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Review

Targeting the hypoxic response in bone tissue engineering: A balance between supply and consumption to improve bone regeneration

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ABSTRACT

Bone tissue engineering is a promising therapeutic alternative for bone grafting of large skeletal defects. It generally comprises an *ex vivo* engineered combination of a carrier structure, stem/progenitor cells and growth factors. However, the success of these regenerative implants largely depends on how well implanted cells will adapt to the hostile and hypoxic host environment they encounter after implantation. In this review, we will discuss how hypoxia signalling may be used to improve bone regeneration in a tissue-engineered construct. First, hypoxia signalling induces angiogenesis which increases the survival of the implanted cells as well as stimulates bone formation. Second, hypoxia signalling has also angiogenesis-independent effects on mesenchymal cells *in vitro*, offering exciting new possibilities to improve tissue-engineered bone regeneration *in vivo*. In addition, studies in other fields have shown that benefits of modulating hypoxia signalling include enhanced cell survival, proliferation and differentiation, culminating in a more potent regenerative implant. Finally, the stimulation of endochondral bone formation as a physiological pathway to circumvent the harmful effects of hypoxia will be briefly touched upon. Thus, angiogenic dependent and independent processes may counteract the deleterious hypoxic effects and we will discuss several therapeutic strategies that may be combined to withstand the hypoxia upon implantation and improve bone regeneration.

1. Introduction

Tissue engineering aims to offer large-scale replacement of damaged organs using implants generated from a combination of cells, growth factors and an appropriate scaffold. The engineering of bone tissue is a promising therapeutic strategy for non-healing large bone defects, as autologous bone transplants, the current gold standard, are limited in quantity and often associated with donor-site morbidity. A key challenge in the clinical application of cell-based tissue-engineered bone implants is the poor diffusion of oxygen into avascular tissue, which is limited to 200 μm at most, necessitating a functional blood vessel network to bring oxygen into larger constructs (Ma et al., 2014b). However, the blood vessels at the implant site are in most cases disrupted by the trauma or surgery that caused the bone defect, leading to hypoxia or even anoxia within the implant. This impaired oxygenation and nutrient supply may lead to low cell survival and failure of the implant. Indeed, studies in cardiac tissues have shown that tissue-engineered constructs of clinically relevant size are devoid of oxygen in the centre, causing massive loss of cell viability and regenerative capacity (Radisic et al., 2006). Analogous herewith, necrotic cells are observed in the centre of implanted large bone constructs (Scheufler et al., 2008). On the other hand, bone cells have the capacity to withstand the temporary hypoxia that occurs during certain stages of bone development or uncomplicated fracture repair (Drager et al., 2015) by activating the hypoxia signalling pathway. Indeed, a recent study has shown that in bone, physiological oxygen concentrations can be as low as 1.3% or 10 mmHg (Spencer et al., 2014), which are much lower than the ambient oxygen levels that we breathe (21% or 160 mmHg). Moreover, activation of the hypoxia signalling pathway is known to increase exponentially as oxygen concentrations fall below 8% or 60 mmHg (Ehrismann et al., 2007). A better understanding of the processes activated by the hypoxia signalling pathway in skeletal cells may therefore provide novel options to improve bone tissue engineering strategies.

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In this review, we will discuss the current knowledge on hypoxia signalling in bone regeneration and we will provide an overview of the different strategies that are being explored in bone tissue engineering to overcome cell death after implantation due to the long-lasting and extensive hypoxia, including pro-angiogenic therapies, prevascularization strategies and cellular preconditioning.

2. Bone tissue engineering

While bone has a tremendous regeneration potential and is one of the few tissues that can heal completely without scar formation, 10% of fractures results in a non-union (Tytherleigh-Strong et al., 1997). In case of congenital defects, like neurofibromatosis 1, or surgical resection, this regenerative potential may become even more challenged and often leads to inadequate healing, which causes an enormous health care burden. The current gold standard treatment consists of bone auto- or allografts, but several problems restrict their usefulness including the limited availability at the harvest site, incomplete integration into the defect and risk of disease transfer (Delloye et al., 2007). A promising therapeutic alternative for autografts in large bone defects is bone tissue engineering, which is a branch of regenerative medicine that aims to create new bone tissue from osteogenic stem or progenitor cells seeded on an osteoconductive carrier or scaffold, often in combination with osteoinductive growth factors.

The bone-forming cells frequently used for tissue engineering are the mesenchymal stromal cells (MSC, also known as mesenchymal stem cells). Bone marrow-derived MSC are used most often, although it are the periosteum-derived cells that primarily contribute to fracture healing and are therefore increasingly being studied (Colnot, 2009; Gomez-Barrena et al., 2011). Indeed, human and mouse periosteum-derived cells were considered much more difficult to obtain than bone marrow-derived cells, but protocols for their isolation have been developed and can now be routinely used (De Bari et al., 2006; van Gastel et al., 2012). These cells highly express classical MSC markers, show trilineage potential *in vitro* and contribute extensively to bone regeneration *in vivo*, making them exceptionally suited for bone tissue engineering strategies (Roberts et al., 2015). Adipose-derived MSC and embryonic or induced pluripotent stem cells are also being actively explored as cell source because of their greater availability. However, their *in vivo* bone-forming potential has not been as rigorously studied as for the bone-derived MSC (reviewed in (Robey, 2011)).

Numerous materials are used as carriers for these cells: they range from biological materials designed to closely mimic the composition of bone (i.e. calcium phosphate and collagen) over synthetic polymers (e.g. polylactic acid, polycaprolactone) to bioactive glass and metals, and many types of composite designs. Most scaffold materials can be chemically treated or biologically functionalized with peptides to enhance their osteoconductivity, degradability and angiogenic response. As the focus of this review is on the hypoxic response as a potential therapeutic target, we refer the reader for more information on biomaterial characteristics and their functionalization to some recent excellent reviews (Bose et al., 2012; Wu et al., 2014).

Finally, certain growth factors that have been described to be crucial for fracture healing are also being used for bone tissue engineering (reviewed in (Gothard et al., 2014)). Perhaps the most important examples are the bone morphogenic proteins (BMPs), notably BMP-2. They are currently approved for spinal fusion and open tibia fractures, although application of high-dosed BMP alone has proven not to be as safe as initially believed (Shields et al., 2006). Angiogenic growth factors, such as vascular endothelial growth factor (VEGF) or placental growth factor, are also crucial for fracture repair and can be used to induce angiogenesis in bone tissue-engineered constructs. Several other factors, like fibroblast growth factors or transforming growth factors, can act on both osteo- and angiogenesis (reviewed in (Stegen et al., 2015)). However, when these factors are used as monotherapy for bone repair, they often have to be applied in high doses and frequently result in adverse side effects such as soft tissue inflammation and swelling, haematoma formation, heterotopic ossification and induction of osteolysis. These complications seriously limit their clinical applicability (Epstein, 2011; Shields et al., 2006). Nevertheless, these growth factors may potentially be used at much lower doses when they are combined with cell-based constructs. It is hypothesized that this reduced dose may be sufficient to stimulate primarily the bone forming potential of the implanted cells, although supporting clinical evidence is still lacking. Alternatively, the use of growth factors can be restricted to the pre-implantation cell expansion phase in order to enhance the regenerative potency of the implanted MSC and thereby prevent possible side effects caused by *in vivo* application of growth factors (van Gastel et al., 2014).

To summarize, bone tissue engineering is an expansive field with new combination approaches being developed continuously. However, the therapeutic success of clinically relevant regenerative implants will largely depend on how the implanted cells are adapted to react to the hostile and hypoxic host environment into which they are placed.

3. The hypoxia signalling pathway

The crucial mediators of hypoxia signalling are Hypoxia Inducible Factor 1 (HIF-1) and HIF-2, heterodimeric transcription factors consisting of an α and β subunit. Both subunits are constitutively expressed in most tissues and cells, but the HIF-α subunit is turned over very rapidly at the protein level under normoxic conditions (Fig. 1). When sufficient oxygen is available, HIF-1α is hydroxylated by proline hydroxylase domain (PHD)-containing enzymes (PHD1, PHD2, PHD3), of which PHD2 appears to be the main effector for HIF-1 activity (Appelhoff et al., 2004; Berra et al., 2003). These enzymes use oxygen and α-ketoglutarate as substrates and ferrous iron and ascorbate as cofactors. Upon hydroxylation of HIF-1α, the Von Hippel-Lindau protein (VHL) ubiquitinates the N- and C-terminal oxygen dependent degradation domains of HIF-1α, causing HIF-1α to be targeted for proteasomal degradation (Maxwell et al., 1999). As a result, HIF-1α is a highly unstable protein in aerobic conditions. When oxygen tension decreases, the PHDs will become less active, resulting in stabilization of HIF-1α which can then bind to HIF-β and exert its transcriptional activity in the nucleus (Huang et al., 1998). Reduced activity of PHDs through lowered oxygen concentration is however not the only mechanism that can induce HIF stabilization. Certain hormones and growth factors, such as angiotensin II, epidermal growth factor and insulin-like growth factor can induce HIF-mediated signalling in normoxic conditions via activation of protein kinase C and phosphatidylinositol-3 kinase (Fukuda et al., 2002; Richard et al., 2000; Zhong et al., 2000). Interestingly, the osteochondrogenic Runt-related transcription factor 2 (Runx2) also directly increases HIF-1α activity in MSC and hypertrophic chondrocytes by competing with VHL (Kwon et al., 2011; Lee et al., 2012). Hypoxia signalling can also be chemically induced by using small molecules that block PHD activity; most of them interfere with cofactor or substrate availability (e.g. iron chelators, α-ketoglutarate analogues) and therefore not only target PHDs, but specific PHD inhibitors are actively being developed.

The HIF complex transactivates genes containing one or more hypoxia response elements (5′-[A/G]CGTG-3′), of which more than 100 have been identified to date. These genes are mainly involved in the regulation of energy metabolism, angiogenic response,

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