Angiogenesis and vascularity for tissue engineering

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1. Introduction

Tissue engineering is a field of medicine that has experienced significant growth in prominence over the past three decades. Though traditional interventions exist for many medical maladies, tissue engineering aims to combat such disorders through the synthesis of body tissues and organs, resulting in functional implants. Tissue engineering takes an innovative approach, often utilizing autologous stem cells for tissue construction or materials that are biocompatible while avoiding immune rejection (Nomi et al., 2002).

Despite its immense successes, a major hurdle still faces tissue engineering. Large volumes of implanted tissue are unable to stimulate the formation of necessary blood vessels required for their survival. In the body, naturally occurring, equivalent vascular networks serve vital functions in gas and nutrient exchange, metabolic processes, and waste expulsion. Though individual, large vessels have been successfully engineered for implant, it is still exceptionally difficult to fashion a stable and sustainable network of vessels for large volumes of tissue (Nomi et al., 2002). Neovascularization after tissue damage requires a level of positive and negative control that has not been successfully replicated in a laboratory environment to date. As such, rapid de novo synthesis of a controlled, established vascular network remains a challenge today.

Angiogenesis is the morphogenetic process of forming new blood vessels from pre-existing ones (Laschke et al., 2006; Dai and Rabie, 2007; Li and Rabie, 2007). This event plays an important, normal physiological role in wound healing, tissue repair, pregnancy, and exercise (Ferrara and Davis-Smyth, 1997), and exists in contrast to vasculogenesis (the formation of the very first blood vessels in the body, and especially predominant in embryological development). Yet, the abuse of angiogenesis, leading to an uncontrolled vascular formation as a consequence of epigenetic influence, nucleotide polymorphisms, or endocrine irregularities can also result in tumor formation (Verbridge et al., 2010). However, angiogenesis is clearly an activity that is central to development and tissue maintenance. Successful modulation of angiogenesis can have profound therapeutic outcomes for organs and tissues deprived of an adequate, stable vasculature. Studies from the last two decades have shown that the manipulation of various factors directly influences angiogenic outcome.
Angiogenesis requires the activity of soluble factors such as Vascular Endothelial Growth Factor (VEGF; outlined in the next section and in Tables 2 & 3), basic Fibroblast Growth Factor (bFGF), Platelet-derived Growth Factor (PDGF), Transforming Growth Factor-β (TGF-β) (Nomi et al., 2002; Dai and Rabie, 2007; Li and Rabie, 2007; Kanczler and Oreffo, 2008; Bates, 2010), Keratinocyte Growth Factor (KGF) (Elia et al., 2010), Hepatocyte Growth Factor (HGF) (Hoot et al., 2010), Ephrin-B2 (Herbert et al., 2009), and Angiopoietin (Han et al., 2010) (Table 1). Morphologically, angiogenesis can be attributed to endothelial cell migration and proliferation as well as pericyte recruitment, migration and differentiation (Egginton, 2010).

| Molecule                           | Known Properties                                                                 | Citations                                                   |
|------------------------------------|----------------------------------------------------------------------------------|-------------------------------------------------------------|
| Basic Fibroblast Growth Factor (bFGF) | Stimulates activity of fibroblasts, neurons, smooth muscle cells, and endothelial cells; acts via tyrosine kinase receptors; induces production of VEGF during angiogenic stimulation. | (Pepper et al., 1992; Lee et al., 2003; Arkudas et al., 2007; Jung et al., 2010; Wu et al., 2010) |
| Platelet-derived Growth Factor (PDGF) | Certain isoforms possess survival and mitogenic functions; implicated in tumor angiogenesis; compared to VEGF, PDGF: (a) has comparable angiogenic activity, (b) produces blood vessels with decreased permeability and leakage, and (c) is thought to produce functionally different blood vessels. | (Li et al., 2010; Wu et al., 2010) |
| Transforming Growth Factor β (TGF-β) | Promotes and inhibits angiogenesis and tumor invasion via stimulation of Hepatocyte Growth Factor (HGF) expression. | (Hoot et al., 2010) |
| Keratinocyte Growth Factor (KGF)    | Member of FGF family; certain isoforms have been implicated in wound healing as well as the inhibition of neovascularization. | (Wang et al., 2010) |
| Hepatocyte Growth Factor (HGF)      | Stimulates endothelial cell growth, migration, scatter, and elongation independently of VEGF. | (Hoot et al., 2010) |
| Ephrin-B2                          | Implicated in arterial/venous differentiation (see section: Modulation of Notch Signaling). | (Herbert et al., 2009) |
| Angiopoietin                       | Signals through Tie receptors; certain isoforms are angiogenic inhibitors, while others promote inhibition of apoptosis; Stabilize blood vessels and reduce leakage. | (Han et al., 2010) |

Table 1. Notable Ancillary Growth Factors and Their Properties

2. VEGF and its receptors: a brief overview

In studying the development of cells, tissues, and organs, Vascular Endothelial Growth Factor (VEGF) has been identified as a key, though not sole, proponent of angiogenesis (Takahashi and Shibuya, 2005; Arkudas et al., 2007; Dai and Rabie, 2007; Yla-Herttuala, 2009;
Bates, 2010; Elia et al., 2010). VEGF exists in six classes (Table 2), lettered A through F, though the vast amount of literature concerning VEGF-A indicates that it is by far the most widely understood of all. VEGF-A exists in at least eight homodimeric isoforms, all conceived by alternative mRNA splicing (Ferrara, 2009) and differing by their amino acid number. Of these, VEGF_{121} and VEGF_{165} are predominant and show great promise in tissue engineering applications.

| VEGF Class | Known Properties | Citations |
|------------|------------------|-----------|
| A          | Induces proliferation of arterial, venous, and lymphatic vascular endothelial cells; stimulates monocyte chemotaxis; hematopoietic effects; eight isoforms in humans. | (Ferrara, 2009) |
| B          | May play role in atrial conduction, but not required for cardiovascular development. | (Olofsson et al., 1996a; Olofsson et al., 1996b; Aase et al., 2001) |
| C          | Involved in embryonic angiogenesis, lymphangiogenesis, & lymphatic vessel maintenance; mitogenic for cultured endothelial cells. | (Kukk et al., 1996; Orlandini et al., 1996; Jeltsch et al., 1997; Yamada et al., 1997; Dumont et al., 1998) |
| D          | Thought to be involved in pulmonary development, endothelial cell mitogen; Function(s) still generally unclear. | (Orlandini et al., 1996; Yamada et al., 1997; Achen et al., 1998; Farnebo et al., 1999) |
| E          | Proteins are encoded by Orf-viruses and predominantly expressed in sheep, goats, and rarely in humans. | (Lyttle et al., 1994; Meyer et al., 1999) |
| F          | Derived from snake venom; enhances the formation of vascular fenestrations in guinea pigs. | (Klein and Catargi, 2007; Matsunaga et al., 2009; Yamazaki et al., 2009) |
| Placental Growth Factor (PlGF) | Induces vascular permeability; supplements VEGF activity during wound-healing; may enhance VEGF-driven angiogenesis. | (Carmeliet et al., 2001; Adini et al., 2002; Hattori et al., 2002; Luttun et al., 2002; Odorisio et al., 2002) |

Table 2. Six General VEGF Classes
The binding of VEGF to any of its many receptors, such as VEGF-R1 (Flt-1) (Shibuya et al., 1990; de Vries et al., 1992), VEGF-R2 (KDR) (Terman et al., 1991), VEGF-R3 (Flt-4) (Fitz et al., 1997), and Neuropilin (NRP-1 and -2) (Soker et al., 1996; Soker et al., 1998; Dallas et al., 2008), has been shown to trigger a signaling cascade that results in the activation of angiogenesis (Stefanini et al., 2009). Not surprisingly, VEGF receptor and ligand placement and density have been implicated in the successful transmission of the signaling cascades required for angiogenesis (Stefanini et al., 2009). Modulating receptors and their densities appear to be a logical avenue for further efforts in modulating neovascularization. Further, Heparin Sulfate Proteoglycan (HSPG) has been implicated in the binding of VEGF to their receptors, but its exact function remains unclear (Ferrara et al., 2003; Lee et al., 2010). Preliminary findings suggest that HSPGs inhibit VEGF binding to its receptors, and consequently, angiogenic activity (Lee et al., 2010).

3. Modulations to VEGF and its delivery

Though VEGF has a significant influence on cell migration, proliferation, and vasodilation, the uncontrolled or sole use of VEGF in vivo has been shown to result in the disordered growth of blood vessels into a dense mass (hemangioma), malignant tumor angiogenesis, and the assembly of leaky vessels (Takahashi and Shibuya, 2005; Bates, 2010). As such, the fundamental function of VEGF in physiological development and maintenance, contrasted by its role in tumorigenesis and vessel instability, is paradoxical. In instances where VEGF is heterogeneously present in a microenvironment, areas of high VEGF expression resulted in abnormal angiogenesis. However, implanting VEGF-transfected myoblasts, with each cell equally producing VEGF over time, led to the formation of a stable, normal vascular network (Misteli et al., 2010). The latter provides a compelling case for carefully-controlled VEGF release, distribution, and kinetics in tissue engineering applications. As discussed below, several current tissue-engineering efforts are positioned to resolve this dilemma.

It should not be assumed that blind administration of VEGF is solely responsible for a successful angiogenic effort. Many studies have illustrated that the promotion of successful angiogenesis depends on a prolonged exposure to a low dose of VEGF (Wernike et al., 2010), while other studies claim that micro-environmental conditions must be taken into account (Ferrara and Davis-Smyth, 1997; Misteli et al., 2010). With regards to the latter, techniques like FACS purification (Misteli et al., 2010) and microdialysis (Hoier et al., 2010; Marcus et al., 2010) are becoming increasingly prevalent in closely monitoring VEGF expression in the microenvironment. Further, many attempts at eliciting controlled angiogenesis also focus on coupling the properties of VEGF with certain other growth factors – most notably bFGF (Arkudas et al., 2007) or KGF (Elia et al., 2010) (see Table 1); stable vessels were formed when VEGF was combined with either of these two factors.

There exist contradictions regarding the parameters of tissue exposure to VEGF. In one comparison, a study highlighting growth factor implementation in orthopedic applications suggested that VEGF delivery for over 14 days may have interfered in the vascularization during bone healing and restoration (Wernike et al., 2010). This suggestion challenges an argument that a longer VEGF exposure (of approximately one month) was necessary for the production of stable, but leaky, vessels as brief exposure to VEGF (less than 15 days) resulted in the formation of unstable vessels. Further, these vessels actually degenerated after VEGF delivery cessation (Tafuro et al., 2009). Disparities such as this are common in the
literature and demonstrate the sheer complexity of VEGF activity in various tissue environments.

The view that successful VEGF delivery and angiogenic response are strictly dose-dependent is challenged by factors such as delivery kinetics, which appear to be critical for proper vasculature (Borselli et al., 2010; Wernike et al., 2010). For instance, a characteristic problem with the bolus delivery of VEGF is the outcome of variable systemic effects (Matoka and Cheng, 2009) such as haemorrhage, hypotension, or flu-like symptoms (Benjamin et al., 1999). This method of delivery also fails to achieve the prolonged supply of physiological low dose of VEGF necessary to produce a mature, lasting vascular network. The lack of lasting vessel formation observed in many therapeutic trials is probably due to difficulties in the delivery of VEGF, a growth factor with an apparently narrow therapeutic window (Hariawala et al., 1996; Lee et al., 2000; Dor et al., 2003; Ozawa et al., 2004), in the optimal time and dose for maintaining sufficient vascularity.

A need exists for biomaterials or stable scaffolds that enable slow, sustained VEGF release (Rocha et al., 2008) for a predictable and functional outcome. Studies in different laboratories have indicated that incorporation of growth factor into slow-release polymer formulations could present a means for better control of dose, location, and duration of active signals in tissue (Edelman et al., 1991; Lee et al., 2000; Sheridan et al., 2000; Ehrbar et al., 2004). The kinetics of VEGF delivery today depends on the physical properties (such as cross-linking and porosity, for instance) of biocompatible conduits such as fibrin-gels (Ehrbar et al., 2004; Arkudas et al., 2007), gelatin microparticles (Patel et al., 2008), collagen / fibronectin hydrogels (Glotzbach et al., 2010), and PLG(A) scaffolds (Murphy et al., 2000)(Rocha et al., 2008; Matoka and Cheng, 2009; Borselli et al., 2010; Golub et al., 2010). Moreover, transfecting or transducing developing cells (muscle precursor cells or myoblasts, for instance) with VEGF and subsequently injecting them into a site can also provide a steady, longer-term delivery of the growth factor (Misteli et al., 2010).

Once VEGF is coupled with a delivery conduit, a more intricate approach to controlling VEGF delivery involves modifying the factor itself. Extensive delivery-mechanics research was conducted with the isoform VEGF121. Though VEGF121 is initially confined within a biomaterial (such as a fibrin-gel matrix) upon implant, plasmin and metalloproteinase degradation of the implant over time allows for the rapid, free diffusion of VEGF121 into the whole body environment (Ehrbar et al., 2004), potentially resulting in angiogenesis that may not be localized. Additionally, the short biological half-life of VEGF impedes its use in long-term applications. A synthetic variant of VEGF121, known as TG-VEGF121, cross-links to fibrinogen by the transglutaminating activity of factor XIII during fibrin-gel polymerization. This covalently tethered TG-VEGF121 is protected from rapid diffusion. Gradual degradation of the fibrin-gel by local fibrinolytic activities results in a local liberation of low levels of TG-VEGF121 into tissue. Experimental animal models have shown that fibrin-conjugated TG-VEGF121 produced more structurally stable vessels than VEGF121 while avoiding vascular leakage (Ehrbar et al., 2004; Ehrbar et al., 2008).

4. Hypoxia-mediated control and modulation of notch signaling

Hypoxia presents another means of employing direct control on VEGF and ancillary angiogenic factors. In the low-oxygen environments of normal muscle, VEGF mRNA experiences decreased degradation and increased expression (Ikeda et al., 1995; Levy et al., 1995; Levy et al., 1996; Tang et al., 2004), though severely hypoxic settings will actually
impede VEGF up-regulation (Milkiewicz et al., 2004). VEGF mRNA stability is made possible by its interaction with HuR, a complex that binds and stabilizes RNAs, consequently regulating gene expression (Levy et al., 1998). The increase in VEGF production can be attributed to the Internal Ribosome Entry Site (IRES), which is accountable for efficient factor synthesis under hypoxic conditions (Stein et al., 1998). Other proteins can also impact VEGF efficiency and activity. Control of expressed VEGF lies with ORP150 (oxygen regulated protein), a chaperone that transports VEGF from the cell’s endoplasmic reticulum to the Golgi apparatus (Kuwabara et al., 1996; Ozawa et al., 2001). An increase in ORP150 levels correlates with an increased production of VEGF during hypoxia (Ozawa et al., 2001). Further, Hypoxia-inducing factor (HIF) is responsible for activating the transcription of genes associated with neovascularization (Covello and Simon, 2004; Ramirez-Bergeron et al., 2006). These proteins represent points of control for VEGF modulation.

Because angiogenesis is the synthesis of vasculature from existing vessels, constant remodeling and modification of vessels takes place in vivo. Existing endothelial cells, such as stalk and tip cells, have receptors that respond to environmental conditions like hypoxia, in which Notch signaling (driven by VEGF-A presence) is most prevalent. The presence of VEGF-A leads to an increase in the presence of Delta-like Ligand 4 (DLL4), a major Notch ligand. It is believed that Notch signaling modulates the ratio of VEGF to its receptors through the inhibition of VEGF-R2.

Manipulation of the Notch signaling pathway presents another means of controlling angiogenesis. Cao, et al., discussed the role of Notch signaling in modulating VEGF activity, resulting in effective pruning and branching of vascular vessels (Cao et al., 2009; Cao et al., 2010). Activation of the Notch signaling pathway inhibits VEGF signaling by down-regulating the VEGF receptor synthesis. With fewer available receptors for free VEGF to bind, endothelial cell proliferation is effectually curbed. This process occurs at the cellular level of the lining endothelium, a major component of vascular vessels, and vascular growth in any particular direction is controlled.

5. Applications in regenerative medicine: notable case studies

Recent advances in understanding the angiogenic process and isolating potent and specific angiogenic growth factors prompted their therapeutic usage. Evidence that VEGF is a specific endothelial cell growth factor suggested its potential in therapeutic angiogenesis. Injection of the VEGF₁₆₅ protein enhanced revascularization of rabbit ischemic hindlimbs (Takeshita et al., 1994). VEGF treatment induced collateral vessel formation, endothelium-dependent blood flow and tissue perfusion. VEGF has been tested for potential beneficial effects on wound repair in diabetic animal models (Greenhalgh et al., 1990; Tsuboi and Rifkin, 1990; Frank et al., 1995). The growth factor treatment regimens accelerated granulation, tissue formation, and wound closure. However, due to the high clearance and/or degradation of the proteins from the administration site, topically administered growth factors would require high dosages and frequent delivery.

An alternative strategy for therapeutic angiogenesis is gene therapy using recombinant angiogenic growth factors. The first study using VEGF₁₆₅ cDNA was performed by gene transfer into the iliac artery of an ischemic hindlimb of a rabbit (Bauters et al., 1994; Takeshita et al., 1996). 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 (Asahara et al., 1996). This technique was further employed in patients with peripheral vascular disease and critical limb ischemia (Isner et al., 1996). Clinical trials showed significant improvement in collateral blood flow, healed ischemic ulcers, and most importantly, salvage of limbs in patients in whom amputation was imminent (Isner, 1998; Isner and Takayuki, 1998). VEGF protein and cDNA have been used for coronary revascularization, resulting in improved myocardial perfusion and increased collateral density (Isner and Losordo, 1999).

As previously discussed, a successful angiogenic outcome is not solely attributed to VEGF implementation alone (discussed in Section 3 and Table 1). Wilcke, et al., showcased the coupling of VEGF with bFGF in a fibrin dermal substitute (Wilcke et al., 2007). This led to a marked improvement in factor delivery, with a notable, prolonged release and resulted in a higher density of newly developed vessels in \textit{in vivo} murine models. Concurrently, an \textit{in vivo} experiment performed by Zacchigna, et al., studied the effect of a VEGF and Angiopoietin 1 (Ang 1) combination (delivered via an adeno-associated viral vector, for long-term protein production and release) on the muscle blood flow (MBF) and vascular permeability of rat skeletal muscle (Zacchigna et al., 2007). The grouping elicited a marked increase in both resting MBF and perfusion post exercise stimulation. To contrast, VEGF expression alone did not enhance the resting MBF and actually reduced tissue perfusion after exercise.

Elcin, et al., researched \textit{in vitro} release kinetics and \textit{in vivo} angiogenic effects of human VEGF-loaded PLGA sponges in rats (Elcin and Elcin, 2006). When compared with control sponges (containing no factor) and bolus injections of VEGF, the use of VEGF-loaded PLGA sponges led to the establishment of neovascularized sites suitable for tissue engineering purposes. Patel, et al., who utilized VEGF-loaded gelatin microparticles infused in biodegradable composite scaffolds, outlined another notable attempt in VEGF delivery modulation in the field of orthopedics (Patel et al., 2008). Findings suggested that modulating the degree of gelatin cross-linking could affect VEGF release into the microenvironment and, consequently, angiogenic outcome. Ennett, et al., studied the temporally regulated delivery of VEGF \textit{in vivo}, hypothesizing that the means of VEGF-loading into a delivery scaffold would have an impact on the factor release kinetics (Ennett et al., 2006). They compared (a) VEGF loaded directly into a PLG scaffold with (b) VEGF pre-encapsulated in PLG microspheres that were later used to fabricate a PLG scaffold. Though pre-encapsulated VEGF microspheres further delayed the factor’s release into the surrounding environment, this approach produced a desirable angiogenic outcome, with significant local angiogenesis and negligible systemic effects.

Pre-encapsulation of VEGF by nanoparticles or microspheres has also been investigated in conjunction with Matrigel hydrogels, PLGA-, and collagen-scaffolds. While it is clear that encapsulation protects the factor(s) within the nanoparticles and microspheres, it also offers a mechanism of controlled release – especially ideal for a potent angiogenic factor like VEGF. Even though pre-encapsulated VEGF-loaded PLGA scaffolds performed better than similarly loaded Matrigel hydrogels (with respect to release profiles in a saline solution; also in comparison with free VEGF \textit{in vitro}, both of the loaded delivery conduits improved angiogenesis \textit{in vivo}. An increase in both endothelial cell counts and red blood cells was noted at the sites of implantation (des Rieux et al., 2010). Further, VEGF-loaded PLGA microspheres combined with collagen elicited strong enhancements to vascular sprouting and activation of endothelial cells \textit{in vivo} and \textit{in vitro}, respectively (des Rieux et al., 2010).
The idea of optimizing delivery agents combined with the notion that co-factor usage is ideal for neovascularization resulted in an experiment that analyzed the co-implementation of VEGF with FGF-2 via an acellular collagen scaffold implant (Nillesen et al., 2007). An implant containing both factors resulted in the highest vessel density and the most mature blood vessels (characteristics of an enhanced, stable vasculature) in a rat model when compared with scaffolds containing either of the factors alone or no factor at all.

6. Conclusion

A significant challenge facing tissue engineering lies in eliciting controlled neovascularization. Controlled neovascularization can have a profound impact in vessel and organ synthesis, as well as in the treatment of damaged tissues. Many current attempts for such control entail the use of VEGF. Attempts at fabricating an ordered vascular network include changes to VEGF through structural modification, the employment of strategies controlling its release, and coupling VEGF to other growth factors. Yet, despite the promises shown by VEGF, significant obstacles remain. Many researchers propose that use of growth factors alone will not ensure stable angiogenesis. Rather, a combination of growth factors (VEGF and bFGF, for example), delivery methods, and modulation of inflammatory responses (via fibroblast, macrophage, cell-adhesion molecule and cytokine manipulation, for instance) and pathways (such as hypoxia and Notch) are thought to facilitate adequate vessel fabrication and stability.

Additionally, investigation into the mechanisms of cellular crosstalk is necessary to better understand angiogenesis in general. Moreover, the refinement and implementation of microenvironment monitoring technologies are vital in ensuring proper vascular development. Such technologies would enable researchers to closely study the impact of gross- or modulated-release of growth factors on the delicate balance required for vascular formation and branching.

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8. References

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