoform-specific VEGF receptor because it binds $VEGF_{165}$, but not $VEGF_{121}$ [84]. Expression of NRP-1 by EC augments the mitogenic and chemotactic activity of $VEGF_{165}$, but not that of $VEGF_{121}$ [86]. Interestingly, NRP-1 is also expressed by many tumor cell lines and various normal non-EC types, suggesting that it could sequester VEGF on the surface of tumor cells and transactivate neighboring EC [84].

Fibroblast growth factor

bFGF, or FGF-2, was the first FGF isolated from bovine pituitary [7, 11]. bFGF acts as a strong fibroblast mitogen and was identified as the first EC mitogen [1]. bFGF is also a chemotactic factor for EC and is highly angiogenic. A second member of the FGF family, acidic FGF (aFGF, or FGF-1), was purified at the same time from bovine brain [13]. Some oncogenes, such as *int-2*, *hst* (or *KS*), and FGF-5 have been found to encode proteins that share homology with the FGF family. The oncogene homologues are mostly secreted, whereas aFGF and bFGF are localized to the HSPG on the cell surface and in the extracellular matrix (ECM) [55]. Heparin and HSPG play an important role in protecting FGF from proteolytic degradation and potentiate its mitogenic activity [97]. Both aFGF and bFGF lack a signal peptide and are normally not secreted. It is not yet clear how they are delivered into the ECM.

bFGF is widely distributed in normal and malignant tissues, whereas aFGF is mainly expressed in neuronal tissues and bone but is also found in tumors [8]. In contrast to VEGF, FGF is not specific for EC. FGF receptors are expressed in EC and in smooth-muscle cells, fibroblasts, myoblasts, and tumor cells [8]. Besides their angiogenic activity, the FGFs are important for wound healing and tissue repair [11]. They stimulate epithelialization and regeneration of cartilage. FGFs play an important role in mesoderm differentiation during embryonic development and are required for limb-bud formation. bFGF is mitogenic for neuronal cells such as oligodendrocytes, astrocytes, and Schwann's cells. bFGF and aFGF promote differentiation of neuroblasts and serve as survival factors for neurons in culture [74].

FGF receptors are expressed by many cell types that respond to FGFs. Like VEGF, FGF family members bind a family of cell-surface catalytic receptors, but they may differ in their signaling pathways [8]. bFGF and aFGF bind to FGF-R1 (flg) and FGF-R2 (bek). More recently, FGF-R3 and FGF-R4 receptor proteins have been discovered [11].

Indirect angiogenic factors

A large group of angiogenic growth factors do not have a direct effect on EC, such as induced proliferation or migration, but they induce angiogenesis in vivo. These factors are considered indirect angiogenic factors and

include platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), and angiopoietin (Ang).

Plate-derived growth factor

PDGF is a potent stimulator of growth and motility of fibroblasts and smooth-muscle cells, but it also acts on EC and neurons. PDGF induces an angiogenic response in vivo but does not affect EC proliferation directly. PDGF is a potent mitogen for vascular smooth-muscle cells. PDGF supports the formation of functional vascularized connective tissue in wound healing during tissue repair. PDGF receptors are found only on a subset of EC, mostly on developing endothelial tubes and on microvascular EC, but not on quiescent EC. It is not yet clear whether PDGF can initiate angiogenesis in vitro or support an ongoing angiogenic process [70].

Transforming growth factor-β

TGF-β inhibits EC proliferation in vitro but promotes angiogenesis in vivo [68]. TGF-β and its receptors are important regulators of EC differentiation and of the establishment and maintenance of vessel-wall integrity [68]. The effects of TGF-β on angiogenesis probably occur via the recruitment of macrophages and fibroblasts that secrete angiogenic factors [68]. TGF-β functions to stabilize the newly formed vessels by recruiting smooth-muscle cells and pericytes as well as promoting their proliferation [16]. It has been shown that when EC are cocultured with mesenchymal cells, TGF-β expression is increased, leading to suppression of EC proliferation. Taken together, these findings suggest that TGF-β has a different function with regard to EC and vessel formation at different stages of the angiogenic process.

Angiopoietin

The angiopoietins (Ang), Ang1 and Ang2, have recently been discovered as ligands for the EC receptor Tie-2 [17]. However, like TGF-β, these factors do not seem to enhance EC proliferation, but they have an important role in angiogenesis in vivo [17]. The first indications of their role in vessel formation came from receptor-gene "knockout" studies before the ligands were discovered [76]. Tie-2-null mice have EC that form tubes but lack branching networks and proper organization into larger vessels, suggesting an indirect angiogenic effect of the ligands. Only Ang1 can activate the Tie-2 receptor to signal for the recruitment of periendothelial support cells such as pericytes and smooth-muscle cells. Ang2 is a competitive inhibitor of Ang1 and it blocks Ang1 signaling through Tie-2. The proposed function for Ang2 is that it serves to inhibit the interaction between EC and the supporting cells so as to facilitate the migration of EC from the vessel to form a new capillary.

Antiangiogenic factors

The angiogenic process is also associated with pathologic conditions, most importantly, with tumor growth and metastasis. Hence, there is considerable interest in molecules that display antiangiogenic activity because of their therapeutic potential. Several naturally occurring antiangiogenic molecules have recently been discovered, including thrombospondin-1, platelet factor-4, fumagillin derivative AGM-1470 (TNP-470), thalidomide, angiostatin, and endostatin [29]. These molecules can inhibit EC proliferation in vitro, disrupt endothelial tubes, and, most importantly, repress tumor growth [64, 65]. Some of the antiangiogenic molecules are currently undergoing clinical trial for the treatment of cancer, atherosclerosis, and retinopathy [31]. Although antiangiogenic factors have recently attracted much attention, their potential for tissue engineering is not yet clear.

Models for the study of angiogenesis

The identification of new angiogenic growth factors has allowed the use of some of these factors for clinical application. However, before they are applied pharmacologically, the total effect of each factor, whether alone or in combination with other factors, should be realized. Various assays have been designed for the measurement of angiogenic potential, ranging from in-vitro EC proliferation assays to complex in vivo studies [50]. Advanced in vivo assays have documented the different aspects of an angiogenic factor but have usually not determined direct effects on the EC. The in-vitro EC proliferation or migration assays demonstrate whether the factor can act directly on EC but cannot predict the extent of their angiogenic effects in vivo. Another in vitro assay measures endothelial tube formation in a three-dimensional bed made of a collagen gel or similar matrices [94]. This assay tests the functionality of EC in response to the angiogenic factor.

The simplest in vivo assay for the measurement of angiogenesis is performed in the chorioallantoic membrane (CAM) [62]. Angiogenic factors such as VEGF and bFGF induce the formation of a heavy capillary network on the CAM. More complex tests are done in the whole animal. The cornea micropocket assay is performed by insertion of the factor into a small incision in the cornea of a rat or mouse [52]. New blood vessels growing into the avascular cornea are then analyzed. The factor can be injected intravenously for assay of its systemic effect. Another novel system used to study angiogenesis consists of a transparent chamber placed in the animal's skin, which allows the visualization and documentation of the kinetics of new blood-vessel growth [98]. With this assay it is possible not only to test

the angiogenic effect of a certain molecule but also to look at the angiogenic process during wound healing or after tissue transplantation.

Therapeutic angiogenesis for ischemia and tissue engineering

Recent advances in our understanding of the angiogenic process and the isolation of potent and specific angiogenic growth factors have encouraged the use of these factors therapeutically [42, 47]. Treatment is divided into two approaches, therapeutic angiogenesis and therapeutic vasculogenesis. The first approach uses an angiogenic factor as a pharmacologic agent. Earlier studies using bFGF protein have shown augmentation of collateral vessel formation in a rabbit ischemic hindlimb model [6] or in infarcted myocardium [95]. Treatment with bFGF resulted in better perfusion of, increased blood flow to, and salvage of the ischemic tissues. Clinical trials using bFGF and aFGF proteins in patients undergoing coronary-artery bypass surgery have shown improvement of perfusion and revascularization [77].

Evidence that VEGF is a specific EC growth factor has suggested its potential in therapeutic angiogenesis. Injection of $VEGF_{165}$ protein has enhanced the revascularization of an ischemic hindlimb of a rabbit [90]. Like bFGF, VEGF treatment has induced collateral vessel formation, endothelium-dependent blood flow, and tissue perfusion. An alternative strategy for therapeutic angiogenesis using recombinant angiogenic growth factors is gene therapy. The first study using $VEGF_{165}$ cDNA was performed by gene transfer into the iliac artery of an ischemic hindlimb of a rabbit [10, 91]. VEGF protein was expressed at the site of injection, augmenting the formation of collateral vessels. Subsequently, intramuscular gene transfer of VEGF cDNA was used in a similar model with similar results [2]. This technique was further employed in patients with peripheral vascular disease and critical limb ischemia [49]. Clinical trials have shown significant improvements in collateral blood flow, healed ischemic ulcers, and, most importantly, salvage of limbs in patients in whom amputation has been imminent [46]. VEGF protein and cDNA have also been used for coronary revascularization, resulting in improved myocardial perfusion and increased collateral density [48].

Therapeutic vasculogenesis illustrates the use of endothelial cells for revascularization. Although vasculogenesis has been thought to be restricted to embryogenesis, recent studies have reported the presence of endothelial progenitor cells (EPC) circulating in the bloodstream [3]. EPC can be recruited to distinct sites and, upon stimulation with angiogenic factors, can differentiate into mature EC and participate in the angiogenic process. Furthermore, the amount of circulating EPC has been increased by ischemia and by treatment with granulocyte/macrophase colony-stimulating factor (GM-CSF), which stimulates hematopoetic stem-cell production [89]. These studies point to EPC recruitment and differentiation as a major component in tissue revascularization. Altogether, the progress recently made in experimental and clinical angiogenic therapy offers practical approaches to tissue vascularization in vivo.

The requirements for ischemic tissue revascularization and for "de novo" vascularization of engineered tissues share many similarities. However, whereas angiogenic processes taking place in ischemic limbs or infarcted myocardium have been studied extensively, studies on the vascularization of bioengineered tissues have not been performed until recently. Efforts have been aimed at incorporating the knowledge acquired on angiogenesis in ischemic tissues into practical approaches to the vascularization of bioengineered tissues.

Bioengineered tissues are usually supported by scaffolds of biocompatible matrices made from natural or artificial sources [45]. The matrix must maintain a mechanical strength sufficient to withstand compressional forces from the surrounding tissue in vivo. Successful vascularization is dependent on the porosity of the supporting matrix. A positive correlation between the pore size of poly-L-lactic acid (PLLA) implants and the rate of vascularization has been observed, although the vascularization was accompanied by the accumulation of fibrous tissue in the implants [60, 75]. In another study the use of polyvinyl alcohol (PVA) as the supporting polymer showed that an increase in implant porosity was correlated with increased diffusion coefficiency and vascularity [79].

Three approaches have been used for vascularization of bioengineered tissue: (1) incorporation of angiogenic factors in the bioengineered tissue, (2) seeding of EC along with other cell types in the bioengineered tissue, and (3) prevascularization of the matrix prior to cell seeding. Angiogenic growth factors may be incorporated into the bioengineered tissue prior to implantation for the attraction of host capillaries and enhancement of neovascularization of the implanted tissue. In one study, VEGF was embedded in alginate beads for the release of bioactive VEGF, which would otherwise be quickly degraded [22]. VEGF-alginate beads were incubated with medium and VEGF activity could be detected in the medium for up to 2 weeks. An alternative approach to the supplementation of angiogenic factors takes advantage of genetically engineered cells that secrete high levels of angiogenic proteins. In a recent study, cultured myoblasts were infected with a recombinant retrovirus encoding for VEGF protein, and myoblasts expressing VEGF were implanted into the tibialis anterior of mouse legs [87]. The newly formed vascular structures consisted of proliferating EC that formed networks of vascular channels and hemangiomas. These structures resembled those formed during embryonic assembly of blood vessels, i.e., vasculogenesis.

We have recently used a new approach to accomplish VEGF delivery into bioengineered tissues (Fig. 1). High levels of VEGF can be produced by Chinese hamster

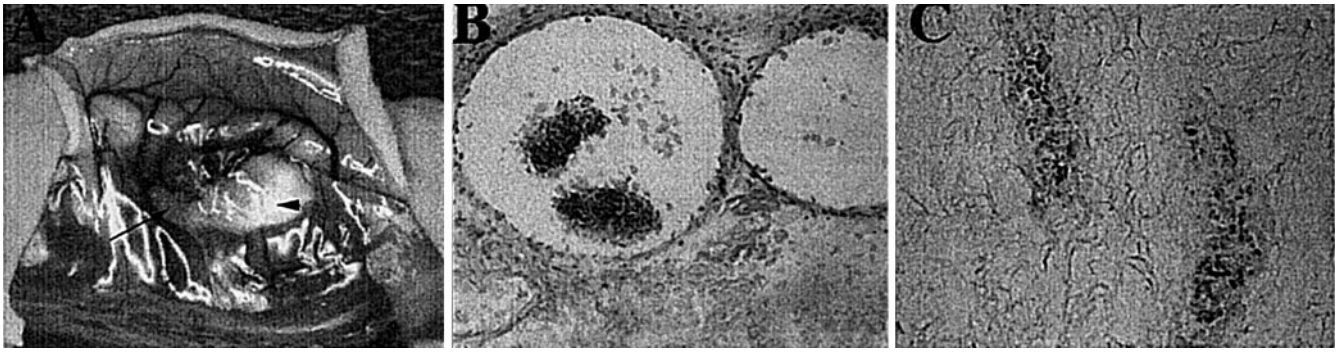

Fig. 1A–C VEGF delivery in vivo for the enhancement of matrix vascularization. Chinese hamster ovary (CHO) cells transfected with $VEGF_{165}$ cDNA were encapsulated in microspheres composed of Ca-alginate and coated with poly-L-lysine. CHO/VEGF encapsulated cells were coimplanted with matrices in mice. The matrices were retrieved after 3 weeks, embedded in paraffin, and sectioned. **A** Macroscopic examination of the implantation site. Increased vascularization is evident at the site of matrix (*arrow*) and encapsulated-cell (*arrow head*) implantation. **B** Cross section of retrieved CHO/VEGF microspheres. The section was immunostained for VEGF, showing the positively stained CHO/VEGF cells within the capsules. **C** Sections of the matrix were immunostained with anti-CD-31 (PECAM) antibodies, which showed colonies of positively stained EC

ovary (CHO) cells transfected with $VEGF_{165}$ cDNA. CHO/VEGF cells were encapsulated in microspheres composed of Ca-alginate and coated with poly-L-lysine. VEGF was secreted in large amounts into the medium of the encapsulated cells for up to 6 weeks. Encapsulated CHO/VEGF cells were coimplanted with cell-seeded matrices in mice. Macroscopic examination of the implantation sites revealed a progressive increase in vascularization near the cell-seeded matrices. Control matrices, which had been implanted without seeding of encapsulated CHO/VEGF cells, showed no change in vascularization. Immunohistochemical analysis showed positive staining of vessels and an increasing number of newly formed capillaries at the implant site only. These results indicate that angiogenic factors such as VEGF can be used to direct EC to the implantation site, where they then form structures that develop blood vessels.

The second approach employs cultured EC, which are incorporated into the bioengineered tissue prior to implantation. We have seeded human penile corpus-cavernosum-derived smooth-muscle cells and EC on biodegradable polymer scaffolds so as to reconstruct penile corporal tissue in vitro and in vivo [66]. Cell-seeded polymers were implanted in the subcutaneous space of nude mice. Histologic examination indicated the organization of smooth-muscle tissue and the accumulation of endothelium lining the luminal structures at 14 days postimplantation. Well-organized tissue structures consisting of muscle cells and newly formed capillaries were observed after 28 and 42 days. Another study showed that incorporation of aortic EC into polyglycolic acid (PGA) matrices resulted in the development of capillaries and lymphatic-like structures in the matrices at 4 weeks after implantation [43]. In addition, unorganized EC and other unusual vascular structures were observed, such as parallel arrays of capillaries, thin sinusoidal structures, and some layered complex structures.

The issue involving the use of EC as a source for tissue revascularization is complex, not only due to the choice of the correct EC type, i.e., arterial versus venous, but also for immunologic reasons. The vasculature EC of the implant are the first cells to be exposed to the host's immune system and may trigger an immunologic response that could lead to rejection. Ideally, the bioengineered tissue should be vascularized with the patient's own EC. Recent advances in our understanding of EC differentiation could provide a novel solution to this problem. It has been found that EC progenitors (EPC), circulating in human peripheral blood, could be isolated and grown in culture [3]. Cultivation of these cells in the presence of VEGF enhanced their differentiation to EC and promoted the formation of endothelial tubes and, subsequently, the expression of endothelial nitric oxide synthetase. In vivo treatment of mice with VEGF resulted in increasing numbers of circulating EPC [4]. Using the cornea micropocket assay, it was shown that VEGF also enhanced the incorporation of EPC into sites of active angiogenesis. Bone-marrow-derived cells have previously been used to coat polytetrafluoroethylene (PTFE, Teflon) vascular grafts [63]. The marrow cells survived for up to 6 months and the grafts showed complete endothelialization and maintenance of patency. Grafts without bone marrow cells were endothelialized only at the anastomotic sites, but other areas were covered with fresh thrombi and were subsequently occluded. These results suggest a new approach to isolation of autologous EC for the vascularization of bioengineered tissues. Alternatively, creation of an "angiogenic trap" at the site of implantation could enhance the recruitment of ECP into the implant.

An alternative direction in the vascularization of bioengineered tissue is the prevascularization of the supporting polymer prior to cell seeding. In this manner the bioengineered tissue becomes organized around the vascular network, providing sufficient tissue perfusion. Prevascularization was achieved by initial implantation of the "empty" polymer to allow the growth of fibrovascular tissue [37]. Subsequently, human hepatocytes were injected into the prevascularized polymer, and

reorganized hepatic parenchyma could be observed after 9 days. This study demonstrates the feasibility of prevascularized matrix support for freshly isolated or in-vitro-cultured cell implantation.

Despite the successes achieved in the bioengineering of tissues consisting of thin layers of cells, such as skin, a major challenge for future tissue engineering is the production of larger organs with more complex structures, such as the kidney. Tissues with a large mass of cells require a vascular network of arteries, veins, and capillaries for the delivery of nutrients to each cell. The development of efficient methods for the vascularization of bioengineered tissues is critical for a successful outcome. Some fundamental guidelines must be followed before the goals of tissue vascularization can successfully be achieved; these are basic principles that have emerged from years of angiogenesis and tissue-engineering research:

1. The support matrix for the bioengineered tissue must be compatible with EC growth and capillary formation [61]. The matrix can be coated with a substance that allows EC adherence and growth, such as collagen, fibronectin, or laminin, among others. The matrix should have a high degree of porosity to allow the penetration of blood vessels into the implant.
2. Angiogenic growth factors are required for EC proliferation and blood-vessel formation. It would be conceivable to incorporate the source of a slow-release form of an angiogenic factor into the bioengineered tissue prior to implantation so as to enhance new capillary growth from the host's vascular network into the implanted tissue [22]. Alternatively, cells within the bioengineered tissue can be genetically altered to secrete angiogenic factors [39, 87]. Secretion of angiogenic factors from the implant can also attract circulating EPC to the implantation site and promote the vascularization of the tissue [4].
3. For the enhancement of tissue revascularization, EC or EPC may be incorporated into the bioengineered tissue. These EC form capillaries within the tissue in vitro and connect to the host's vascular network. The combination of EC seeding into the bioengineered tissue with the insertion of a constitutive source for angiogenic factor secretion may be advantageous.
4. The angiogenic process in the bioengineered tissue must be controlled before a functional vascular network can be obtained. Sustained overproduction of angiogenic factors such as VEGF may result in deformed, nonfunctional blood vessels [87]. The generation of new vessels should follow the kinetics of normal development in the vasculature [16, 34]. A combination of concentrations and various periods of exposure to different angiogenic factors is recommended. For example, high concentrations of VEGF may be required for the initial differentiation, proliferation, and recruitment of EC to form endothelial tubes. Subsequently, proliferation should subside and the recruitment of pericytes and smooth-muscle cells can be augmented by TGF-β. Vascular maturation and stability requires another set of growth factors, such as angiopoietin and PDGF. Finally, for the avoidance of hypervascularization of the bioengineered tissue, antiangiogenic molecules such as angiostatin and endostatin may be used [32].

Many obstacles must be overcome before entire tissue-engineered solid organs can be produced. Recent developments in angiogenesis research may provide important knowledge and essential materials for the accomplishment of this goal.

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