Vascularization in Bone Tissue Engineering

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Bone is a highly vascularized tissue, and its development, maturation, remodeling, and regeneration are dependent on a tight regulation of blood vessel supply. This condition also has to be taken into consideration in the context of the development of artificial tissue substitutes. In classic tissue engineering, bone-forming cells such as primary osteoblasts or mesenchymal stem cells are introduced into suitable scaffolds and implanted in order to treat critical-size bone defects.

Keywords: vascularization ; tissue engineering ; bioprinting ; bone ; mesenchymal stem cell ; endothelial cell

1. Introduction

Bone can be divided into an external layer, named cortical bone, and an internal layer, referred to as cancellous bone. Cortical bone shows extreme mechanical stiffness with relatively low porosity [1][2]. In contrast, cancellous bone shows very high porosity and only about 10 percent of the mechanical stiffness of cortical bone. Within the cortical bone, osteons form functional units that have the so-called haversian canals in the center, which contain nerves and blood vessels [3][4]. These osteons with the associated haversian canals are not found in cancellous bone and are also not necessary for the blood vessel supply due to the high porosity of this bone compartment.

The vascular endothelial growth factor (VEGF) plays a decisive role in the initiation of vascularization. It is formed by hypertrophic chondrocytes and mesenchymal stem cells and, as a potent angiogenic growth factor, induces the sprouting of preexisting surrounding blood vessels into the bone tissue [5]. It can therefore be assumed that VEGF is the decisive factor for the coupling of angiogenesis and osteogenesis. In mouse experiments, it could be shown that the inhibition of the VEGF function has a negative effect on bone angiogenesis and, thus, a negative effect on bone formation [6].

When a bone is fractured and blood vessels are damaged, hematoma forms and inflammatory cells migrate into the fracture site. The ingrowth of new blood vessels is essential for the formation of a soft callus consisting of fibroblasts and chondrocytes. The soft callus is, by way of enchondral ossification, converted into a rigid, calcified hard callus. Further mineralization and remodeling processes within the callus eventually lead to the repair of the damaged bone [7]. Here, too, it could be shown that VEGF plays an important role. For example, in a rabbit model, the application of recombinant VEGF to the fracture area led to an improvement in neovascularization and bone healing [8]. In contrast, the inhibition of VEGF in a mouse model resulted in decreased angiogenesis, decreased callus mineralization, and an inhibition of bone healing [9].

Due to the great importance of vascularization in both bone formation and in bone healing, it is clear that this aspect must also be taken into account in the tissue engineering of bone tissue (BTE). In the clinical context, bone defects can be treated with autologous or allogeneic bone grafts, with autologous bone replacement being the gold standard. Critical-size bone defects can be caused by trauma, tumor, osteomyelitis, avascular bone loss, and non-union fracture, and are thus very common. Bone transplantation is performed in an estimated 2.2 million procedures yearly worldwide [10]. Smaller defects are bridged by non-vascularized bone grafts (e.g., iliac crest), while larger defects require pedicled or free tissue transfer [11][12]. However, the use of autologous bones is restricted due to the limited availability and due to the fact that an additional bone defect is created. In the case of allogeneic bone transplantation, additional limitations include possible rejection by the host and the possibility of disease transmission [13][14].

2. Induction of Vascularization by Angiogenic Growth Factors

Angiogenesis represents a mechanism by which new blood vessels are formed from a preexisting vascular network. In contrast, vasculogenesis means that new blood vessels are formed de novo by endothelial progenitor cells. During embryogenesis, these cells differentiate to mature endothelial cells and form a primitive vascular plexus that further matures into a hierarchical network with vessels of different calibers [15]. Under physiological as well as under pathophysiological conditions such as the growth of solid tumors, angiogenesis is initiated by the expression and

secretion of pro-angiogenic diffusible soluble growth factors (GFs). Numerous pro-angiogenic growth factors have been identified in recent decades [16]. Prominent representatives are members of the vascular endothelial growth factor (VEGF) family [17], fibroblast growth factor (FGF) [18][19], angiopoietins (Ang) [20], transforming growth factor (TGF) [21][22], platelet-derived growth factor (PDGF) [23][24], and some interleukins [25][26]. Under normal conditions, the vasculature in the adult human body is relatively quiescent. However, during wound repair as well as in the context of solid tumor growth, angiogenesis in the respective tissues is dramatically stimulated. Angiogenic growth factors are expressed and secreted by a great variety of different cell types in response to different stimuli. Expression of VEGF, for example, can be induced by other GFs [27], activated oncogenes [28][29], and tissue hypoxia [30]. After secretion of the angiogenic GFs, they bind to their respective receptors on the cell surface of endothelial cells in neighboring blood vessels and induce several characteristic angiogenic cell responses. In order to trigger angiogenesis from neighboring vessels, the extracellular matrix and basement membrane of the blood vessel have to be degraded. This is achieved by inducing the expression of matrix metalloproteinases in endothelial cells. In addition, angiogenic GFs also induce the migration, invasion, and proliferation of endothelial cells, finally leading to the formation of a new lumen-containing blood vessel capillary, which matures by recruitment of vessel stabilizing mural cells such as pericytes or smooth muscle cells [31].

In order to induce in vivo neovascularization in tissue engineering applications, angiogenic GFs were mixed in hydrogels or coated on solid scaffolds that served as drug-release systems upon implantation. For example, Minardi and colleagues used multiscale microspheres, composed by a nanostructured silicon multistage vector (MSV) core and a poly(dl-lactide-co-glycolide) acid (PLGA) shell for controlled release of the angiogenic factor PDGF-BB [32]. The release kinetics of PDGF-BB was sustained over two weeks and a robust neovascularization was induced upon subcutaneous implantation in mice. Similarly, a VEGF release system was established by Chen and colleagues [33]. In this system, heparin cross-linked demineralized bone matrices were loaded with VEGF. This scaffold also showed sustained release of the angiogenic GF and induced angiogenesis in a subcutaneous implantation model. Angiogenic GFs were also used to induce vascularization in critical-sized bone defects. For example, Quinlan and colleagues incorporated VEGF in alginate microparticles [34]. These particles were then incorporated in collagen-hydroxyapatite scaffolds and implanted in a rat calvarial defect model. In these experiments, VEGF showed a sustained release rate and improved vascularization as well as bone formation in bone defects. The importance of VEGF in bone tissue engineering and bone regeneration and its central role in the coupling of angiogenesis and osteogenesis is highlighted by several recent review articles [35][36][37].

A great variety of natural [38][39][40], semi-synthetic [41][42], and synthetic [43][44][45][46] hydrogels have been used during the past decade for the release of a great variety of different GFs in order to improve neovascularization in BTE. From these studies, it became clear that an effective angiogenic therapy is strictly dependent not only on the right GF or the right combination of different GFs, but also on a temporal and dose controlled release of the angiogenic GFs. In the case of VEGF, for example, it was shown that a high initial burst of release, leading to an unphysiologically high concentration of VEGF in the implanted construct and the surrounding tissue, is associated with the appearance of functionally impaired blood vessels. In this case, the high concentration of VEGF leads to the formation of immature leaky blood vessels, whereas lower doses of VEGF induced the formation of mature functional blood vessels within the implanted construct [47].

As further development of the vascularization strategy on the basis of single recombinant angiogenic GFs, release systems were developed in which two or more GFs are released. For example, two angiogenic GFs, VEGF, and PDGF-BB were used with the aim to induce the formation of blood vessels with increased inherent stability and maturity [42][48]. Angiogenic GFs have also often been combined with osteogenic factors in order to enhance bone tissue formation in BTE applications. For example, Suárez-González and colleagues have loaded porous beta tricalcium phosphate (β-TCP) scaffolds with VEGF and a biologically active peptide derived from human bone morphogenetic protein (BMP)-2 [49]. They were able to show a controlled sequential delivery of both factors over a time course of 60 days. Moreover, by using a sheep intramuscular implantation model, in vivo biological activity of both factors was demonstrated. Similarly, Kuttappan and colleagues combined BMP-2 with VEGF or bFGF and were able to show increased vascularization and bone formation in a rat critical sized calvarial defect model [50].

3. Induction of Vascularization by Biofabrication of Vessel Networks

The classical BTE concept is based on scaffolds that are randomly seeded with bone-forming cells and cells that provide the vasculature upon implantation, as already discussed in the previous sections. However, the prevascularization strategy that is based on the targeted fabrication of blood vessels employs the technique of 3D-bioprinting.

3D-bioprinting represents an additive manufacturing process for the production of artificial tissues that combines biomaterials, cells, and growth factors that are deposited in a layer-by-layer manner [51][52]. This technique allows precise control of the spatial distribution of cells within the bioprinted construct and opens the way to printing multiple cell types in

a biomimetic manner to produce an artificial tissue that corresponds more closely to the native target tissue than would have been possible with classical tissue engineering.

The technology of bioprinting has already been used to print of blood vessels in 3D-tissue constructs and can be considered to represent a new kind of prevascularization strategy. There are two major routes of vascularization reported in the literature: the bioprinting of vessel-forming cells (endothelial cells) and the printing of sacrificial structures that can be removed, leaving behind a microchannel that can be populated by endothelial cells.

Endothelial cells are bioprinted in high density in suitable bioinks such as fibrin or collagen in the form of lines or branched structures in the form of native blood vessels. Upon in vitro culture of these bioprinted constructs, self-organization of endothelial cells and lumen formation can be detected. These endothelial structures resemble early blood vessel capillaries in such a way that lumina are confined by a single layer of endothelial cells.

4. Surgical Strategies for Vascularization

A major issue with all the above strategies is the time needed until the replaced tissue is vascularized by the host. Tissues are oxygenized by diffusion over a distance of 200 µm, meaning that cells in the middle of the construct will invariably undergo hypoxic cell death [53][54][55]. In order to create stable constructs that reliably integrate in the host tissue, it is paramount to deliver nutrients to these cells by neovascularization. Neovascularization can take the form of angiogenesis, which describes a process in which host vessels proliferate and penetrate the construct, or vasculogenesis, which implies that vessels are developed in the implant. In order to avert hypoxic cell death, which occurs during the first days after implantation [56], surgical strategies have been devised in order to vascularize the implant immediately after implantation.

The “in vivo bioreactor” principle unites traditional reconstructive surgery techniques and tissue engineering by relying on the host body as a bioreactor [57]. Imaging data are used to calculate the form and size of the defect. Chambers are then created, which correspond to the defect, filled with the preferred construct (cells, scaffold, growth factors), and implanted at a defect-remote site in the body. After a period of prelamination, the flap containing the construct is transferred into the defect and the vascular pedicle of the flap containing the construct is microsurgically anastomosed to recipient vessels. In this way, immediate and complete perfusion of the construct is achieved [53][58]. This technique is well-established experimentally in large animals (pig and sheep) and has been successfully performed in humans. In 2004, a subtotal replacement mandible consisting of a titanium mesh cage filled with bone mineral blocks coated in bone morphogenic protein 7 and bone marrow aspirate containing undifferentiated precursors was grown inside the latissimus dorsi muscle of a patient with previous subtotal mandibulectomy due to neoplasm. The muscle-construct unit was microsurgically transferred to the recipient site after seven weeks, and it showed good integration and a favorable aesthetic and functional outcome [59]. This approach avoids raising bone donor tissue, but it necessitates raising a muscle flap, which also represents considerable donor site morbidity. Another drawback is the technical complexity, need for two major surgeries, and a treatment period of several months.

Alternatively, and in order to avoid the harvest of a flap, an arteriovenous (AV) loop can be created. This approach involves anastomosing an artery with a vein, and transferring this “neopedicle” to the construct chamber, where it has to undergo microvascular sprouting in order to infiltrate the construct [60]. This approach was extensively verified experimentally [61][62] in large animal models [63] and clinically [64]. This technique also requires considerable time for the engineered tissue to vascularize by sprouting, necessitating hospitalization of several months [65], and thus limiting its clinical use.

To accelerate vascularization in the AV-loop chamber, growth factors [66] and stem cells [67] have been incorporated in the construct with promising results. These data suggest that combining other vascularization strategies in an AV-loop concept is a promising avenue of further research.

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