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Review article

The scope and sequence of growth factor delivery for vascularized bone tissue regeneration

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ABSTRACT

Bone regeneration is a complex process, that *in vivo*, requires the highly coordinated presentation of biochemical cues to promote the various stages of angiogenesis and osteogenesis. Taking inspiration from the natural healing process, a wide variety of growth factors are currently being released within next generation tissue engineered scaffolds (in a variety of ways) in order to heal non-union fractures and bone defects. This review will focus on the delivery of multiple growth factors to the bone regeneration niche, specifically 1) dual growth factor delivery signaling and crosstalk, 2) the importance of growth factor timing and temporal separation, and 3) the engineering of delivery systems that allow for temporal control over presentation of soluble growth factors. Alternative methods for growth factor presentation, including the use of gene therapy and platelet-rich plasma scaffolds, are also discussed.

1. Introduction

Bone tissue is remarkable with respect to its high capacity for self-renewal as well as its ability to naturally cycle through stages of resorption in order to maintain skeletal integrity [1]. However, of the 15.3 million fractures occurring annually in the United States, an estimated 10% are classified as non-union, fractures, which: 1) clinically have no observable sign of healing over time, 2) show a lack of progression in radiological appearance, and 3) have ceased biological healing without uniting [2–5]. A wide variety of materials have been explored as scaffolding to bridge the defect and encourage bony wound healing. However, success has been limited to date given that few scaffold materials are naturally (and simultaneously) osteoconductive (physically viable templates for bone tissue ingrowth), osteoinductive (capable of inducing differentiation of stem cells towards an osteogenic lineage) and osteogenic (containing osteoprogenitor cells) [6–8]. To further complicate matters, there is also a need to establish a vascular network within the scaffold, which will ultimately provide necessary oxygen and nutrients to regenerated bone tissue as well as waste removal functionality [9]. Notably, the inclusion of biochemical cues, such as growth factors, within the scaffold has been found to aid in the success of such implants by helping recruit and instruct cells within the regenerating niche [7,10].

The selection of the biochemical cues to be presented within bioengineered scaffolding has been inspired by the signals naturally presented within the bone microenvironment [11]. For example, bone morphogenetic proteins, and in particular, bone morphogenetic protein 2 (BMP-2), which is naturally present during bone healing [9] are commonly included in synthetic bone scaffolds to induce osteoblastic differentiation and increase osteoblast proliferation [12]. Platelet derived growth factor (PDGF), which is released by clotting platelets during fracture healing, is frequently encapsulated and released to promote the recruitment of mesenchymal stem cells (MSCs) [13] (which among other things, are cells that can differentiate into osteoblasts, and function as neovasculature-stabilizing pericytes [14], as well as promote regeneration through the secretion of immunomodulatory cytokines and trophic growth factors [15]). Furthermore, the release of angiogenic growth factors, including PDGF and vascular endothelial growth factor (VEGF), has been shown to play a major role in angiogenesis (the development of new blood vessels in the wound space) by increasing endothelial cell recruitment and subsequent tubule formation

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[16] in a process, which mimics that observed during ossification in development [17].

Despite the promising results from controlled delivery of individual growth factors, broad clinical translation for critical sized bone defects has yet to be achieved, suggesting that the promotion of successful healing requires a more sophisticated solution [18]. For instance, the controlled release of PDGF is well known to support regeneration by promoting nascent blood vessel formation and by recruiting and inducing mitogenesis of MSC or smooth muscle cells [13,19–21]. However these effects appear to be limited to the early phases of cell infiltration and tissue regeneration, and few differences can be found at later time points between control and PDGF-eluting scaffolds in terms blood vessel number, extracellular matrix (ECM) production, and osteoblast density [22,23]. Similarly, although scaffolds releasing BMP have shown enhanced bone defect repair *in vivo* [24–26], vascular network formation in these constructs appears to be limited [27]. These observations suggest that, while growth factors do indeed offer some distinct, predictable advantages, release of individual growth factors may not fully address the aforementioned inadequacies of synthetic scaffolds alone [28].

In contrast to the current practice of releasing individual growth factors from synthetic scaffolding in an attempt to produce engineered tissue regeneration, natural, *in vivo* bone healing is a highly ordered, sophisticated and complex process that requires the coordinated presentation of multiple growth factors that direct the action of the numerous cell types involved in bone regeneration [8,29,30]. Accordingly, the delivery of a single growth factor may very well be a gross oversimplification of the incredibly complex signaling that guides bone tissue healing. In fact, it is becoming increasingly apparent that natural bone defect repair consists of a number of distinct, sequential stages of coordinated cellular action. This process begins with an inflammatory response to injury and hematoma formation that is upregulated by inflammatory cytokines such as IL-1, IL-6 and TNF-α [30,31]. Angiogenic factors, including VEGF and PDGF then initiate the infiltration of the hematoma with vasculature that carries nutrients and attracts cells to the injury site [32,33]. Once recruited into the wound space, cells, such as endothelial cells and MSCs secrete a series of additional growth factors that regulate and initiate further healing stages, including the transformation of the hematoma into a chondrogenic (cartilaginous) callus and simultaneous attraction of cells for the next phase of regeneration [15]. The next wave of infiltrating cells includes chondroclasts and osteoblastic progenitors that resorb the chondrogenic callus and begin forming new bone [29]. Overall, the numerous biochemical cues that coordinate these sequential stages of regeneration to produce organized tissue *in vivo* more closely resemble a highly orchestrated set of time-dependent "instructions" than a simple "mixture" of growth factors [8,29,30].

This review will summarize current research intending to more closely replicate the complex process of bone regeneration through the engineered and orchestrated stage-wise (or sequenced) release of growth factors.

2. Growth factor delivery

2.1. Single growth factor delivery and bone regeneration

Prior studies involving the delivery of single growth factors provide information regarding the processes that each of these signals is involved in directing *in vivo*. Indeed, these studies may even provide important clues as to how these signals fit within the overall regenerative process. Accordingly, this section will generally review several key findings from the delivery of single growth factors, with mention of both the positive results observed to date as well as highlighting notable limitations.

As mentioned previously, BMP-2 has been frequently incorporated in scaffolds for bone regeneration because of its well-recognized ability to promote bone formation [34,35]. At the cellular level, BMP-2 is a potent inducer of osteoblast proliferation as well as a differentiator of mesenchymal stem cells towards an osteoblastic phenotype [36] (Fig. 1). Accordingly, BMP-2 increases bone formation at the defect site for applications ranging from spinal fusion [37] to dental and craniofacial regeneration [38]. While these studies are evidence of the usefulness of BMP-2 for inducing mineralized tissue, there are large variations in the success of human response to BMP-2 treatment. For example, in one clinical investigation, stimulation of bone regeneration stimulation following administration of BMP-2-soaked collagen sheets ranged from excellent bone formation, to no bone formation with fibrous tissue in patients receiving the same delivery construct [12]. This may be explained by the rapid delivery of BMP-2 from collagen-soaked scaffolds, mediated primarily by protein desorption, with no barriers to diffusion to create sustained release rates [39]. As noted in other studies, when BMP-2 (or other growth factors) are delivered *via* bolus administration, they rapidly diffuse from the site of administration with a lack of temporal and spatial control, resulting in only transient cellular stimulation with supra-physiological concentrations of the growth factor [40]. In an attempt to present proteins in a more physiologically relevant manner, several methods to sustain BMP-2 release have been explored, including its encapsulation within (and release from) polymers, its expression through delivery of plasmids, and its entrapment within scaffold coatings [41–43].

While basic sustained release provides a more extended signal to cells to direct bone regeneration, a major shortcoming of delivering only BMP-2 is the limited capacity for BMP-2 alone to initiate vascular network formation [27]. As previously described, vascular infiltration is a required and necessary phase of overall bone healing that precedes ossification [30,44]. Hence, in addition to (and, in the context of vascularization, more significantly than) BMP-2, delivery of several known angiogenic growth factors has also been explored during bone regeneration to promote vessel development and growth. These include VEGF, a potent mitogen for endothelial cells [17], and platelet endothelial growth factor (PDGF), which has been shown to act as a chemoattractant for cell types that stabilize growing vasculature [28,45] (Fig. 1). Moreover, PDGF has also been observed to upregulate VEGF production for continued angiogenesis [46]. Beyond VEGF and PDGF, additional angiogenic factors observed in the bone regeneration niche include fibroblast growth factor (FGF), which plays a role in the sprouting of new capillaries [47], and insulin like growth factor (IGF) a growth factor known to play a role in adult neoangiogenesis [44]. Each of these angiogenic factors has also been delivered to enhance bone healing and, specifically, the vascularization stage of regeneration. Notably, several studies suggest that delivering angiogenic factors alone for bone regeneration leads to the inability to produce organized bone regeneration [27,48,49]. Indeed, when releasing VEGF alone, no bone formation was observed in either ectopically or orthotopically implanted scaffolds [27].

2.2. Dual growth factor delivery

To create bone scaffolds with a better likelihood of vascularity, the simultaneous incorporation of both an angiogenic factor (to jumpstart vessel formation) and an osteogenic factor (typically BMP-2, to induce bone formation) has been explored. A controlled, "dual release" of two growth factors at the same time could be argued to be closer to mimicking the natural healing process. There is indeed some evidence that the delivery of an osteogenic and angiogenic factor can enhance bone formation in some circumstances when compared to the delivery of an osteogenic factor alone [49,50]. For example, Huang et al. have shown that the delivery of both VEGF and BMP-2 from scaffolding seeded with human bone marrow stromal cells (hBMSCs) resulted in significantly greater amounts of bone formation than when BMP-2 was delivered alone from the same subcutaneously implanted scaffold at 15 weeks using a murine severe combined immunodeficiency model [49]. More

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