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Bioengineering application using co-cultured mesenchymal stem cells and preosteoclasts may effectively accelerate fracture healing

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A B S T R A C T

Fracture non-union is the most challenging complication following fracture injuries. Despite ongoing improvements in the surgical technique and implant design, the treatment efficacy of fracture non-union is still far from satisfactory and currently there is no optimal solution. Of all of the methods used for the treatment of non-union, bone tissue bioengineering using scaffolds and mesenchymal stem cells (MSCs) is the most widely studied and has emerged as a promising approach to address these challenges. However, there are several critical limitations, such as the low survival rate of MSCs under an inflammatory, ischemic environment. Accumulating studies have demonstrated that preosteoclasts not only play a role in the remodeling of the callus, but also participate in the entire process of fracture repair. The close crosstalk between preosteoclasts and MSCs stimulates the recruitment, proliferation, and differentiation of osteoblasts and improves the osteogenic differentiation of MSCs. With no *in vivo* study reported thus far, we hypothesize that the administration of preosteoclasts together with MSCs at a certain ratio may effectively accelerate fracture healing and provide a new and promising therapeutic strategy for the clinical management of fracture non-union.

Introduction

Current situation of fracture non-union

Fractures are among the most common disorders worldwide and are associated with a heavy healthcare and societal burden [1,2]. Among the complications following the treatment of fractures, fracture non-union is the most challenging, with a reported incidence of 5–30% of long bone fractures and even more in cases of compromised soft tissue conditions and comorbidities, such as diabetes [3,4]. Fracture non-union impairs not only a patient's psychological health and quality of life but also their financial situation. Based on previous studies, the cost of non-union treatment is almost twice that for normal fracture healing, up to US$25,000 per patient [5]. The current "gold standard" treatment for patients suffering from fracture non-union is to perform bone grafting using either an allograft or autograft. However, there are several important drawbacks of bone grafting, which include limited bone volume and morbidity at the harvest site, including local hematoma and infection [6]. Despite ongoing improvements in surgical technique and implant design, the treatment efficacy of fracture non-union is still far from satisfactory and currently there is no optimal solution [1,3,4,7]. Thus, there remains an urgent need to develop new methods to accelerate bone healing.

Bioengineering using scaffolds and mesenchymal stem cells (MSCs) in managing fracture non-union

Of all of the methods used to treat non-union, bone tissue bioengineering using scaffolds and MSCs is the most widely studied and has emerged as a promising approach to address these challenges [8–10]. MSCs have the potential to differentiate into a variety of mature cell types, including osteocytes, chondrocytes, and adipocytes, and play a key role during fracture healing. Accumulating studies have demonstrated that MSCs promote new bone formation and initiate fracture healing and have clinical potency in the management of fracture non-union [11,12]. The bioengineering scaffolds are also reported to mimic the extracellular matrix and promote bone regeneration during fracture healing. Indeed, several scaffolds have been used in bone tissue engineering research and pre-clinical trials [13,14].

Using stem cells alone to repair a bone defect is not physiological. Several critical limitations remain for current bioengineering techniques using scaffolds and MSCs. First, due to the inflammatory, ischemic environment with oxidative stress at the injury site, it is difficult for implanted MSCs to survive. Toma et al. reported that less than 0.44% of MSCs survive by day 4 after engraftment, which severely limits their therapeutic effect [15]. Second, although this technique has shown some promise, how to enhance the osteogenic differentiation of the

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implanted MSCs remains largely unsolved. Several studies have attempted to solve this problem using a combination of osteogenic growth factors such as bone morphogenic protein 2 (BMP2) [16]. However, the high cost of commercially available growth factors limits their applications [14]. Second, a large number of cells, such as osteoclasts, are involved in fracture healing. Using only MSCs and scaffolds cannot mimic the microenvironment in the fracture area [17].

The origin and identification of preosteoclasts

Osteoclasts are large, multinucleated, tartrate resistant acid phosphatase positive (TRAP +) cells that gradually differentiate from hematopoietic precursors cells (TRAP + mononuclear cells), specifically those from the monocyte-macrophage lineage. Under the influence of the bone-associated environment, particularly the stimulation of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL), monocytes differentiate into macrophages, preosteoclasts, and finally mature osteoclasts that are capable of resorbing bone [18–20]. To harvest preosteoclasts, pure monocytes/macrophages from bone marrow or peripheral blood should be cultured with M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 3 days. To identify preosteoclasts, TRAP activities of the cultured preosteoclasts using a commercial kit should be detected [20].

Preosteoclasts and osteoclasts in fracture healing

Typical fracture healing consists of three interrelated phases: the inflammation phase, the callus formation phase, and the callus remodeling phase. Preosteoclasts and osteoclasts are abundant in the fracture callus and callus chondro-osseous junction during endochondral ossification. The role of osteoclasts in fracture healing has generally been believed to be limited primarily to the callus remodeling phase, during which mature woven bone and underlying cartilage matrix are removed by osteoclasts, eventually recreating the typical bone structure and the Haval system based on mechanical stress applied to bone [21–24]. However, several studies have demonstrated that preosteoclasts not only play a role in callus remodeling but also participate in the entire process of fracture repair.

A recent study showed that sphingosine-1-phosphate secreted by osteoclasts promotes osteoblast activity and bone formation in mice [25]. Osteoclasts also produce BMP6, platelet-derived growth factor-BB (PDGF-BB), and Wnt10b *in vitro*, which stimulates the recruitment, proliferation, and differentiation of osteoblasts [26,27]. Ota et al. demonstrated that transforming growth factor beta 1 (TGF-β1) promotes osteoblast migration by increasing the expression of chemokine (C-X-C motif) ligand 16 (CXCL16) and leukemia inhibitory factor in osteoclasts [28]. Tartrate-resistant acid phosphatase (TRACP), a bone morphogenetic protein synthesized by osteoclasts, increases the expression alkaline phosphatase (ALP) by osteoblasts and is a pathway in anabolic bone remodeling [29]. According to Walia et al., *Ctsk* (*cathepsin K*)-deletion mice use a positive feedback loop that increases the number of osteoclasts, resulting in an increase in bone formation [23]. Osteoclasts also play an important role in vascularization. Takeshita et al. found that CTHRC1 (collagen triple helix repeat-containing 1), a protein secreted by osteoclasts, targets stromal cells to stimulate osteogenesis [30]. Xie et al. showed that preosteoclasts enhance angiogenesis during bone remodeling by releasing platelet-derived growth factor subunit B (PDGF-B) [20]. Meanwhile, Cackowski et al. showed that osteoclasts promote the secretion of matrix metalloproteinase 9 (MMP9) and promote vascularization to accelerate bone repair [31].

Preosteoclasts and osteoclasts also participate in immune regulation during fracture healing [32]. Immediately after fracture, the cascade of bone repair processes initiates a local immune response. Bone repair is affected in patients with immune disorders and in immune-impaired mouse models [33,34]. Kiesel et al. reported that osteoclasts cross-present antigens and form $CD8^+$ $FoxP3^+$ T regulatory (Treg) cells that have an immune-regulative function [35], consistent with another study that demonstrated that osteoclasts derived from normal bone mineral secrete immune-regulative cytokines (IL-10 and TGF-β) and change $CD4^+$ T cells to immunosuppressive $CD4^+$ Treg cells [36] and may play an important and positive regulatory role in the inflammatory microenvironment around the fracture site.

In summary, osteoclasts and preosteoclasts play vital roles in promoting osteoblast proliferation and differentiation, vascularization, and immune regulation, which theoretically increase bone formation and promote fracture healing.

Hypothesis

Successful fracture healing is based on coordinated cross-talk between MSCs and preosteoclasts. During the process of fracture healing, inflammation, callus formation, and callus remodeling phases partially overlap. Previous studies have suggested that seed cells used in the bioengineering management of fracture include stem cells, osteoblasts, and vascular endothelial cells. We believe that preosteoclasts, which naturally exist in the fracture area and play vital important roles, should also be considered potential seed cells. Indeed, Annamalai et al. performed indirect co-culture of BMP2-loaded microspheres and macrophages with isolated adipose-derived MSCs and demonstrated that macrophages with certain subtypes produce a strong osteogenic response, comparable to direct supplementation of the culture medium with BMP2 [37]. However, thus far, *in vivo* bioengineering applications using co-cultured MSCs and preosteoclast have not been studied. Having focused on the biological mechanism of fracture healing for years [38–43], we hypothesize that administration of preosteoclasts together with MSCs at a certain ratio at an early stage can increase the overlap of the three phases of fracture healing and further accelerate the process of bone regeneration, which may provide a new and promising therapeutic strategy for bone tissue engineering and the clinical management of fracture non-union.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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