Vascularization in Tissue Engineering: Angiogenesis versus Inosculation

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Key Words

Angiogenesis • Inosculation • Scaffold • Tissue engineering • Vascularization

Abstract

Background/Purpose: The key challenge in tissue engineering is the establishment of an efficient vascularization for tissue constructs guaranteeing long-term survival and function. Vascularization may be achieved by the stimulation of angiogenesis or the inosculation of preformed microvascular networks within the implants to the host microvasculature. The present review provides an overview of these two concepts applied in tissue engineering. **Methods:** A literature search was performed in PubMed for publications focusing on vascularization, angiogenesis and inosculation in tissue engineering. **Results:** Several strategies have been proposed to stimulate the ingrowth of new blood vessels into tissue constructs. These include the modification of the chemical composition and architecture of scaffolds, their bioactivation by incorporation of growth factor delivery systems or by cell seeding as well as the stimulation of stem cell recruitment. However, because angiogenesis is a time-consuming process, all of these approaches cannot prevent ischemic cell death within larger 3-dimensional tissue constructs during the initial phase after implantation. To overcome this problem, in vitro or in situ prevascularization has emerged as a novel concept in tissue engineering. This bears the advantage that preformed microvascular networks within tis-

sue constructs simply have to inosculate with the host microvasculature at the implantation site to get completely blood-perfused within a short period of time. **Conclusions:** During the last years, considerable progress has been made in the development of promising vascularization strategies in tissue engineering. Particularly the inosculation of preformed microvascular networks has the great potential to markedly improve the survival of tissue constructs after implantation. The optimization of this vascularization strategy may pave the way for a broad clinical use of tissue engineering applications in the future.

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Introduction

Tissue engineering is a rapidly growing field of research, which is driven by the urgent need for tissue substitutes and transplantable organs in daily clinical practice. In fact, the generation of functional tissue constructs by combining the principles of engineering and life sciences may dramatically change human health care in the future, offering the exciting possibility to restore, maintain or improve tissue function at any time independently from available donor organs [1].

The classical tissue engineering approach involves the isolation and seeding of organospecific cells or multipotent stem cells on different scaffold biomaterials. The scaffolds serve as artificial extracellular matrices for the

Fig. 1. Schematic drawing of the two principal vascularization strategies in tissue engineering, i.e. angiogenesis and inosculation. The angiogenesis approach is characterized by the ingrowth of vascular sprouts from the host microvasculature (red vessels) into an implanted tissue construct, which finally form a new microvascular network. In case of the inosculation approach, a pre-formed microvascular network (blue vessels) is generated within a tissue construct prior to its implantation. This bears the advantage that the preformed microvessels simply have to develop interconnections (inosculation) to the host microvasculature to get fully blood-perfused within a short period of time. These interconnections may develop within the construct (internal inosculation, encircled by dashed line) or within the surrounding host tissue (external inosculation, encircled by closed line).

cells to attach, to proliferate and finally to form tissue constructs for implantation into a defect site [2, 3]. A major prerequisite for the long-term survival and function of these constructs is their sufficient vascularization after implantation. In particular, the cells in the center of large

3-dimensional tissue constructs are crucially dependent on a vascular supply of their own, because their oxygen and nutrient demand cannot be covered only by diffusion processes from the host tissue [4]. Accordingly, the development of efficient vascularization strategies for engineered tissue constructs is a key challenge [5], which has to be mastered in order to pave the way for a broad clinical use of tissue engineering applications.

During the last few years, two principal vascularization strategies have emerged in the field of tissue engineering (fig. 1). The first strategy focuses on the ingrowth of newly formed blood vessels into implanted tissue constructs from the surrounding host tissue by stimulating angiogenesis [6]. On the other hand, a rapid blood supply of tissue constructs may be achieved by inosculation [7]. In this case, preformed microvascular networks are generated within tissue constructs prior to their implantation. At the implantation site, these networks simply have to develop interconnections with the host microvasculature to get fully reperfused within a short period of time.

The present review gives an overview of studies focusing on angiogenesis and inosculation in tissue engineering. Moreover, it highlights the most interesting approaches, which may be used for the optimization of these processes with the central aim to achieve a rapid and sufficient vascularization of tissue constructs.

Angiogenesis in Tissue Engineering

The ingrowth of newly formed blood vessels into an implanted tissue construct is a highly dynamic process. The first step in this process is the activation of the host microvasculature at the implantation site by angiogenic growth factors, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor [8]. These factors may originate from different sources. They may be produced by cells of the host tissue itself due to tissue injury during the implantation procedure or in consequence of an inflammatory response to the implant [7]. On the other hand, it is possible to generate tissue constructs with artificial protein delivery systems or different cell types, which are capable of releasing angiogenic growth factors [9–11].

Upon angiogenic activation, the endothelial cells of the host microvasculature start to produce matrix metalloproteinases, resulting in the degradation of their basement membrane [12]. This is the prerequisite for their subsequent migration into the surrounding interstitium,

which is morphologically reflected by the formation of vascular buds and sprouts. The sprouts progressively grow into the implanted tissue construct and interconnect with each other to develop new blood-perfused microvascular networks [13]. The wall of these networks is finally stabilized by the production of extracellular matrix compounds and the recruitment of smooth muscle cells or pericytes [14].

Accordingly, successful vascularization of a tissue construct via angiogenesis is dependent on the coordinated sequence of various humoral and cellular mechanisms and, in particular, the close interaction between the host tissue and the implant. This multistep process of angiogenic vascularization offers different possibilities to stimulate and accelerate the formation of vascular networks in tissue constructs.

Stimulating Angiogenesis in Tissue Constructs

Several approaches, which may improve vascular ingrowth into implanted tissue constructs, are currently under investigation. These include (i) the modification of the chemical composition and architecture of scaffolds, (ii) their bioactivation by incorporation of growth factor delivery systems or by cell seeding and (iii) the stimulation of stem cell recruitment at the implantation site.

During the last few years, different studies have shown that the vascularization of a scaffold is crucially dependent on its chemical composition [13, 15, 16]. Of interest, there is a close relation between the inflammatory and the angiogenic host tissue response to an implanted scaffold biomaterial. For instance, Rücker et al. [13] found that poly-L-lactide coglycolide (PLGA) scaffolds induce slight inflammation after implantation into the dorsal skin fold chamber of Balb/c mice. This is associated with a marked angiogenic host tissue response and a good vascularization of the implants after 14 days. In contrast, collagen-chitosan-hydroxyapatite hydrogel scaffolds of identical architecture induce severe inflammation, resulting in a complete lack of ingrowth of newly formed microvessels into the implants. Additional studies have shown that polyurethane scaffolds, which exhibit an excellent *in vivo* biocompatibility, are characterized by a poor vascularization [17, 18]. These findings indicate that scaffold biomaterials with slight proinflammatory properties may be appropriate for the stimulation of the angiogenic process at the implantation site.

Besides the chemical composition, the architecture of scaffolds is a further important determinant for adequate

vascularization. Druecke et al. [19] systematically analyzed the effect of pore size on the vascularization of poly(ether ester) block copolymer scaffolds. Of interest, they demonstrated that blood vessel ingrowth was markedly improved in scaffolds with large pores of 250–300 μm when compared to implants with smaller pore sizes. During the last few years, sophisticated techniques have been established for the fabrication of scaffolds exhibiting a highly interconnected porous structure with controllable pore sizes [19–21]. By now, it is even possible to generate scaffolds with distinct porosity levels. For instance, Yang et al. [22] applied a rapid prototyping technique for the fabrication of ceramic scaffolds with submicrometer pores to improve cell/surface interactions, pores of tens of micrometers to support the ingrowth of bone tissue and corridors of 100–600 μm to enable vascularization. The use of novel microfabrication techniques further offers the exciting possibility to induce directional blood vessel ingrowth into polymer scaffolds along preformed network structures with a vascular geometry [4, 23].

A common strategy to improve scaffold vascularization is the stimulation of the angiogenic host tissue response at the implantation site by incorporation of angiogenic growth factors into the implants. For this purpose, VEGF [24, 25], basic fibroblast growth factor [26, 27], platelet-derived growth factor [28] and angiogenin [29] are the most frequently used factors. They can be covalently immobilized [30, 31] or loaded on the scaffold surface via collagen coating [32]. Moreover, it is possible to fill porous scaffolds with growth factor-containing gels [10]. Alternatively, the factors may be bound to nanoparticles [33] or encapsulated into microspheres with defined degradation rates [34–38]. This enables the fabrication of implants containing several growth factor delivery systems with controlled release rates for each factor [9]. This bears the advantage that the angiogenic process is stimulated much more efficiently than by the use of one growth factor alone. In fact, studies could demonstrate that the incorporation of multiple growth factors into scaffolds resulted in a markedly improved vascularization and the development of mature pericyte-coated microvascular networks inside the implants [10, 39, 40]. Gérard et al. [41] recently reported that the proangiogenic effect of growth factors can be further improved by combining them with copper sulfate. Finally, scaffolds may also be enriched with plasmid DNA to stimulate the local cellular production of angiogenic growth factors at the implantation site [42, 43].

An improved vascularization of scaffolds is also achieved by seeding them with differentiated tissue-specific cells [11, 44] or multipotent stem cells [45, 46]. These cells contribute to the vascularization process via different mechanisms. On the one hand, the cells inside a construct stimulate the ingrowth of blood vessels by releasing angiogenic growth factors. In fact, after implantation into a defect, they initially lack a blood supply of their own and, thus, suffer from hypoxia. This is typically associated with the intracellular upregulation of hypoxia-inducible factor (HIF)-1 α and HIF-1 α -mediated expression of VEGF [47]. On the other hand, in contrast to tissue-specific cells, stem cells exhibit the capacity to differentiate into vascular cells and to self-assemble into novel microvessels [48]. Schumann et al. [11] recently seeded both cell types, i.e. osteoblast-like cells and bone marrow-derived mesenchymal stem cells, on PLGA scaffolds and analyzed their vascularization during a 14-day implantation period. Of interest, blood vessel ingrowth into the constructs was markedly improved when compared to nonseeded control scaffolds due to an increased expression of VEGF with comparable results for both seeded cell types. These findings indicate that the vascularization was mainly driven by the production of VEGF rather than by the vessel-forming capacity of the bone marrow-derived mesenchymal stem cells. Other studies could demonstrate that this vascularization strategy may be further optimized by seeding scaffolds with genetically modified cells, which continuously secrete angiogenic growth factors, independently from their state of hypoxia [49, 50].

Furthermore, it is possible to promote the development of new blood vessels by stimulating the homing of circulating stem cells. These cells have been shown to be recruited to ischemic sites via the stromal-cell-derived factor 1/chemokine receptor type 4 axis [51]. Accordingly, incorporation of stromal-cell-derived factor 1 into PLGA and polycaprolactone scaffolds results in an accumulation of stem cells at the implantation site, which is associated with an enhanced angiogenic host tissue response to the implants [52, 53]. Besides, the recruitment of stem cells into tissue constructs may also be stimulated by cell seeding. For instance, Tasso et al. [54] generated porous ceramic cubes seeded with mouse mesenchymal stem cells and found after implantation into syngeneic mice that in contrast to nonseeded control scaffolds these constructs were capable of recruiting circulating endothelial progenitor cells and pericyte-like cells.

Finally, recent progress in the field of computational modeling now offers the possibility to simulate the in-

growth of new blood vessels into scaffolds under highly standardized conditions [55–57]. Accordingly, this *in silico* approach may crucially contribute to the future optimization of scaffold architecture as well as growth factor incorporation and cell seeding of scaffolds.

Inosculation in Tissue Engineering

The pivotal problem in vascularizing implanted tissue constructs through angiogenesis is the fact that the development of new blood vessels is a time-consuming process, which can only be accelerated to a limited extent. Studies estimate that the physiological growth rate of microvessels is not faster than approximately 5 $\mu\text{m}/\text{h}$ [58, 59]. Accordingly, even highly successful proangiogenic strategies will not be able to prevent cell death in the center of large 3-dimensional tissue constructs during the initial days after implantation.

These considerations have led to the introduction of prevascularization approaches in the field of tissue engineering. The basic idea of these approaches is the generation of preformed microvascular networks within tissue constructs prior to their implantation. In this case, the networks simply have to develop interconnections to the blood vessels of the host tissue at the implantation site, which is also termed inosculation [7]. This bears the major advantage that the implants are fully blood-perfused within a short period of time, independently from their size and 3-dimensional design.

There are different possibilities for the prevascularization of tissue constructs. *In vitro*, scaffolds may be seeded with endothelial cells or endothelial progenitor cells, which have the capacity to self-assemble spontaneously into capillary-like structures [60, 61]. However, these structures are often unstable. To overcome this problem, the cells may be transfected with genes, which enhance their survival [62, 63]. Because such a genetic manipulation is always associated with an oncogenic risk, a better alternative may be the coseeding of scaffolds with endothelial cells and vessel-stabilizing cell types. Koike et al. [64] incorporated human umbilical vein endothelial cells (HUVECs) and 10T1/2 mesenchymal precursor cells into fibronectin type I collagen gels, which were then implanted into mice. By this, they could demonstrate that the HUVECs formed interconnected blood-perfused vessels, which were covered with 10T1/2 cells expressing the mural-cell marker α -smooth muscle actin. These vessels were stable and functional for 1 year. In contrast, constructs that were seeded with HUVECs

alone only showed a minimal perfusion and already regressed after 60 days.

Prevascularized tissue constructs may also be generated in situ by implanting scaffolds into a well-vascularized implantation site of the body, which then serves as a natural bioreactor [65, 66]. Thereby, a microvascular network develops within the implants by the ingrowth of microvessels from the surrounding host microvasculature. After establishing a sufficient vascularization, the constructs are explanted and transferred into the final defect site. This approach bears the advantage that fully functional blood vessels are formed in the implants without the need of complex cell isolation, seeding and cultivation procedures. However, it requires the implantation of scaffolds for prevascularization, their removal and the final insertion into the host defect. Thus, it should be considered that each of these surgical interventions may be associated with intra- and postoperative complications.

Once a prevascularized tissue construct has been successfully generated in vitro or in situ, it should rapidly develop interconnections to the blood vessels of the host tissue after implantation. This involves the growth of vascular sprouts originating from the host microvasculature towards the connecting ends of the implants' preformed microvessels and vice versa. Recent studies indicate that this growth is directed by specialized endothelial cells at the tips of sprouts, which extend multiple filopodia along VEGF-A gradients [67, 68]. These cells are followed by proliferating endothelial stalk cells, resulting in the elongation of the sprouts. Finally, the inosculation of encountering sprouts is crucially dependent on the balance between Notch and Wnt signaling, which controls the stability of new vessel connections [69].

In the past, most studies suggested that inosculation takes place inside prevascularized grafts due to the regression of preformed microvessels and invasion of microvessels of host origin [70, 71] (fig. 1). However, more recent studies show that the preformed microvessels can also grow outside the implants, promoting external inosculation in the surrounding host tissue and, thus, improving vascularization of the implantation site [65] (fig. 1). In addition, the sprouting angiogenic activity of the preformed microvessels also contributes to an increased microvessel density in the center of the implants [65]. These findings indicate that preformed microvascular networks are highly dynamic and actively contribute to the establishment of a sufficient blood supply to tissue constructs after implantation.

Promoting Inosculation of Prevascularized Tissue Constructs

Although the inosculation of preformed microvessels represents a promising vascularization strategy in tissue engineering, experimental studies show that even this approach cannot guarantee an adequate blood perfusion of tissue constructs during the very first days after implantation [60, 65]. This is not surprising, because inosculation is also dependent on angiogenic processes, such as the angiogenic activation of the preformed microvessels and the host microvasculature and their growth towards each other. Thus, there is a reasonable need for the establishment of novel strategies, which further optimize the inosculation of prevascularized tissue constructs.

We recently analyzed the effect of cultivation of in situ prevascularized tissue constructs prior to their implantation [72]. Of interest, we found that short-term cultivation for 3 days in Dulbecco's modified Eagle's medium reduced the number of α -smooth-muscle-actin-positive preformed microvessels and increased the cellular production of VEGF within the constructs. After implantation, this resulted in an accelerated inosculation of the preformed microvessels when compared to those of non-cultivated control implants. In another study, we additionally embedded the prevascularized tissue constructs in Matrigel during the cultivation period [73]. By this, we could stimulate the outgrowth of preformed microvessels into the proangiogenic extracellular matrix, which promoted external inosculation of the constructs with the host microvasculature and markedly improved the implants' vascularization during the first 14 days after implantation.

Taken together, these findings demonstrate that inosculation can be optimized by modification of the angiogenic activity of preformed microvascular networks within tissue constructs. On the other hand, it may also be possible to improve the angiogenic host tissue response and, thus, to accelerate the directed growth of microvessels from the host microvasculature towards the implants. Nonetheless, despite these approaches the natural inosculation of two microvascular networks will always be a process that takes hours to days [72, 73]. Accordingly, inosculation strategies may be combined in the future with preconditioning methods, which increase the ischemic tolerance of cells inside tissue constructs during the initial phase after implantation [74]. Alternatively, prevascularized tissue constructs comprising a vascular pedicle may be generated by flap fabrication [75] or by using an arteriovenous loop [76]. This approach bears the

major advantage that the blood vessels of the constructs can be directly anastomosed to the host microvasculature by means of microsurgical techniques during the implantation procedure, resulting in an immediate restoration of blood perfusion within the implants.

Conclusions and Future Perspectives

By now, there is no doubt that the major precondition for the successful transfer of tissue engineering applications into clinical practice is the establishment of effective strategies, which guarantee an adequate blood supply to implanted tissue constructs. Accordingly, sophisticated approaches have been developed, which improve the

ingrowth of new blood vessels into tissue constructs. However, because angiogenesis is a time-consuming process, these approaches alone cannot prevent ischemic cell death within larger 3-dimensional tissue substitutes during the initial days after implantation. The generation of preformed microvascular networks within the implants and their subsequent inosculation at the implantation site represent a promising alternative to overcome this problem. Nonetheless, recent studies indicate that there is still a need to optimize such prevascularization strategies. Novel insights into the regulatory mechanisms of angiogenesis and network formation as well as further progress in the fields of computational modeling, biomaterial research and microsurgery will contribute to achieve this goal in the near future.

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