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Investigation of angiogenesis in bioactive 3-dimensional poly (D,L-lactide-co-glycolide)/nano-hydroxyapatite scaffolds by *in vivo* multiphoton microscopy in murine calvarial critical bone defect

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ABSTRACT

Reconstruction of critical size bone defects remains a major clinical challenge because of poor bone regeneration, which is usually due to poor angiogenesis during repair. Satisfactory vascularization is a prerequisite for the survival of grafts and the integration of new tissue with existing tissue. In this work, we investigated angiogenesis in 3D scaffolds by *in vivo* multiphoton microscopy during bone formation in a murine calvarial critical bone defect model and evaluated bone regeneration 8 weeks post-implantation. The continuous release of bioactive lentiviral vectors (LV-*pdgfb*) from the scaffolds could be detected for 5 days *in vitro*. *In vivo*, the released LV-*pdgfb* transfected adjacent cells and expressed PDGF-BB, facilitating angiogenesis and enhancing bone regeneration. The expression of both *pdgfb* and the angiogenesis-related genes *vWF* and *VEGFR2* was significantly increased in the *pdgfb* gene-carrying scaffold (PHp) group. In addition, microCT scanning and histomorphology results proved that there was more new bone ingrowth in the PHp group than in the PLGA/nHA (PH) and control groups. MicroCT parameters, including BMD, BV/TV, Tb.Sp, and Tb.N indicated that there was significantly more new bone formation in the PHp group than in the other groups. With regard to neovascularization, 8 weeks post-implantation, blood vessel areas (BVAs) were 9428 ± 944 μm^2, 4090 ± 680.3 μm^2, and none in the PHp, PH, and control groups, respectively. At each time point, BVAs in the PHp scaffolds were significantly higher than in the PH scaffolds. To our knowledge, this is the first use of multiphoton microscopy in bone tissue-engineering to investigate angiogenesis in scaffolds *in vivo*. This method represents a valuable tool for investigating neovascularization in bone scaffolds to determine if a certain scaffold is beneficial to neovascularization. We also proved that delivery of the *pdgfb* gene alone can improve both angiogenesis and bone regeneration Acronyms.

Statement of Significance

Reconstruction of critical size bone defects remains a major clinical challenge because of poor bone regeneration, which is usually due to poor angiogenesis during repair. Satisfactory vascularization is a prerequisite for the survival of grafts and the integration of new tissue with existing tissue. In this work, we investigated angiogenesis in 3D scaffolds by *in vivo* multiphoton microscopy during bone formation in a murine calvarial critical bone defect model and evaluated bone regeneration 8 weeks post-implantation. To verify that *pdgfb*-expressing vectors carried by the scaffolds can promote angiogenesis

Abbreviations: 3D, 3-dimensional; LV, lentiviral vector; vWF, Von Willebrand factor; VEGFR2, vascular endothelial growth factor receptor 2; *pdgfb*, platelet-derived growth factor-b; PLGA/nHA, Poly(D,L-lactide-co-glycolide)/hydroxyapatite nanoparticle; PH, PLGA/nHA; PHp, PLGA/nHA/LV-*pdgfb*; BVAs, blood vessel areas; BMD, bone mineral density; BV/TV, the ratio of bone volume to tissue volume; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number; MPM, multiphoton microscopy; TPEF, two-photon excited fluorescence; SHG, second harmonic generation signal.

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in 3D-printed scaffolds *in vivo*, we monitored angiogenesis within the implants by multiphoton microscopy. To our knowledge, this is the first study to dynamically investigate angiogenesis in bone tissue engineering scaffolds *in vivo*.

1. Introduction

Bone is a dynamic and highly vascularized tissue with a unique capacity to heal and remodel continuously during the lifetime of an individual [1]. It is generally believed that spontaneous bone regeneration occurs in response to relatively small defects; however, large bone defects, caused by traumatic injury, tumor removal, or genetic disorders, have to be restored by various grafts, including autografts, allografts, and artificial grafts made from biomaterials [2–4]. Due to the problems of autografts, such as significant donor site morbidity, hemorrhage, deep infection, and chronic pain [5–7], and of allografts from human cadavers and donors, like risk of pathogen transmission and immunological rejection [8–10], artificial grafts are promising alternatives, which can be made from biodegradable materials, three-dimensionally (3D) printed, genetically modified, and biomechanically engineered [11–13].

Bone regeneration is a process that involves blood vessel formation and different types of cells and growth factors [14–16]. Angiogenesis in tissue-engineering scaffolds is essential for supplying cells with oxygen and nutrients, removing waste products, and ultimately functionalizing implanted scaffolds [17–19]. To enhance the osteoinductivity of scaffolds for bone regeneration, the scaffolds have been impregnated with various growth factors [15,20,21], such as bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), and fibroblast growth factors (FGFs). However, the short half-life and high cost of growth factors limit their clinical applications [22–24]. To tackle these drawbacks, alternative growth factor delivery strategies for sustained bioactivity have been developed, such as those involving genes modification [25,26].

Platelet-derived growth factor-BB (PDGF-BB) is a chemoattractant and mitogen for mesenchymal and osteogenic cells, which stimulates expression of angiogenic molecules, all of which play pivotal roles in bone healing [27–29]. PDGF-BB has been widely studied, both preclinically and clinically, and has been approved by the FDA for promoting bone regeneration, particularly in periodontal tissues [30–33]. Recent work showed that PDGF-BB promotes angiogenesis by stimulating endothelial cell migration and proliferation *in vivo* [34–36]. Therefore, inducing *de novo* formation of vasculature in scaffolds with PDGF-BB is one way to potentially enhance tissue growth in bone tissue engineering.

Release of transfection vectors able to express growth factor genes from scaffolds is a way to improve tissue regeneration [37–39]. Viral vectors are commonly used for this purpose because of their high gene delivery efficiency and stability [40,41]. Recent rapid advances in multiphoton microscopy (MPM) imaging techniques have made it possible to simultaneously visualize cells, extracellular matrix, and surrounding vascular networks *in vivo* [42,43]. There have been reports that osteoblasts and osteoclasts can be visualized in live bone by using intravital two-photon microscopy [44,45]. However, there are a few studies on the dynamics of angiogenesis in bone tissue-engineering scaffolds using intravital MPM due to strong tissue scattering and absorption of blood [46,47].

In this study, we designed porous PLGA/nHA 3D scaffolds containing *pdgfb*-expressing lentiviral vectors (PLGA/nHA/LV-*pdgfb*, PHp) to achieve better angiogenesis and bone regeneration in the repair of critical bone defects. PHp scaffolds released transfectable *pdgfb* expression vectors that caused PDGF-BB to be expressed and secreted from nearby transfected cells and promote the recruitment of endogenous stem cells and osteoprogenitors to the defect, enhancing angiogenesis and bone regeneration. To verify that *pdgfb*-expressing vectors carried by the scaffolds can promote angiogenesis in 3D-printed scaffolds *in vivo*, we monitored angiogenesis within the implants by multiphoton microscopy. To our knowledge, this is the first study to dynamically investigate angiogenesis in bone tissue engineering scaffolds *in vivo*.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide-co-glycolide) (PLGA) (L/G ratio 75:25, MW 66000-107000) and hydroxyapatite nanoparticles (nHAp; average diameter <200 nm) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 1,4-dioxane was purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Stericup filters were bought from Merck Millipore Corporation (Darmstadt, Germany). The PDGF-BB cDNA was purchased from Sino Biological Inc. (Beijing, China). anti-PDGF-BB mouse polyclonal antibody and PDGF-BB recombinant protein were obtained from BioVision, Inc. (San Francisco, USA). Unless specified, all cell culture reagents were obtained from Life Technology (NY, USA). The lentivirus vectors encoding GFP and Tomato were from Dr. Chiju Wei (Shantou University, China) and Dr. Sam S. Gambhir (Stanford University, USA), respectively.

2.2. Lentivirus (LV) production

The *pdgfb* cDNA was cloned into a lentiviral expression vector at a custom multiple-cloning site downstream of the cytomegalovirus promoter. Lentivirus was prepared in 293FT cells by transfection with virus packaging plasmids (pLP1, pLP2, and pVSV-G) using the standard calcium phosphate method with chloroquine (final concentration 25 μM) [48,49]. After 48 h of transfection, culture supernatants were harvested and filtered with a Millipore Stericup filter (0.45 μM). Viral particles were concentrated by ultracentrifugation (OPtima L-100XP, Beckman Coulter, America) for 2 h at 25,000 rpm, resuspended in phosphate buffered saline (PBS), aliquotted, and stored at −80 °C. To determine the titer of lentivirus, HEK293T cells were incubated with serially diluted lentivirus solution for 24 h, and the lentivirus titer was calculated as previously described [49].

2.3. Fabrication of 3D PLGA/nHA scaffolds and LV immobilization

Porous 3D PLGA/nHA scaffolds were fabricated by the rapid-prototype method [50]. PLGA was dissolved in 1,4-dioxane to create a homogeneous solution, and nHA powder was added to the solution to a PLGA:nHA ratio of 10:1 (w/w). The mixed solution was stirred vigorously using a magnetic stirrer until a uniform paste was formed. The paste was used to fabricate 3D scaffolds using a 3D low-temperature printer (Tissue Form II, China) with a computerized nozzle that deposited the paste layer-by-layer, bottom to top, according to a predesigned model. The scaffolds