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Chapter 6

General Discussion

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Currently, the world is experiencing an exceedingly high demand for functional bone grafts to repair bone defects. It is perceived as a more promising approach to develop bone grafts using the tissue engineering approach than harvesting autografts from patients. Through tissue engineering bone grafts can be functionalized with specific properties so as to repair bone defects with various complications, such as bone defects in patients with diabetes or metabolic bone disorders, large bone defects beyond their self-healing capacity or locally infected bone defects. Therefore, functionalized bone grafts, with osteoinductivity and/or antibacterial activity, have been intensively studied and developed.

Many biomaterials with potential osteoinductivity have been reported and their osteoinductivity was mainly evaluated by heterotopic bone formation in animal models. Glass cylinders [1] and poly-hydroxyethylmethacrylate [2] were the first synthetic materials associated with heterotopic bone formation. Composites, consisting of polylactide and HA particles have however recently proven to be osteoinductive too [3, 4]. In the family of metals, porous titanium has shown osteoinductivity alone [5], coated with a thin layer of calcium phosphate [6] or in a construct with a calcium phosphate ceramic [7]. In contrast to the limited number of reports on osteoinduction by polymers and metals, ceramics – particularly CaP based biomaterials– have shown osteoinductive potential in a variety of studies: HA [8, 9], β -TCP [10, 11] and BCP – the mixture of HA and TCP [12, 13]. This is possibly because that the presence of a calcium phosphate source is a prerequisite for heterotopic bone formation to occur. The liberation of Ca^{2+} , PO_4^{3-} , HPO_4^{2-} from the material into the surroundings may cause precipitation of carbonated apatite that incorporates calcium, phosphate and other ions (Mg^{2+} , Na^+ , CO_3^{2-}), as well as proteins, and other organic compounds [14, 15], which are necessary for bone formation.

Apart from the chemical composition of the material, the geometry and macrostructural properties have shown to play an important role in bone formation. In the case of macrostructure, porosity is of great importance. Bone formation has never been observed on a dense sintered ceramic, which does not degrade *in vivo*, whereas a ceramic with the same chemical composition, but containing pores, induced bone formation [16, 17]. Generally, the importance of pores inside bone graft substitutes is related to the invasion of the material by blood vessels, that bring along growth factors and oxygen as well as osteogenic cells with the capacity to differentiate into osteoblasts [18]. Moreover, with the presence of “protective areas” in the form of pores, heterotopic bone formation can occur without being disturbed by high body fluid refreshments or mechanical forces due to implant movement.

In addition to chemical composition and macrostructural properties, material surface properties at micro- and nanoscale have shown to be of great importance for the

osteoinductive potential of the material. Since the micropores (defined as pores with a diameter smaller than 10 μm) [14, 16] enlarge the surface area, mineral deposition from the body fluids are expected to be more pronounced – which may be beneficial for osteoinduction to occur.

No matter what chemico-physical properties these potential osteoinductive materials possess, all of them were designed to form new bone by recruiting more osteogenic cells and/or growth factors. It is therefore logical to develop bone substitutes by directly adding osteogenic cells and/or growth factors to them via tissue engineering. Bone grafts loaded with osteogenic cells are regarded as materials with osteogenicity—the ability to produce bone independently. In this thesis, we focus on osteoinductive bone grafts — the bone substitutes loaded with growth factors.

Among all growth factors, BMPs are the most intensively studied. Both BMP2 and BMP7 are approved by the FDA to be used in the treatment of a variety of bone-related conditions including spinal fusion and nonunion [19]. However, multiple questions regarding a suitable carrier for BMPs, dosage, repeat exposure, carcinogenesis and long-term results have hampered the potential benefits these molecules could offer for bone formation [20]. The present way of delivering BMP2 clinically, the superficial adsorption of BMP2 onto bone filling materials [21], causes burst release and consequently the transient high local concentration of BMP2. This kind of delivery of BMP2 is often associated with various potential side effects such as an excessive stimulation of bone resorption and the induction of bone formation at unintentional sites [22]. To maximize its osteoinductivity, BMP2 needs to be sustainably delivered to target sites at a low concentration [23]. Guided by this theory and given the necessity of a source of calcium and phosphate in the process of bone formation, we used CaP as the carrier of BMP2 to develop CaP-based slow-delivery system of BMP2, BMP2-cop.BioCaP (chapter 2) and BMP2-BioCaP (chapter 3). The release kinetics of BMP2 in these materials showed a steady controlled release mode. BMP2-cop.BioCaP proved to function as an effective osteoinducer to improve bone regeneration in CSBDs, which are 8mm rat cranial defects in this thesis. Furthermore, as an independent osteoinductive bone substitute, BMP2-BioCaP granules showed significant ectopic bone formation in subcutaneous sites in rats.

Since BMPs are not bony tissue-specific [24], their localized (vs. systemic) and release-controlled (vs. uncontrolled) delivery is necessary to prevent any undesired and uncontrolled ectopic bone formation in non-bony tissues in the body [25]. Besides BMP2-cop.BioCaP (chapter 2) and BMP2-BioCaP (chapter 3), there are therefore many other systems developed to achieve a localized and release-controlled BMP2 delivery system

Alginate, is a non-immunogenic polysaccharide found abundantly in the surface of seaweeds, have been used in a wide range of tissue engineering applications due to its gel-forming properties [26]. BMPs can be encapsulated into alginate matrices and its release mode can be modulated by different parameters such as particle size, viscosity and chemical composition. Liew et al. [27] in a recent investigation found that particle size affected the extent of burst release and the higher the viscosity the slower the encapsulant release.

Unlike natural polymers and collagen, synthetic biodegradable polymers pose no danger of immunogenicity or possibility of disease transmission. A number of synthetic biodegradable polymeric delivery systems for BMPs were developed [28, 29]. Particularly, Nano- and micro-particles from synthetic polymers have attracted much attention for the localized and release-controlled delivery of growth factors due to their attractive tendency to amass in sites of inflammation [30]. In a recent example of a combined localized and release-controlled delivery system, poly D, L-lactide-co-glycolic acid (PLGA) nanospheres immobilized onto prefabricated nanofibrous poly L-lactic acid (PLLA) scaffolds were used to load and deliver rhBMP-7 [31, 32]. BMP7 delivered from nanospheres-scaffolds induced significant ectopic bone formation while passive adsorption of the protein into the scaffold resulted in failure of bone induction either due to the loss of protein bioactivity or its rapid release from the scaffolds upon implantation *in vivo*.

We have to bear in mind that not only material properties themselves, but also other factors have an great impact when studying osteoinduction: for instance the animal model and implantation site. Yang and co-workers tested the performance of sintered BCP ceramics in five different animal models at heterotopic locations in a single study. Until day 120, in rats, rabbits and goats, only dense fibrous connective tissue encapsulating the ceramics and loose connective tissue inside the pores were observed – without signs of bone formation. However, in dogs and pigs, bone formation was found in implants retrieved as early as 45 days after implantation. Extensive amounts of bone were found at day 120 mainly in the pores of the materials implanted in pigs [12]. This study showed that larger animals yielded more bone than smaller ones, with exception of the goat where no bone formation was observed.

Several studies have also investigated the osteoinductive capacity of a material, depending on the implantation site. No obvious bone formation was found after four months of subcutaneous implantation of a BCP ceramic in goats, whereas intramuscularly, bone was induced in seven out of ten implants in the same animals [33]. These studies suggest that at intramuscular locations, bone formation occurs more frequently – or at least at a higher rate. In this thesis, although we used the more challenging animal model – the rat and the more challenging implantation sites – subcutaneous implantation, we still gained satisfactory

bone regeneration using our materials – BMP2-cop.BioCaP (chapter 2) and BMP2-BioCaP (chapter 3). This supports our hypothesis on the osteoinductivity of our materials.

Although these models were chosen in such a way that they resembled the clinical situation as closely as possible, only clinical trials will be able to provide the proof for the relevance of osteoinductivity in human patients. Although ectopic bone formation in animals is used to judge if a material is osteoinductive material, we need to further consider the influence of the animal model and implantation sites when studying osteoinductive materials

To repair bone defects with local infection, it is necessary to produce bone-filling-materials with both osteoinductivity and antibacterial properties. The most common way is to encapsulate both osteoinductive agents and antibiotics into carrying materials. Mostly, both the encapsulated osteoinductive agent and antibiotic are released simultaneously and are largely dependent on the permeability and the degradability of the carrying materials. Calcium sulfate, a widely used bone-defect-filling material, is also frequently adopted as an antibiotic carrier for the treatment of infected bone defects [34]. It has many advantages such as low price, a high level of biodegradability, good biocompatibility [35] and high osteoconductivity. Wang et al. used calcium sulfate to carry BMP2 and vancomycin using internal co-encapsulation [36]. In a bone defect in the proximal tibia, this material significantly augmented new bone formation compared to the control [36]. Besides the advantages of calcium sulfate, we should also bear in mind is that this kind of material usually forms a solid block and lacks of porous structure, which may hinder the ingrowth of bone tissues.

Compared to simultaneous release of osteoinductive agents and antibiotics, it is more preferable to deliver the osteoinductive and antibacterial drugs from the bone-filling materials according to the aim of the clinical application and optimal delivery mode of each drug. For example, an antibacterial drug is encapsulated into a carrying material while in the same system an osteoinductive drug superficially adsorbed onto its surface, or vice versa. The two carrying modes can realize different aims: the former mode is mainly aimed for promoting bone regeneration with a prevention of potential infection, while the latter mode is mainly aimed for suppressing an existing bacterial activity and thereafter promoting bone regeneration. Most of the current studies with a mixed carrying mode for BMP2 and antibacterial drugs focused on bone regeneration with the prevention of potential infection. Song et al. developed a pHEMA [(poly(2-hydroxyethyl methacrylate))/nHA (nanocrystalline hydroxyapatite) composite [37]. In this composite, nHA was added to enhance the osteoconductivity of the composite. The encapsulated vancomycin was released in a sustained manner over 2 weeks, which could significantly inhibit the growth of *Escherichia coli*. The BMP2 preabsorbed onto the pHEMA-nHA-vancomycin composite was continuously released over 8 days, which induced osteogenic differentiation of C2C12 cells [38]. In critical rat femoral segmental

defects, the authors showed that the pHEMA-nHA-vancomycin-BMP2 composites could achieve full bridging with substantially mineralized callus and partial restoration of torsional strength [39]. On the other hand, as abovementioned, the superficially adsorption is less favorable for the osteoinductive efficiency of BMP2. In another study, the authors tried to modify the carrying material to slow down the release of the superficially adsorbed BMP2. Zhou et al. used zein, a major starch storage protein found in corn, as a carrying material for antibacterial HACC and BMP2 [40]. 10% HACC was encapsulated into zein, which showed a strong antibacterial effect without significantly compromising cell proliferation. Different amounts of mesoporous silica SBA-15 nanoparticles were added into zein in order to provide large and highly ordered pores and uniform tunable channels [41]. The release of the superficially adsorbed BMP2 was significantly slowed down with the higher ratio of mesoporous silica SBA-15 nanoparticles. In a radial bone defect model (20 mm in length and 5 mm in diameter) in rabbits, zein-HACC-S20-BMP2 composite almost fully repaired the bone marrow cavity after 12 weeks. The authors concluded that Silica/HACC/zein scaffolds with both antibacterial and osteoinductive activities had an immense potential in orthopedics and other biomedical applications [42].

Unlike the mode mentioned above, in chapter 3 we designed the BMP2-BioCaP/HACC complex with an osteoinductive drug encapsulated into a carrying material and an antibacterial drug superficially adsorbed onto its surface, aiming to suppress existing bacterial activity and thereafter promoting bone regeneration. HACC, a strong antibacterial drug was adsorbed on the BMP2-BioCaP granules so as to achieve a burst release, which was designed to rapidly kill residual bacteria in infected bone defects without resulting in bacterial resistance. On the other hand, to function as an effective osteoinductive agent, BMP2 needs to be released slowly and continuously at a low concentration [23]. Based on this theory, we developed BMP2-BioCaP granules [43] where BMP2 was internally incorporated into the BioCaP granules. BMP2 was therefore released slowly and continuously with the undergoing degradation of BioCaP granules *in vivo*. The bone formation observed in the BMP2-BioCaP/HACC complex also supports the optimal delivery mode of BMP2. We therefore conclude that BMP2-BioCaP/HACC complex is a sequential release system with a burst release of a powerful antibacterial agent-Hydroxypropyltrimethylammonium chloride chitosan (HACC) followed by a controlled release of BMP2. This release system is in line with the optimal delivery mode of both HACC and BMP2, which makes BMP2-BioCaP/HACC complex be able to rapidly kill residual bacteria and thereafter induce new bone formation so as to repair infected bone defects.

Limitations and future perspective

At this point the question remains on how to progress growth factor-based bone tissue engineering strategies into widespread adoption in clinical practice. For this to happen, a number of limitations and further investigations need to be considered. Firstly, it is important to bear in mind that our osteoinductive biomaterials (BMP2-cop.BioCaP and BMP2-BioCaP) are available in the shape of granules. They can induce satisfactory bone formation in defects with intact bony walls. However it is very difficult for them to induce bone formation in an environment without supporting bony walls, such as alveolar ridge in need of augmentation or bone nonunion. Their clinical application in bone tissue engineering is therefore limited and further effort is needed to produce defect-matching scaffolds, for example by applying 3-dimensional printing technique.

Secondly, although bone formation in an ectopic model, such as subcutaneous pockets in rats, is considered as golden standard to confirm the osteoinductivity of a biomaterial, it fails to exam if the biomaterial can functionally repair bone defects. In other words, we also need bone-defect models to evaluate if the repaired bone tissue can perform the original function.

Thirdly, because the pathogenesis of infections in infected bone defects is a complex process and involves interactions between the pathogen, biomaterial, and host, the *in-vitro* assays do not account for host defense and some other *in-vivo* factors. In our ongoing studies, *in-vivo* infected-bone-defect models will be utilized to evaluate the antibacterial efficacy of BMP2-BioCaP/HACC complex.

Conclusion

In summary, it is clear that current strategies discussed in this thesis are less than ideal, but they are useful explorations in the field of bone tissue engineering. Based on the discoveries in this thesis, we have not only favorable preclinical outcomes for our osteoinducer, the osteoinductive and antibacterial biomaterials, but also a reliable radiological method — CBCT to clinically evaluate bone regeneration. It builds a solid foundation for the translation of our osteoinductive and antibacterial biomaterials into clinical practice.

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