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Engineering more than a cell: Vascularization Strategies in Tissue Engineering

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Summary

Host integration and performance of engineered tissues have been severely limited by the lack of robust strategies to generate patent vascularization and tissue perfusion. This review highlights a selection of exciting developments in vascularization approaches for tissue engineering research. Current strategies for vascularization in tissue engineering are related to growth factor signaling and delivery, cell transplantation, bioactive smart matrix materials, and directed fabrication. Application of these techniques to in vivo models has resulted in a number of robust host vascular responses, especially with synergistic and engineered bioactive systems. The future outlook of the field includes refinement and development of new technologies for vascularization and combining these techniques with functional repair models for metabolically active tissues and relevant disease states.

Introduction

Tissue engineering quickly grew as a field through the 1990's on its promise to deliver manufactured organs and tissue constructs to address the organ transplantation shortage [1]. As we progress into the second decade of the 21st century, the organ shortage still persists and progression of tissue engineering approaches from mere combinations of cells and materials to true living and integrated tissues have stalled due in part to the lack of robust strategies to generate patent vascularization and integration with host vasculature. To provide sufficient oxygen tension for survival, metabolically active tissues must reside within 150 to 200 μm of a capillary lumen [2]. Without a perfusing blood supply as a central component of any tissue engineering design, engineered tissue scalability, survival, and integration are extremely limited. With the exception of a few poorly vascularized tissues such as skin epidermis and cartilage, many of the initial targets of tissue engineering such as artificial pancreas as well as cardiac and hepatic tissues have yet to be realized. Advancement to engineering these higher-order levels of tissue architecture has required a deeper understanding of the mechanisms of vascular development and the patterns of interaction between multiple cell types on molecular, cellular, and tissue scales [3]. Functional tissues are permeated with hierarchical blood vessel, nerve, and lymphatic networks and may additionally contain epithelial ductwork. Much like the wiring, plumbing, and ventilation systems which are central to the function of a modern building, these supportive infrastructures are critical to native tissue survival and integration

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with organism-level physiology. Engineered tissues on any meaningful scale or complexity must incorporate aspects of functional infrastructure, with vasculature being the most immediately critical for survival and adequate function of transplanted cells. It is little wonder then, that a great impetus has been placed on vascularization as an integral aspect of the regenerative medicine paradigm.

Current Tissue Engineering Approaches to Vascularization

In its fundamental definition, tissue engineering or regenerative medicine is not limited to building artificial tissue in the lab, but ultimately encompasses treatments to enhance or restore function to diseased and damaged tissue [1]. Driving many of the cell and growth-factor based strategies to vascularize engineered tissue constructs *in vitro* is the clinical research aimed at restoring circulation to ischemic tissue in a variety of pathologies. With the development of recombinant angiogenic and vasculogenic growth factors, initial clinical studies were undertaken to deliver growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in myocardial and peripheral limb ischemia (Fig. 1A). The prohibitive cost and complexity of maintaining high levels of recombinant protein over a sufficient time frame in the zone of ischemia quickly led to the experimental evaluation of gene therapy and delivery of regenerative cell types as well as combination therapies. A large body of evidence with more than 1000 patients enrolled in placebo-controlled trials established the relative safety of angiogenic gene therapy, [4] and ongoing studies have generally established the safety of cell-based therapies. Advances in the clinic such as evidence of improved cardiac output and reduced incidence of non-healing ulcers are overshadowed by the fact that rigorous phase II and III trials have failed to unequivocally demonstrate improvements in high level benchmarks such as exercise stress testing.

Promising results from combination therapies co-delivering [5] or time-release staggering multiple growth factors [6] support the idea that a robust vascular healing response requires coordination of the correct signaling factors, dosing, and exposure time points. Incorporation of biomaterial delivery vehicles aims to solve some of the complex pharmacokinetics, but this approach is ultimately hindered by a lack of knowledge of optimal dosage and timing and the inability of delivered signals to override the “background noise” of a pro-inflammatory environment. Research into “master switch” upstream activators such as HIF-1α [7-8] that activate an entire pro-vascular signaling cascade are an exciting direction to the growth factor delivery field. But even if regenerative medicine can one day fully recapitulate the pharmacokinetics of an endogenous healing or developmental vascularization response, ultimately the question arises as to why the natural healing mechanisms failed to begin with or in the case of myocardial infarction, why no native regenerative repair process occurs at all. Various disease states, aging, and scar-tissue formation are obvious blockades to natural endothelial repair mechanisms, and growth factor signaling alone may be an intrinsically limited strategy when delivered in the context of diseased or non-healing tissue [9].

New approaches to treating ischemia are focusing on delivery of regenerative cells alone (Fig. 1B) and in combination with growth factors and biomaterial scaffolds (Fig. 1E). For cell therapy, we are beginning to understand the importance of delivering multiple progenitor cell-types in creating functional tissues. Advances in gene and cell delivery techniques have yielded astonishing results in animal models such as polymeric nanoparticle gene delivery vehicles combined with human embryonic (hEST) and mesenchymal stem cells (MSC) that showed significant vascularization and engraftment [10].

While current tissue engineering approaches aim to overcome the native tissue dysfunction by delivering effective regenerative cells in conjunction with the appropriate matrix and signaling molecules, the monumental challenge of integrating engineered tissues with the host

vasculature remains significant. The challenge of clinically delivering functional and vascularized large-scale tissue substitutes creates a 'chicken-or-the-egg' paradox. Is a functional vasculature required before regenerative cells can be transplanted, or are the regenerative cells needed to give rise to the new vasculature simultaneously as functional tissue develops? Researchers must attempt to either: connect and perfuse a pre-fabricated functional critical-sized tissue, form a pre-vascularized site and subsequently add in functional tissue, or simultaneously form vasculature alongside functional tissue.

From the standpoint of *in situ* tissue formation, tissue engineering research has progressed to the point of predictably and repeatedly producing patent, stable vasculature in a variety of animal models through transplantation of a combination of endothelial and mesenchymal cells or progenitors encapsulated in biological extracellular matrix (ECM) [11] and Matrigel™ implants [12]. The driving force behind this remarkable advancement is a mimicry of embryonic vasculogenesis where angioblasts and mesenchymal stem cells organize into a network to form a pericyte-stabilized capillary bed [13]. The ability to recapitulate this capillary network formation using adult cells obtained in routine sampling procedures of the blood and bone marrow represents a useful and feasible pool from which to further develop clinically relevant vascularized tissue constructs. An alternative source of vasculogenic cells in adult patients are endothelial progenitor cells (EPC) which can be mobilized to circulation from the bone marrow by administration of granulocyte colony stimulating factor (G-CSF) and home to sites of ischemia, inflammation, and biomaterials with artificial EPC capturing motifs [14]. EPC are an intriguing cell type for treating ischemic conditions that have shown promising functional recovery in animal models [15] and human trials [16-17].

Matrigel™, a decellularized matrix derived from mouse sarcoma cells, has been a common component for both *in vitro* endothelial tube formation and *in vivo* 3D network vascularization. However, Matrigel™ is a poorly controlled and highly uncharacterized environment from an engineering perspective, containing a mélange of growth factors and matrix-associated bioactive signals. Unfortunately, because of its tumoral and xenogenic origin, Matrigel™ is ultimately a sub-optimal choice for development of clinically-relevant therapies. Major effort is being concentrated on development of fully synthetic or well-defined biological matrices with potent pro-angiogenic properties manifested either through encapsulated co-culture systems of endothelial and mesenchymal cells or cell-free smart materials (Fig. 1C) directly recruiting vascular ingrowth from the surrounding host tissue.

Novel vascular-inductive biomaterial systems include schemes for directly conjugating growth factor to a degradable matrix and releasing it in a cell-demanded manner. One such system that has shown promising results incorporates bioactive ligands into a synthetic polyethylene glycol (PEG) hydrogel. PEG hydrogels for vascularization have been developed with different crosslinking reaction schemes. Popular renditions include 4-arm PEG-vinyl sulfone (PEG-VS) crosslinked by Michael-type addition [18] and PEG-diacrylate (PEGDA) crosslinked by photoinitiated free-radical polymerization [19]. Both systems are functionalized with protease (MMP)-cleavable peptide sequences, domains for cell adhesion (RGD peptide), and tethered growth factors. These PEG-based matrices have been used to promote both *in vitro* [20] and *in vivo* [19,21] vascular network formation from encapsulated cells or vessel ingrowth from the surrounding tissue. Engineered natural fibrin-based matrices offer an alternative to the PEG systems, with recombinant variants of growth factors designed to directly bind fibrin, and are remarkably potent inducers of vessel ingrowth from surrounding tissue [22]. These engineered matrices that directly bind growth factor and release it in a proteolytically-dependent or "ondemand" manner induce more stabilized and longer-lasting vasculature compared to diffusive growth factor release. However, even these induced stabilized vessels are reported to regress in time in the absence of true physiological demand [18]. Yet another promising artificial matrix idea utilizes self assembled peptide amphiphile nanofibril matrices with

heparin sulfate binding sites to present bioactive ligands and growth factors to promote *de novo* subcutaneous vascularization [23]. Research in engineered matrices has progressed for tissue engineering models including cardiac progenitor differentiation [24], pancreatic islet encapsulation [25], and epithelial morphogenesis [26]. We can expect future research to combine engineered vascular-inductive matrices with repair or replacement of metabolically active tissues *in vivo*.

An alternative strategy to inducing vascular organization into a scaffold is to fabricate vascular conduits directly prior to implantation (Fig. 1D). Several clever engineering techniques to generate endothelial-lined channels in tissue engineered constructs have emerged. One simple yet effective technique involves close-packed modular cylindrical collagen matrices coated in endothelial cells to generate endothelial lined channels in a random packed array. These channels remodel *in vivo* to generate a vascularized graft [27]. Another self-assembly technique uses microtissue building blocks made from human artery-derived fibroblasts coated with human umbilical vein endothelial cells (HUVEC) to mold a small diameter vascular graft with high levels of ECM deposition [28]. In theory, such a system could also be used for inducing vascularization of a tissue-engineered construct. Cell sheet technology is another vascular design technique that employs a process of alternatively stacked monolayers of HUVEC and myoblasts to create highly vascularized implants of myoblasts *in vivo* with robust endothelial networks [29].

Developing along-side the effort to create clinically-useful and well-characterized pro-vascular matrices are approaches to merge this technology with relevant tissue-specific replacement models. For example, pancreatic islets are highly vascularized spherical clusters of endocrine cells in the pancreas which include the insulin producing β-cells. Islet transplantation is a promising therapeutic option with freedom from exogenous insulin injection for type-1 diabetes, yet current transplantation techniques are severely limited due to high islet morbidity associated with poor engraftment and reperfusion. Current efforts to improve islet transplantation therapy include gene therapy to overexpress angiogenic growth factors [30-31] in transplanted islets and seeding of islets into pre-vascularized Matrigel™ [32] and collagen [33] implants.

Engineering mechanically sound and functional cardiac tissue for the repair of myocardial infarction and associated ischemic heart disease, the leading cause of death in developed countries, is one of the most promising and grand targets for tissue engineering. Progress in development of engineered cardiac tissue has not always addressed the need for vascularization and engineered tissues suffer from necrotic cores and little or no integration with the host tissue [34]. Incorporation of HUVEC and mouse embryonic fibroblasts (MEF) in cardiac patches leads to a strong vascular network formation *in vitro* and which, if formed preceding implantation in rat heart tissue, shows vastly improved integration and perfusion than patches without HUVEC and MEF [34-35]. Taking the process one step further, neonatal cardiac cell patches containing angiogenic factors pre-vascularized for 1 week in the omentum and subsequently transplanted into infracted heart tissue showed improved structural, electrical, and cardiac output over non-vascularized controls [36]. Alternatively, microvascular segments stabilized in collagen and transplanted into ischemic myocardium formed vascularized cardiac patches with improved left ventricular function [37].

Finally, the most direct approach to providing the necessary cues and allowing cells and tissues to control the ultimate shape of the engineered tissue and associated vasculature is direct fabrication of functioning tissue. Technologies to exert spatial control over the placement and organization of individual cells and tissue microstructures include 3D tissue printing [38-39] and lithographic fabrication [40-41] of vascular-inductive matrices have emerged as viable

options. Microfluidic devices are also becoming employed to create controlled *in vitro* systems for studying underlying mechanisms and testing new ideas [42].

Methods for validation and analysis of vascularization techniques

It is important to measure and validate the architecture and function of induced vasculature. Induced vasculature often has little resemblance to native tissue architecture, and has the potential to look and behave like tumor vasculature [43]. Furthermore, induced vasculature may suffer from poor perfusion and functionality. Several robust analysis strategies have been developed for both *in vivo* and *in vitro* models [44].

In vitro analysis techniques

Three dimensional culture of endothelial cell types or co-culture of endothelial and mesenchymal progenitors (MSC or 10T1/2 embryonic fibroblasts [11]) will self-organized to form tubule networks in pro-vascular environments [44]. The addition of a mesenchymal cell type serves to stabilize the endothelial tubes. Furthermore, if these cell types are cultured on the surface of microcarrier beads that are encapsulated in the matrix, endothelial tubes sprout from the beads [44-45]. These bead-initiated sprouts can be easily quantified for number, length, and branching and used as a screening and analysis tool. Three dimensional tissue culture of small sections of aorta from rat or mouse also sprout endothelial tubes from the aortic tissue in pro-vascular environments and are an alternative to single cell culture [46]. Lastly, the chorioallantoic membrane (CAM) is a vascularized membrane on developing chicken embryos that is widely used as a pseudo-*in vivo* system for testing vascularization effects [44].

In vivo analysis techniques

Traditionally, histological techniques including lectin- and immunostaining have been used to quantify induced vascularization *in vivo*. Three-dimensional architecture and function (vessel perfusion) are key measures that are difficult to determine from histological sections. One highly effective technique for quantitative analysis of three-dimensional vascular architecture is perfusion with the silicone rubber radio-opaque injection compound Microfil ® (Flow Tech, Inc.) to create a cast of the vasculature. The vascular cast can then be scanned in three dimensions at high resolution (sub 10 μm) with microCT and analyzed with a number of algorithms for density, branching, and connectivity [46-47]. One drawback to the microCT method is that it is a terminal procedure. Development of vasculature over time in live animals can be observed by intravital microscopy with window-models and dorsal skin-fold chambers [44]. Blood flow to ischemic limbs can be observed in live animals over time by laser Doppler perfusion imaging [21] (Moor Instruments) or with GFP-cells [44] and infrared dye tracer studies and in vivo fluorescence imaging (IVIS ®, Caliper Life Sciences, Inc.).

Conclusions / Future Outlook

Current research is tackling the problem of vascularization with four distinct strategies as illustrated in Figure 1:

1. Direct modulation of existing tissue by growth factor or cytokine signaling
2. Delivery of endothelial and mesenchymal progenitors to form a self-assembled network
3. Delivery of vascular-inductive engineered materials
4. Controlled methods to directly incorporate vessel conduits into the engineered tissue
5. Combination strategies incorporating materials, cells, and/or growth factors.

It is clear that further undertakings in tissue engineering must consider vascularization as a key design parameter. All of the discussed methods have shown moderate success in animal models. Future directions of tissue engineering research need to apply what has been learned about vascular induction and combine it with the design of functional tissue substitutes to take the field to the next level. In particular, synergistic and coordinated interactions among cells (including stem cells), biomaterials engineered to respond to local and systemic biological stimuli, and bioactive molecules will be required to attain the goal of functional tissue engineered constructs integrated with the host.

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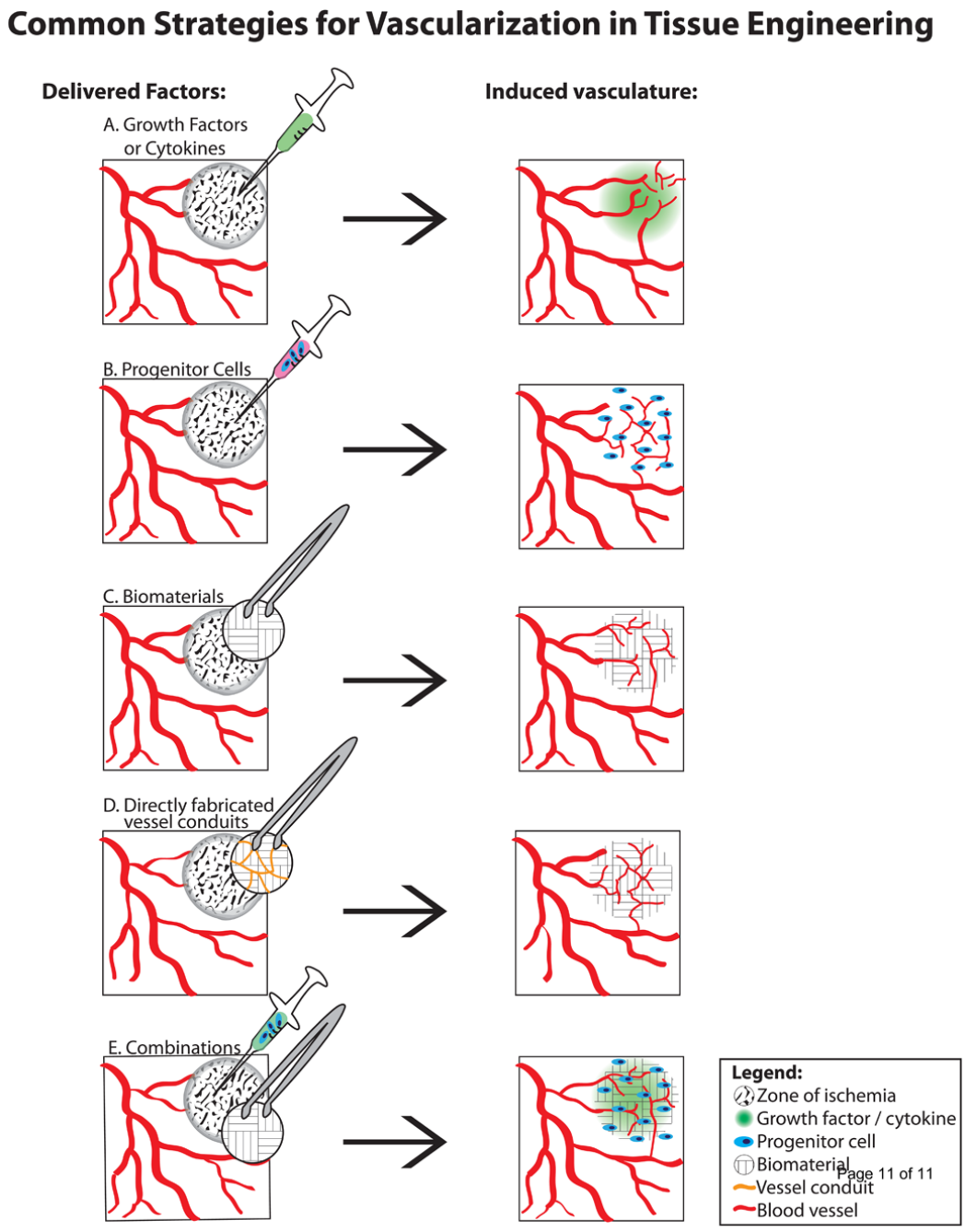

Figure 1.

Effective strategies for inducing vascularization in engineered tissues include delivery of:
A. Growth factors such as VEGF and bFGF as recombinant proteins or gene vectors. EPC-mobilizing cytokines such as G-CSF
B. Progenitor cells such as EPC and MSC
C. Biomaterials such as bioactive PEG hydrogels
D. Vessel conduits or endothelium-lined channels directly fabricated into an implant.
E. Combination therapies such as growth factor binding scaffolds with cells