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Delivery of dimethyloxallyl glycine in mesoporous bioactive glass scaffolds to improve angiogenesis and osteogenesis of human bone marrow stromal cells

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ABSTRACT

Development of hypoxia-mimicking bone tissue engineering scaffolds is of great importance in stimulating angiogenesis for bone regeneration. Dimethyloxallyl glycine (DMOG) is a cell-permeable, competitive inhibitor of hypoxia-inducible factor prolyl hydroxylase (HIF-PH), which can stabilize hypoxia-inducible factor 1α (HIF-1α) expression. The aim of this study was to develop hypoxia-mimicking scaffolds by delivering DMOG in mesoporous bioactive glass (MBG) scaffolds and to investigate whether the delivery of DMOG could induce a hypoxic microenvironment for human bone marrow stromal cells (hBMSC). MBG scaffolds with varied mesoporous structures (e.g. surface area and mesopore volume) were prepared by controlling the contents of mesopore-template agent. The composition, large-pore microstructure and mesoporous properties of MBG scaffolds were characterized. The effect of mesoporous properties on the loading and release of DMOG in MBG scaffolds was investigated. The effects of DMOG delivery on the cell morphology, cell viability, HIF-1α stabilization, vascular endothelial growth factor (VEGF) secretion and bone-related gene expression (alkaline phosphatase, ALP; osteocalcin, OCN; and osteopontin, OPN) of hBMSC in MBG scaffolds were systematically investigated. The results showed that the loading and release of DMOG in MBG scaffolds can be efficiently controlled by regulating their mesoporous properties via the addition of different contents of mesopore-template agent. DMOG delivery in MBG scaffolds had no cytotoxic effect on the viability of hBMSC. DMOG delivery significantly induced HIF-1α stabilization, VEGF secretion and bone-related gene expression of hBMSC in MBG scaffolds in which DMOG counteracted the effect of HIF-PH and stabilized HIF-1α expression under normoxic condition. Furthermore, it was found that MBG scaffolds with slow DMOG release significantly enhanced the expression of bone-related genes more than those with instant DMOG release. The results suggest that the controllable delivery of DMOG in MBG scaffolds can mimic a hypoxic microenvironment, which not only improves the angiogenic capacity of hBMSC, but also enhances their osteogenic differentiation.

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1. Introduction

Bone tissue engineering consists of developing bioactive scaffolds to support osteogenic cells and applying bioactive molecules for bone reconstruction [1–4]. One of the concerns in performing bone tissue engineering is that the nutrient supply and cell viability at the centre of the scaffolds are often severely hampered, since the diffusion distance of nutrients and oxygen for cell survival is within 150–200 μm [5]. Furthermore, studies have shown that <5 mm of tissue layers can be formed on the scaffold surface using the tissue engineering approach, and that cell density in the centre of the scaffold tends to be low, and necrosis may occur [5]. To solve these issues, growth factors such as bone morphogenetic proteins (BMP-2 and BMP-7) and vascular endothelial growth factor (VEGF) were loaded in the scaffolds to stimulate cell differentiation and tissue growth [6–9]. However, BMP and VEGF are expensive and must be subjected to very mild conditions to prevent loss of bioactivity. In addition, how to efficiently deliver growth factors in bioactive scaffolds has been another issue. Polymer scaffolds, such as polylactic acid (PLA) and polycaprolactone (PCL) scaffolds have been used for growth factor loading with controllable release kinetics [10–12], but it is known that these polymers have inherent disadvantages as bone repair materials because of their inadequate osteoconductivity. Therefore, PLA and PCL scaffolds are often prepared in the form of composites with bioactive inorganic materials

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in most cases [13,14]. Furthermore, the organic solvents involved in the process of polymer scaffold preparation significantly affect the bioactivity of growth factors. However, traditional bioactive inorganic scaffolds (e.g. hydroxyapatite, β-tricalcium phosphate and 45S5 bioglass®) may lack sufficient nanopore structures to deliver growth factors in a controllable way after being sintered at high temperatures [15].

Recently, mesoporous bioactive glass (MBG) has aroused intense interest in bone tissue engineering [16–21]. Compared with non-mesopore bioactive glass (NBG), MBG has significantly improved specific surface area and nanopore volume, leading to enhanced *in vitro* bioactivity and shortened degradation time [16,22–24]. MBG has great potential for bone tissue engineering and drug delivery applications because of its superior bioactivity and well-ordered mesoporous structure [25–30]. However, MBG scaffolds have a limited mesopore size of <10 nm. It is known that growth factors such as BMP and VEGF have a complex molecular shape and relatively large size (molecular weight >5 kDa). It is difficult to completely load BMP and VEGF into the mesopores of MBG scaffolds. To overcome these drawbacks, the delivery of smaller molecules in MBG scaffolds may be an alternative method of stimulating angiogenic/osteogenic differentiation and further promoting bone growth.

Dimethyloxallyl glycine (DMOG) is a small molecular drug, which is known as a cell-permeable, competitive inhibitor of hypoxia-inducible factor prolyl hydroxylase (HIF-PH). HIF-PH hydroxylates a specific proline residue in HIF-1α at normal oxygen tensions. This reaction promotes binding of HIF to the Von Hippel–Lindau tumor suppressor (pVHL), leading to the degradation of HIF. DMOG is reported to counteract the effect of HIF-PH and stabilize HIF-1α expression under normoxic conditions [31]. HIF-1α is a key transcriptional regulator of vasculogenesis and angiogenesis. DMOG is therefore expected to induce a hypoxic microenvironment and act as a pro-angiogenic compound [32,33]. It is of great interest to deliver DMOG in the scaffold system to stabilize HIF-1α, which may combine the inherent bioactivity with the improved angiogenic property of MBG scaffolds for better bone regeneration. To the present authors' knowledge, there is no report on DMOG delivery in biomaterials, particularly in the tissue engineering scaffolds. Furthermore, the potential effect of DMOG on the biological response of cells is yet to be elucidated. Therefore, the aim of the present study is to investigate the influence of mesoporous structure on the delivery of DMOG, and explore the effect of DMOG delivery on angiogenesis and osteogenesis for hBMSC in the scaffold system.

2. Materials and methods

2.1. Preparation and characterization of porous MBG scaffolds

Porous MBG scaffolds (molar composition: 80Si–15Ca–5P) with varied mesoporous properties were prepared using co-templates of non-ionic block polymer P123 (EO_{20}–PO_{70}–EO_{20}) and polyurethane sponges by controlling the contents of P123. P123 is used to produce mesoporous structures (mesopore size, several nanometers), and polyurethane sponges are used to create large pores (large pore size, several hundred micrometers) as described in previous publications [25,34]. Briefly, to prepare MBG scaffolds with varied mesoporous properties, various contents (0, 2, 8, 10, 12 and 14 g) of P123 (Mw = 5800, Aldrich), 20.1 g of tetraethyl orthosilicate (TEOS, 98%), 4.2 g of $Ca(NO_3)_2{\cdot}4H_2O$, 2.19 g of triethyl phosphate (TEP, 99.8%) and 3 g of 0.5 M HCl were dissolved in 180 g of ethanol and stirred at room temperature for 24 h. The polyurethane sponges (25 pores per inch, ppi) were cleaned with ddH_2O, dried and completely immersed in this solution for 10 min, then transferred to a petri dish to allow evaporation at room temperature for 24 h. This procedure was repeated three times. Once the samples were completely dry, they were calcined at 650 °C for 5 h to obtain MBG scaffolds with varied mesoporous properties. The MBG scaffolds were named 0g-P123@MBG, 2g-P123@MBG, 8g-P123@MBG, 10g-P123@MBG, 12g-P123@MBG and 14g-P123@MBG, as shown in Table 1. Since there is excessive P123 in 14g-P123@MBG, which cannot be completely removed, the prepared 14g-P123@MBG scaffolds are black. Therefore, 14g-P123@MBG scaffolds were not used for further analysis.

The large-pore structure, phase composition and inner mesoporous microstructure of the prepared MBG scaffolds were characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and X-ray diffraction (XRD). Brunauer–Emmett–Teller and Barret–Joyner–Halenda analyses were used to determine the specific surface area, the nanopore size distribution and the nanopore volume by N_2 adsorption–desorption isotherms.

2.2. Loading and release of drug DMOG in MBG scaffolds

To investigate the delivery of DMOG in MBG scaffolds with varied mesoporous properties, 0g-P123@MBG, 8g-P123@MBG, 10g-P123@MBG and 12g-P123@MBG scaffolds were selected for further analyses. DMOG powders were first dissolved in phosphate buffered saline (PBS) in the concentration of 1 mg ml^{-1}. Each MBG scaffold 5 mm in diameter and 5 mm high was soaked in 1 ml of DMOG–PBS solution for 24 h. The solution was then collected and centrifuged at 8000*g* for 15 min. The loading amount of DMOG was determined by UV analysis by calculating the different concentration of DMOG–PBS before and after loading by a depletion method. The DMOG-loaded MBG scaffolds were gently flushed by ddH_2O to remove the excessive DMOG and dried at 37 °C overnight.

For the DMOG releasing analysis, the DMOG-loaded MBG scaffolds were soaked in 2 ml PBS at 37 °C for different periods. At each time point, 1 ml of PBS was collected to analyze the released DMOG by UV analysis, and 1 ml fresh PBS was added back. The cumulative release of DMOG from MBG scaffolds was calculated. The test was performed for three samples from each group.

2.3. Isolation and culture of human bone marrow stromal cells (hBMSC)

Human bone marrow was sourced from patients undergoing knee replacement surgery at the Department of Orthopaedics at the Prince Charles Hospital (PCH) in Queensland, Australia. Informed consent was given by all participants, and the research protocol was approved by the Ethics Committee of the PCH and the Queensland University of Technology. The hBMSC used in the present study were isolated from the bone marrow by density gradient centrifugation using Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) according to procedures described previously [35]. The cells were then plated into the culture flasks in growth medium containing low glucose Dulbecco's Modified Eagle's Medium (Gibco®, Life Technologies Pty Ltd., Australia), 10% fetal bovine serum (*In Vitro* Technologies, Australia) and 1% penicillin/streptomycin (Gibco®, Life Technologies Pty Ltd., Australia). Upon reaching 70–80% confluence, the attached hBMSC were further expanded. The medium was changed twice a week until the cells were confluent. Only early passage cells were used in this study.

2.4. Cell seeding on MBG scaffolds loaded with or without DMOG

Prior to seeding with cells, the prefabricated MBG scaffolds were sterilized by dry heat under 180 °C for 30 min and placed

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