Osteoinductive and Antibacterial Biomaterials for Bone Tissue Engineering

Wang, D.

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Chapter 1

General Introduction

Critical-sized bone defect

Bone defects, resulting from trauma or tumor resections, are common clinical problems that affect hundreds of thousands of patients worldwide. They are usually associated with pain, stiffness of the surrounding joints and disability, which often prevents employment and therefore imposes an economic burden on the patient and on society [1]. Thanks to self-healing mechanism, humans and animals tend to spontaneously repair bone defects. However, if the sizes of the defects are beyond the self-healing capacity, they cannot repaire without medical intervention. Such defects are called critical-sized bone defects (CSBD), which is defined as the smallest size intraosseous wound that will not spontaneously heal completely with bone tissue, or the defects will heal by connective tissue during the lifetime of the animal [2]. Although tremendous efforts have been made to repair CSBD, current strategies encounter a variety of limitations. Further development of effective repair of CSBD is therefore needed in the field of orthopedic, oral and maxillofacial surgery.

Bone tissue engineering

Bone tissue engineering is an interdisciplinary field that combines the knowledge and technology of material engineering and biological factors to regenerate damaged bone tissues[3]. It is based on the understanding of bone structure, bone mechanics, and tissue formation as it aims to induce new functional bone tissues. In other words, to successfully regenerate or repair bone, knowledge of the process of bone defect healing is quite essential.

Bone defects heal by a process that recapitulates many of the events of both intramembraneous and endochondral bone formation, and it uniquely heals without the formation of scar tissue [4, 5]. Initially, hematoma formation is accompanied by an inflammatory response and the recruitment of many of the signaling molecules involved in the regulation of new bone formation (i.e., ILs, TNF-α, FGFs, BMPs, PDGF, VEGF, etc.). At cortex and periosteum level, intramembranous bone formation immediately occurs. The external soft tissues stabilize the fracture by the formation of a callus, which subsequently undergoes chondrogenesis, and then a process highly similar to endochondral ossification. More specifically, after the callus forms, chondrocyte proliferation decreases as the tissues begin to mature (i.e., hypertrophy) and calcify the matrix. In-growing blood vessels carry chondroclasts, which are responsible for resorbing the calcified cartilage and osteoblastic progenitors, which begin the process of new bone formation. The mechanical continuity of the cortex is achieved via subsequent remodeling of the newly formed bone. In the process of repairing bone defects, several key factors are highlighted: (1) a biocompatible scaffold that closely mimics the natural bone extracellular matrix niche, (2) osteogenic cells to lay on the bone tissue matrix, (3) morphogenic signals that differentiate mesenchymal stem

cells into osteogenic cells, and (4) sufficient vascularization to meet the growing tissue nutrient supply and clearance needs.

Bone grafts

Bone grafts must comply with some or all these factors to be able to facilitate bone regeneration to various degrees. If the grafts have the ability to facilitate the migration and proliferation of osteoblasts and progenitor cells [6], they are considered to be osteoconductive material. A solo scaffold is usually an osteoconductive graft. When a scaffold is constructed with osteogenic and/or vasculo-genic growth factors, they are endowed with osteoinductivity─the ability to induce progenitor cells to differentiate down osteogenic lineages [6]. Only when a biomaterial consists of a scaffold, osteogenic cells and growth factors, it is considered to be osteogenic.

Autografts are osteogenic, because they can provide all the elements for bone regeneration such as osteoconductive 3-dimensional scaffolds, osteogenic cells and osteoinductive growth factors [7]. Autografts are therefore regarded as the “gold standard” for bone defect repair. However, autografts need to be harvested from the iliac crest or other sites in patients, and thus it requires a second operation at the site of the tissue harvest [8], which makes patients in danger of significant donor site injury and morbidity, deformity and scarring [9-11]. Another drawback of autografts is their uncontrollable and variable spontaneous resorption, which may compromise the outcome of reconstructing the curvature of the local sites [12].

Allografts represent the second most common bone-grafting technique. They are often from a cadaver. Allogeneic bone is also biocompatible, and is available in various forms, including demineralized bone matrix, morcellised and cancellous chips, cortico-cancellous and cortical grafts, and whole-bone segments, depending on the host-site requirements. In comparison to autografts, allografts are associated with risks of immunoreactions and transmission of infections [13, 14]. Since donor grafts are devitalized via irradiation or freeze-drying processing, they have reduced osteoinductive properties and no cellular component [15, 16]. Furthermore, the bone grafting market is experiencing an unmet supply and great demand; there is currently a shortage in allograft bone graft material [17].

Xenografts are composed of tissue taken from another species. The antigenic potential of xenografts can be diminished or eliminated by chemical treatment. One of the most widely used xenografts in clinical dentistry is deproteinized bovine bone (Bio-Oss®, Geistlich, Switzerland). It is derived from a bovine source and is treated by a chemical extraction process to remove all the organic components and pathogens [18].

Thanks to technological evolution and better understanding of bone-healing mechanisms, many synthetic calcium phosphate (CaP) grafts have been developed to mimic the content and structure of natural bone for repairing bone defects. According to the chemical compostition, CaP can be classified as either hydroxyapatite, beta-tricalcium phosphate, biphasic calcium phosphate, carbonated apatite or calcium deficient hydroxyapatite [19]. Moreover, CaP can be fabricated into different application forms, such as pastes, granules, blocks and composites, which can be classified as ceramic or cements. Ceramics are defined as the inorganic, non-metallic solid materials prepared by sintering [20]. The sintering process removes volatile chemicals and increases crystal size, resulting in a porous and solid material. On the other hand, cements consist of a mixture of calcium phosphates which can be applied as a paste and harden in situ due to a precipitation reaction.

Both xenograft and synthetic CaP have physical and chemical structure similar to that of natural bone [21], which offers them excellent osteoconductivity. However, they lack intrinsic osteoinductivity [22].

Acquiring and evaluating osteoinductivity

For most clinical cases, osteoconductive bone grafts can achieve excellent bone defect reparation. However, there are still a significant number of eligible individuals with CSBD suffering from diabetes, local osteoporosis and metabolic bone disorder which can compromise bone healing. Bone grafts with enhanced ability to repair bone defects are therefore needed. Although cell-based therapies are attracting increasing attention, they are still in their infancy regarding the safety and efficacy for humans. In contrast, growth factor-based therapy is more advantageous in safety, feasibility and practical potential for clinical application in the immediate future. Therefore, we believe it is the safest and most practical method to endow grafts with osteoinductivity by introducing growth factors. One substantial group of growth factors to offer osteoinductivity to bone grafts is the bone morphogenetic proteins (BMPs). BMPs have been found in the demineralized bone matrix more than 40 years ago. They belong to the TGF-β superfamily and promote the differentiation of osteoprogenic cells and induce osteogenesis [23]. The osteoinductive properties of BMPs make them promising candidates to promote bone formation, which has been confirmed in animal studies and clinical trials [24-26]. Some of them are therefore approved by the food and drug administration (FDA) as a medical device [23]. These are recombinant human bone morphogenetic protein-2 (rhBMP-2) and recombinant human bone morphogenetic protein-7 (rhBMP-7).

To evaluate the osteoinductivity of biomaterials, we need to thoroughly understand osteoinduction. It could be divided into 3 principles [27]: (1) mesenchymal cell recruitment; (2) mesenchymal differentiation to bone-forming osteoblasts and (3) ectopic bone formation *in vivo*. Based on this theory, the osteoinductivity *in vitro* can be

determined by evaluating the effect of biomaterials on the differentiation of mesenchymal stem cells. Their osteoinductivity is confirmed only when mesenchymal stem cells are induced to osteogenic differentiation, which can be characterized by alkaline phosphatase (ALP), osteocalcin (OCN) expression and mineralization. ALP [28] and OCN [29] are involved in the process osteoid formation and bone mineralization. OCN is considered a specific marker of osteoblast function [30]. Alizarin red-S is usually used to stain calcite dolomite to show the mineralization [31], which is the reliable signal for osteogenic differentiation. *In vivo* the osteoinductivity of a material is usually demonstrated by bone formation after being implanted in CSBC [32] or ectopic sites such as subcutaneous pockets and intramuscular sites [27].

Antibiotic delivery vehicles for treatment of infected bone defects

Reparation of bone defects can be challenging not only because of their sizes, but also when they are combined with local infections. For example, in bone defects caused by trauma, up to 50 % infectious complications have been reported [33, 34] with the tibia being most often affected [35]. Furthermore, the subsequent chronic osteomyelitis and/or non-union represent a major source of disability and decreased quality of life for the individual patient, and a socio-economic problem for public health systems.

Thorough elimination of local infection is the prerequisite of repairing bone defects[36] and therefore much focus has been put on infection control. Systematical administration of antibiotics is not preferable for local infection control, because it is difficult for systemically administered antibiotics to cross bone tissue with relatively avascular bone surrounding it before reaching the infected defect site. This not only diminishes its effectiveness, but also increases the chances for the induction of bacterial resistance [37].

Logically, these two central shortcomings could be ameliorated with the use of locally delivered antibiotics. Various delivery modes have been developed for infection control. At the clinical level, the mainly applied antibiotic delivery vehicle is in the form of poly (methyl methacrylate) (PMMA) beads. After they had been first clinically applied in the early 1970s [38], they gradually established themselves as a standard option for the local delivery of antibiotics to bone cavities and this trend continues to this very day. Although PMMA beads loaded with hydrophilic antibiotics were successfully applied in the past [39-41], numerous clinical limitations are associated with their use. These include: (1) their non-biodegradable nature and the need for a secondary surgery to remove them [42]; (2) an often insubstantial amount of the released antibiotic following the initial, burst release phase [43], which has led to the promotion of pathogenic resistance to such therapies in the past [44]; (3) proneness to biofilm formation, which hinders the antimicrobial action [45]; and (4) moderate toxicity resulting from the absorption of MMA monomers and the carboxylesterase-mediated conversion of MMA to methacrylic acid [46]. On top of this, a

comprehensive clinical study has yet to prove that PMMA beads are more effective than the systemic antibiotic delivery in treating orthopedic infections [47]. The major clinically available alternative capable of sustained release are calcium sulfate cements, which suffer from other weaknesses, mainly their rapid degradation, which is faster than the bone ingrowth rate and can lead to mechanical failure of the implant [48].

We therefore believe that the ideal antibiotic delivery vehicle to repair infected bone defects should possess the following properties: (1) biodegradable and its degradation rate should match with the ingrowth rate of new bone; (2) can be adjusted to carry proper amount of antibiotics and deliver antibiotics in appropriate mode; (3) the vehicle itself is also a functional bone substitute.

Outline of the thesis:

BMP2 is well-known as an effective osteoinductive agent. This thesis is about introducing BMP2 into different carriers to develop various osteoinductive biomaterials with different properties to repair CSBD and infected CSBDs.

To enhance bone regeneration in CSBD, most bone-defect-filling materials in clinics need to be mixed with autografts to obtain osteoinductivity. Due to the obvious limitation in using autografts, we therefore developed a BMP2-coprecipitated, layer-by-layer assembled biomimetic calcium phosphate particle (BMP2-cop.BioCaP) as a potential osteoinducer. In **chapter 2**, we combined BMP2-cop.BioCaP with clinically often used biphasic calcium phosphate (BCP) to repair an 8mm rat cranial defect. Our hypothesis was that BMP2-cop.BioCaP could introduce osteoinductivity to BCP and so function as effectively as autografts for the repair of CSBD.

BMP2-cop.BioCaP cannot be applied as an independent bone-defect-filling material due to its rapid degradation. We therefore developed a BMP2-incorporated biomimetic calcium phosphate granule (BMP2-BioCaP). In **chapter 3**, we combined it with a strong antibacterial agent ─ hydroxypropyltrimethyl ammonium chloride chitosan (HACC) to fabricate an osteoinductive and antibacterial biomaterial─BMP2-BioCaP/HACC complex for repairing infected CSBD. It was designed with a sequential release system: burst release of HACC and followed by a controlled release of BMP2. We hypothesized that this BMP2-BioCaP/HACC complex could rapidly eliminate residual bacteria and thereafter induce new bone formation in subcutaneous pockets in rats.

Osteoinductivity is essential not only for bone-defect-filling materials to repair CSBD, but also for coatings on metallic biomaterials to improve their osseointegration in patients with comprised surrounding bone tissue. In **chapter 4**, we reviewed that introducing BMP2 to

calcium phosphate coatings endow metallic implant surfaces with osteoinductivity so as to enhance and accelerate their osseointegration.

In clinical practice, unlike animal studies mentioned above, bone regeneration in defects and osseointegration of implants can hardly be evaluated by histological analysis. However, it is essential to have good validity and reliability of radiological evaluating system to assess post-operative integration bone-defect-filling materials and osseointegration of metallic implants. In **chapter 5**, we therefore evaluated the accuracy of cone beam computed tomography (CBCT) by comparing the same measurements gained by CBCT with those on their corresponding histological sections.

In **chapter 6**, the main conclusions of this thesis are discussed and placed in a broader perspective. The limitations of this thesis are also addressed.

Objectives of the thesis

The aims of this thesis are as follows:

1. To introduce osteoinductivity to clinically used BCP by a novel osteoinducer and evaluate if it can improve bone regeneration in CSBD. Furthermore, to explore the mechanism of the osteoinducer enhancing bone regeneration.
2. To develop an antibacterial and osteoinductive biomaterial for treatment of infected CSBDs and to evaluate its antibacterial activity and osteoinductivity *in vitro* and *in vivo*.
3. To review the biological process of osseointegration and offer an overview of the coatings designed for improving osseointegration of metallic biomaterials
4. To evaluate the accuracy of measuring bone thickness surrounding dental implants and the reliability of assessing existence and completion of osseous integration of augmentation material using CBCT.

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