CHAPTER 6

General Discussion
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Bone is a highly dynamic tissue responsible for the mechanical support of soft tissues and muscle, body shape and movement. Bone undergoes a constant remodeling process, meaning that bone tissue is continuously renewed in order to adapt to different mechanical forces and to repair fatigue-related injuries (1). Bone tissue has a remarkable regeneration potential. Under normal physiological conditions and when the bone injury is small, bone can heal by itself without scar tissue formation. However, injuries with a significant bone loss, where the distance between the ends of the fractured bone is large, and where there is low mechanical stability, the bone healing process is compromised leading to delayed unions, non-unions, and large bone defects (2).

Large bone defects in the skull can result from trauma, infection, tumour resection, and congenital disorders. Cranioplasty is usually performed to restore the protective function of the skull, with a secondary goal of restoring cranial aesthetics. Autologous bone graft is still the gold standard for the treatment of large bone defects and it is mostly harvested from the iliac crest. However, the harvesting procedure has some complications including haemorrhage, nerve and vascular lesions, and prolonged or chronic post-operative pain. In addition, limited amount and quality of bone that can be harvested restricts its use in large bone defects. Disease transmission as well as the viability of the graft and material rejection are the major limitations of the use of allografts and xenografts, other types of bone grafts used to treat large bone defects. Moreover, bone substitutes such as titanium implants, polyether-ether-ketone (PEEK), and polymethyl methacrylate (PMMA) are used for the treatment of large bone defects.

PMMA is the most widely used alloplastic material due to its high mechanical strength, stability, and relatively low cost (3). Although the use of PMMA in large cranial defects has positive outcomes, the potential release of non-reacted toxic monomers and cytotoxic issues related to the use of PMMA are still a matter of debate. Moreover, PMMA has been reported to increase bone resorption leading to the aseptic loosening of a PMMA implant. PMMA biomaterial implanted into a defect in the skull will be surrounded by osteoblasts present within the bone tissue. In addition, mesenchymal stem cells (MSCs) will be recruited at the implant site following PMMA implantation. Thus, MSCs and osteoblasts are highly important for the in vitro evaluation of possible cytotoxic effects of PMMA. Better understanding about the possible adverse effects of implanted PMMA and its contact with bone tissue is highly relevant for the clinical outcome as well as for the patient. Therefore, in chapter 2 we investigated whether PMMA affects the
osteogenic differentiation potential of human adipose stem cells (hASC) and/or human osteoblasts (hOBs), mimicking PMMA-cell contact when PMMA is implanted into a bone defect. In addition, we investigated whether PMMA enhances the production of osteoclast factors by hASCs and/or hOBs. We found that PMMA is not cytotoxic, and does not interfere with the osteogenic differentiation potential of hASCs and hOBs, and that PMMA does not enhance production of osteoclast regulatory markers by hASCs and hOBs in vitro. We demonstrated that PMMA does not inhibit bone formation and that activation of osteoclasts by production of osteoclast-related factors due to PMMA is unlikely. These results support the notion that PMMA is highly suitable to treat patients with cranial bone defects.

Despite the availability of different bone substitutes and the development of new orthopaedic devices, the treatment of large bone defects including cranial defects is still a challenge for orthopedic and maxillofacial surgeons (4, 5). Bone tissue engineering using the combination of MSCs seeded on osteoconductive scaffolds with osteoinductive growth factors represents a promising strategy for the treatment of large bone defects (6). The combination of MSCs, inductive factors, and scaffold form a bio-active bone construct, which once implanted into a defect may enhance and contribute to the repair of bone. Different bone progenitor cell types are currently being used for bone tissue regeneration purposes, such as embryonic stem cells, human umbilical vein endothelial cells, induced pluripotent stem cells (iPSCs), adult stem cells (bone marrow stromal cells, bone marrow-derived MSCs, ASCs, muscle-derived MSCs), and dental pluripotent stem cells (7-10). ASCs are a promising source for bone tissue engineering due to their abundance, accessibility, and osteogenic differentiation potential (11, 12).

The physiological process of bone repair is highly complex. Bone repair occurs through two different mechanisms: direct and indirect fracture healing. Direct or primary fracture healing occurs through intramembranous bone formation by direct transformation of mesenchymal cells into osteoblasts resulting in the development of flat bones of the skull including the cranial suture lines. In addition, bone fractures treated by rigid fixation are characterized by low interfragmentary movement that can heal by primary fracture healing (2). Indirect or secondary fracture healing consists of both endochondral an intramembranous bone healing (13). Endochondral bone formation involves the differentiation of mesenchymal progenitor cells into chondrocytes, which deposit hyaline cartilage that is later mineralized and replaced by bone. Endochondral bone formation occurs during development of long bones as well as in fractures treated for example by cast immobilization where rigidly stable conditions are not required.
Instead, healing of bone is enhanced by a certain amount of motion and mechanical loading (2, 14).

Secondary fracture healing is the most common process that occurs after bone fracture, and is the most studied process to understand the regenerative mechanism of bone (2, 15). However, knowledge about the phases that occur during bone repair are limited and based on studies performed only on large bones which does not fully represent the healing process of other bones such as the skull. Increased knowledge about the cellular and molecular mechanism that occur during bone repair, especially in the skull, may lead to the development of successful tissue engineering strategies to treat critical size cranial defects. Secondary fracture healing is characterized by hematoma formation followed by an inflammatory response where different immune cells are attracted to the injury site, and where different cytokines are released. Thus, immune cells as well as cytokines are relevant as they trigger the initiation of bone repair.

Pro- and anti-inflammatory cytokines released after bone fracture are key factors present at the start of the process of fracture healing. Cytokines such as tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), interleukin-6 (IL-6), and interleukin-17F (IL-17F) are present during the inflammatory response during fracture healing (16-20). They are also likely to be released after the implantation of a bone tissue engineered construct into a bone defect (21). For instance TNF-α is expressed in the first 24-72 h after bone fracture. TNF-α is chemotactic, thereby recruiting cells necessary for bone regeneration (21). In addition, local administration of a low dose of TNF at the fracture site shortly after injury promotes fracture healing by up-regulating the innate immune response (22, 23). Thus, TNF-α may be beneficial for bone repair. IL-6 is known to be released in the first 72 hours after bone fracture, and to rapidly decline thereafter (17, 24). IL-6 is secreted by multiple cell types such as osteoblasts, and stimulates osteoclast formation and bone resorption, thereby playing a role in bone homeostasis (25, 26). However, the exact role of IL-6 during fracture healing is still unknown, even though IL-6 knockout mouse studies indicate that lack of IL-6 delays bone healing (27). IL-17F, a cytokine secreted by T-helper cell 17 (Th17), has been shown to be expressed during the early phase of fracture healing, i.e. 3 days post-fracture. Moreover, IL-17F has been shown to stimulate osteoblast maturation in vitro and to strongly induce osteogenic differentiation of MSCs (20, 28). The T helper type 2 (Th2) cytokine IL-4 is also present during fracture healing (18), and is considered anti-inflammatory as it inhibits the production of IL-1, TNF-α, and prostaglandin E₂ (PGE₂) by monocytes (29). IL-4 also inhibits bone resorption (30), and is a chemoattractant for osteoblasts (31). In chapter 3, the effects of the pro-inflammatory cytokines TNF-α, IL-6, IL-8, and IL-17F, and the anti-inflammatory cytokine...
IL-4 on proliferation and osteogenic differentiation of hASCs were studied. The cytokines were added individually to hASCs during 72 h mimicking the early stage of the physiological process of bone repair. We found that hASCs respond to the different cytokines by changes in osteogenic differentiation. Each cytokine analyzed had a specific effect in a specific time frame. The stimulatory effect of IL-6 on ALP activity and mineralization in hASCs suggest that this cytokine may enhance osteogenic differentiation of MSCs (32). IL-4 showed inhibitory effects on osteogenic differentiation observed by decreased bone nodule formation. Thus, IL-6 but not IL-4 may be suitable to induce osteogenic differentiation of MSCs as a strategy for enhancing bone repair.

In Chapter 3, we described the effect of pro and anti-inflammatory cytokines which are known to be released during the inflammatory response of bone repair. However, cytokines may also act through their effect on immune cells thereby affecting MSCs behavior during bone healing. Inflammatory cells such as neutrophils and macrophages migrate to the fracture site within the first 24 h after bone fracture. Neutrophils are attracted by platelet-derived factors as well as by different signals released from the damaged tissue. Neutrophils release different cytokines such as IL-1β, TNF-α, IL-6, IL-10, monocyte chemotactic protein-1 (MCP1) and CXCL1 to attract monocytes to the injury site (33, 34). Neutrophils at the bone injury site may contribute to bone repair by forming extracellular matrix (ECM) needed for bone synthesis. In addition, the role of macrophages in the bone fracture may be dependent on how these cells have been activated in response to certain signal (polarization). Macrophages are categorized as M1 which produce IL-1, IL-6, TNF-α to maintain the inflammatory environment, and M2 macrophages which initiate an anti-inflammatory response, and secrete IL-10 and VEGF to recruit MSCs and promote angiogenesis (35). Thus, the role of immune cells present during bone injury should also be taken into account when studying mechanisms of bone repair for tissue engineering approaches.

To study the importance of cytokines for tissue engineering purposes, experiments have to be performed by using a combination of cytokines, and not individually, since cytokine-signaling pathways in different cell types, a.o. MSCs, are not direct, but rather may have indirect effects by modulating other cytokines. For example, it is unlikely to find strong direct effects on proliferation and/or osteogenic differentiation of ASCs by using IL-6 alone. Thus, one could erroneously conclude that IL-6 is not important when using ASCs. However, IL-6 receptor is crucial to mediate IL-6 signaling in ASCs (32,36).

Hematoma formation at the fracture site occurs by the disruption of blood vessels, and it is characterized by low oxygen concentration. Thus, hypoxia is a another key event in the first
stages of fracture healing (37, 38). It is also present after the implantation of a bone tissue engineered construct in vivo, where an inflammatory response takes place (39-41). The stimulatory or inhibitory effect of the combination of pro- and anti-inflammatory cytokines in a hypoxic environment must be taken into account when designing a bone tissue engineered construct, since 1) cytokines as well as hypoxia are hallmarks of the early stages of fracture healing, 2) both cytokines and hypoxia will likely be present in any implanted tissue-engineered construct in vivo, and 3) cytokines and hypoxia interact at the level of signal transduction. Under hypoxia mTOR is inactivated, which may occur as part of the cell program to maintain energy homeostasis (42). In addition, both the osteogenic effect of IL-6 on human BMSCs and the anti-osteogenic effect of IL-4 on BMMSCs have been ascribed to mTORC1 activation (43, 44). In chapter 4, some aspects of the initial phase of fracture repair, i.e. cytokines and hypoxia, were investigated to better predict cytokine modulation of MSC-aided bone healing for bone tissue engineering purposes to treat large bone defects. For this, the effect of IL-4, IL-6, and their combination on osteogenic differentiation, angiogenic stimulation potential, and mTORC1 activation by hASCs were studied under normoxic and hypoxic culture conditions. IL-4 alone, but not in combination with IL-6, reduced osteogenic differentiation and angiogenic stimulation potential of hASCs under normoxia and hypoxia, indicating that this cytokine may inhibit bone healing and regeneration. However, the effects of IL-4 were mitigated in the presence of IL-6. The inhibitory effect of IL-4 is likely to occur through a pathway different to mTOR. Thus, for a better understanding of bone healing it is important to move towards more complex in vitro systems, taking into account factors such as oxygen tension and combinations of cytokines.

The successful translation of a bone tissue engineered construct into clinical applications is still limited. We propose that the limited success of a bone tissue engineered construct is due to a lack of understanding of the role of the inflammatory response in tissue regeneration, and the lack of proper research with in vitro models that resemble the in vivo microenvironment of bone healing and formation. A bone tissue engineered construct will have to withstand the effect of cytokines and hypoxia present in the inflammatory microenvironment, once the construct is placed into a bone defect (40). In addition, MSCs incorporated into a scaffold will interact, and the scaffold substrate may play a role by modulating the response of MSCs towards cytokines and hypoxia. Scaffolds are a key component of a bone tissue engineered construct as they can mimic the in vivo extracellular matrix, and affect MSCs behavior such as cell growth, proliferation and osteogenic differentiation. In addition, the substrate may interact with MSCs influencing their secretome and osteogenic differentiation potential (45). Therefore, in Chapter 5 we investigated
the direct effect of a 3-day stimulation period with a cocktail of cytokines, i.e. TNF-α, IL-4, IL-6, and IL-17F, on osteogenic differentiation and VEGF expression in hASC cultured on BCP scaffolds under hypoxia. In this chapter we also evaluate the results of studies with hASCs cultured on BCP as well as the effects of cytokines on hASCs cultured on tissue culture plastic (standard two-dimensional (2D) cell culture conditions). Our results showed that a cytokine cocktail combined with hypoxia affects MSC behavior, and the effect is substrate-dependent. This implicates that cytokines combined with hypoxia do affect the results of screening of novel biomaterials for bone tissue engineering.

We conclude, that PMMA, a commonly used material for the treatment of large cranial defects, is not cytotoxic and has not adverse effects on hASCs and osteoblasts. Thus, our studies support the concept that PMMA is suitable to treat patients with large cranial defects. In this thesis, in vitro studies were performed using culture conditions mimicking the in vivo environment of fracture repair. Pro and anti-inflammatory cytokines, used individually, have both enhancing and reducing effects on osteogenic differentiation of hASCs, when applied for 72 h. IL-6 may be suitable to induce osteogenic differentiation of MSCs as a strategy for enhancing bone repair. IL-4 inhibited osteogenic differentiation and angiogenic stimulation potential of hASCs, which is likely to be mediated by a pathway different from mTOR. Therefore, IL-4 alone might be deleterious for bone healing and regeneration. However, the inhibitory effects of IL-4 are mitigated in the presence of IL-6, underlying the importance of the presence of IL-6 during bone repair. In addition, we show that using cytokines in combination affects MSC behavior in an hypoxic environment, and that the effect is substrate-dependent. The outcome of these and future studies will have implications for bone tissue engineering strategies in the field of orthopedics and craniomaxillofacial surgery. Our data provide new insights in the mechanisms involving MSC-mediated bone tissue repair, and how mimicking some aspects of the inflammatory microenvironment of bone repair, i.e. cytokines, hypoxia, and a 3D-culture system, may lead to more approachable tissue engineering models for the treatment of large bone defects. Implementation of developmental biology insights will impact future tissue engineering approaches. Future strategies may include the target of appropriate cytokines that enhance the osteogenic differentiation potential of MSCs mediating early bone repair, as well as angiogenesis.

Bone regeneration is a highly complex process, therefore further research in the role of cytokines, hypoxia, biomaterials, and immune cells present within the bone microenvironment after the implantation of a bone construct, is needed. These studies and others to follow will help
to reveal the most critical factors involved during bone repair and formation, and thereby leading to the development of successful bone tissue engineered constructs.
REFERENCES


