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Effect of nano-structured bioceramic surface on osteogenic differentiation of adipose derived stem cells

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ABSTRACT

Tissue engineering strategies to construct vascularized bone grafts potentially revolutionize the treatment of massive bone loss. The surface topography of the grafts plays critical roles on bone regeneration, while adipose derived stem cells (ASCs) are known for their capability to promote osteogenesis and angiogenesis when applied to bone defects. In the present study, the effects of hydroxyapatite (HAp) bioceramic scaffolds with nanosheet, nanorod, and micro-nano-hybrid (the hybrid of nanorod and microrod) surface topographies on attachment, proliferation and osteogenic differentiation, as well as the expression of angiogenic factors of rat ASCs were systematically investigated. The results showed that the HAp bioceramic scaffolds with the micro-/nano-topography surfaces significantly enhanced cell attachment and viability, alkaline phosphatase (ALP) activity, and mRNA expression levels of osteogenic markers and angiogenic factors of ASCs. More importantly, the biomimetic feature of the hierarchical micro-nano-hybrid surface topography showed the highest stimulatory effect. The activation in Akt signaling pathway was observed in ASCs cultured on HAp bioceramics with nanorod, and micro-nano-hybrid surface topographies. Moreover, these induction effects could be repressed by Akt signaling pathway inhibitor LY294002. Finally, the in vivo bone regeneration results of rat critical-sized calvarial defect models confirmed that the combination of the micro-nano-hybrid surface and ASCs could significantly enhance both osteogenesis and angiogenesis as compared with the control HAp bioceramic scaffold with traditional smooth surface. Our results suggest that HAp bioceramic scaffolds with micro-nano-hybrid surface can act as cell carrier for ASCs, and consequently combine with ASCs to construct vascularized tissue-engineered bone.

1. Introduction

The massive bone defects due to traumatic injuries, cancer, and congenital defects, require the use of large bone grafts [1]. It has been shown that implantation of large bone grafts without adequate vascularity usually results in apoptosis and cartilage formation [2,3]. Therefore, it is considered that both sufficient angiogenesis and osteogenesis is necessary to regenerate the massive defects. A tissue-engineered graft made of bioactive scaffolds with mesenchymal stem cells (MSCs) is proposed as a potential bone graft for massive bone defects, due to its improvement on angiogenesis and osteogenesis in vivo.

As a key factor, biomaterial scaffolds play an important role in bone tissue engineering. Previous studies have demonstrated that the structure of scaffolds could determine its bioactivity and osteoinductive ability. It has been reported that the parameters of micropores size, porosity and interconnectivity could be directly related to bone mineralization and vascularization [4,5]. Moreover,

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numerous studies showed that the micro-/nano-structured surfaces on metal implants could control fundamental cell behaviors including proliferation, migration and differentiation [6–10]. Moreover, it was also showed that microtopographical surface on metal implant could regulate secretion of angiogenic factors by osteoblasts in vitro, which might facilitate angiogenesis in vivo [11]. Previous studies also demonstrated that nanofibrous structures on the polymer scaffolds could induce osteogenic differentiation and be applied for bone tissue engineering [12–15]. Recently, we successfully fabricated the interconnective macroporous hydroxyapatite (HAp) bioceramics with micro-/nano-structured surfaces, which could significantly promote cell attachment, proliferation and osteogenic differentiation of osteoblast (MC3T3-E1) and bone marrow stromal cells (bMSCs) via activation of extracellular signal-related kinases (ERK), and p38 mitogen-activated protein kinase (MAPK) signaling pathways [16,17]. Our studies suggest that this macroporous bioceramic scaffold with micro-/nano-structured surface may possess great potential for constructing vascularized tissue-engineered bone.

As a promising alternative for bMSCs, adipose derived stem cells (ASCs) have been widely evaluated in bone tissue engineering [18,19]. It has been demonstrated that ASCs possessed several advantages over bMSCs, including a less invasive harvesting procedure, an increased number of stem cell progenitors yielded from an equivalent amount of tissue harvested, increased proliferation and differentiation capacities, and better angiogenic/osteogenic properties [20–23]. Furthermore, it was shown that osteogenic differentiated ASCs when seeded on decellularized bone allografts could promote osteogenesis and angiogenesis compared to non-differentiated ASCs and nondifferentiated/differentiated bMSCs [1]. Recently, it was reported that nano-structured topography on metal discs could stimulate osteogenic differentiation of ASCs in vitro [24]. However, the effects and underlying mechanisms of micro-/nano-structured surfaces on ASCs including regulating the expression of angiogenic factors is largely unknown, which need to be clarified. Moreover, ASCs seeded bioceramic scaffolds with micro-/nano-structured surface for bone defect repair has not been reported. Our hypothesis is that the micro-/nano-structured HAp bioceramic surface is able to induce osteogenic differentiation and angiogenesis as it did on MSCs, and the ASCs loaded HAp scaffolds with micro-/nano-structured surfaces can be used to repair bone defects.

To proof our hypothesis, the highly interconective macroporous HAp bioceramic scaffolds with nanosheet, nanorod, and micro-nano-hybrid (the hybrid of nanorod and microrod) surface topographies were fabricated as described in our previous studies [17], and the effects of these three surface topographies on attachment, proliferation, and differentiation of ASCs as well as underlying mechanisms, were systematically investigated in order to identify the optimal surface topography for in vivo study. Then, the HAp bioceramic scaffolds with the optimal surface topography were selected and loaded with ASCs to investigate in vivo osteogenesis and angiogenesis in a rat critical-sized calvarial defect model.

2. Materials and methods

2.1. Fabrication and characterization of the nano-structured HAp scaffolds

In the present study, the HAp bioceramic scaffolds with nanosheet (T1), nanorod (T2) and micro-nano-hybrid (the hybrid of nanorod and microrod, T3) surfaces were fabricated via hydrothermal treatment as described in our previous studies [16,17,25]. Briefly, the interconnective macroporous HAp scaffolds with diameter of 9 mm and height of 3 mm were fabricated for in vitro study, while the scaffolds with diameter of 5 mm and height of 3 mm for in vivo study. The HAp scaffolds with nanosheet, nanorod and micro-nano-hybrid surfaces were achieved via hydrothermal treatment of the á-TCP ceramic scaffolds in 0.2 M NaH_2PO_4, 0.2 M Na_3PO_4 and 0.2 M $CaCl_2$ aqueous solution, respectively. Besides, the macroporous HAp ceramic scaffolds with similar pore size and traditional smooth surface were fabricated using HAp powders as raw materials and used as control group. The surface morphology of the samples and the pore size of the scaffolds were observed by scanning electron microscopy (SEM, JEOL, Japan).

2.2. Rat ASCs isolation and culture

All animal procedures including the sequent animal study in vivo were approved by the Animal Research Committee of Shanghai Ninth People's Hospital affiliated to Shanghai Jiao Tong University, School of Medicine. ASCs were obtained from subcutaneous adipose tissue in the inguinal groove of 6-week-old Fisher 344 rats as described in previous study [26]. Briefly, adipose tissue in the inguinal groove was isolated and was digested with 0.1% collagenase type I (Sigma, USA) with intermittent shaking at 37 °C for 60 min. Finally, the floating adipocytes were separated from the stromal cell fraction by centrifugation, and then, the pellets were cultured in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin, in a humidified 37 °C and 5% CO_2 incubator. After the first passage, the growth medium was replaced with osteogenic medium (DMEM, 10% FBS, 50 μg/mL L-ascorbic acid, 10 mM glycerophosphate and 100 nM dexamethasone). The ASCs of passage 2–4 were used in the experiments. As for in vitro studies, The HAp bioceramic scaffolds were placed in 24 well plates, and then, were seeded with ADSCs at a density of 2×10^4/scaffold.

2.3. Morphology, adhesion and growth of the seeded ASCs

Rat ASCs were cultured on HAp bioceramic scaffolds with nanosheet (T1), nanorod (T2), micro-nano-hybrid (T3) and traditional smooth (T0) surfaces in 24-well plates. At 6 h after cell seeding, the samples were collected for actin assay and SEM assay. As for actin assay, the samples were fixed in 4% paraformaldehyde for 30 min and rinsed with phosphate buffered saline (PBS) for three times, and then treated with 0.1% Triton X-100 to permeabilize the cells for 20 min followed by blocking with 1% BSA for 20 min. Finally, the actin cytoskeletons were labeled by Phalloidia-TRITC (Sigma, USA) for 30 min, meanwhile the cell nuclei was contrast-labeled by 4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma, USA) as described in our previous study [27]. The actin cytoskeletons of cells were detected by confocal laser scanning microscope (CLSM, Leica, Germany). As for SEM assay, the sample for each group was fixed in 2.5% glutaraldehyde overnight at 4 °C, washed with PBS for three times, and then dehydrated by increasing the concentration of ethanol. Finally, these samples were dried by hexamethyldisilazane, sputter-coated with gold and examined by SEM.

2.4. Cell proliferation assay

ASCs were cultured on HAp bioceramic scaffolds T0 ~ T3 for 1, 4 and 7 days, respectively. At each time point, three pieces of cell/scaffold complexes for each group were washed with PBS, and then, 400 μL DMEM with supplement 40 μL 5 mg/mL MTT (Amresco, USA) solution was added and incubated at 37 °C for 4 h. Finally, the medium was replaced with 400 μL dimethyl sulfoxide (DMSO, Sigma, USA) to dissolve MTT formazan, and the absorbance was measured at 490 nm by ELX Ultra Microplate Reader (Bio-tek, USA). All experiments were performed in triplicate.

2.5. Alkaline phosphatase (ALP) activity assay

ALP staining was performed using ALP staining kit (Beyotime, China) at day 10 after ASCs seeded on HAp bioceramic scaffolds T0 ~ T3. Moreover, ALP activity was quantitatively determined at days 4, 7 and 10 as described in our previous study [27]. Briefly, the cells were collected and resuspended in lysis buffer with 0.2% NP-40. Each sample was equivalently mixed with p-nitrophenyl phosphate (pNPP, 1 mg/mL, Sigma, USA) in 1 M diethanolamine buffer and quantified by absorbance at 405 nm (Bio-tek, USA) according to series of p-nitrophenol (pNP) standards. Besides, total cellular protein content for each sample was determined with the Bradford method as described in our previous study. Finally, ALP activity was expressed as pNP (mM) per milligram of total cellular proteins. All experiments were performed in triplicate.

2.6. Quantitative real-time PCR assay

Total RNA was isolated from ASCs cultured on HAp bioceramic scaffolds T0 ~ T3 at days 4, 7 and 10, respectively. At each time point, total RNA was isolated with Trizol reagent (Life Technologies, USA) according to manufacturer's instructions. Then, complementary DNA (cDNA) synthesis was performed using a PrimeScript 1st Strand cDNA Synthesis kit (Takara, Japan). Real-time PCR analysis was performed with the Bio-Rad real-time PCR system (Bio-Rad, USA) on osteogenic markers of runt-related transcription factor 2 (Runx2), bone morphogenetic protein 2 (BMP-2), collagen type 1 (COL1), bone sialoprotein (BSP), osteopontin (OPN), and osteocalcin (OCN), as well as angiogenic factors of vascular endothelial growth factor (VEGF), angiopoietin-1 (ANG-1), placental growth factor (PLGF), and stromal cell derived factor-1 (SDF-1). mRNA levels were normalized to glyceraldehyde-3-phosphatedehydrogenase (GAPDH) using the comparative cycle threshold (CT) method. The primer sequences used in the present study are listed in Appendix Table. All experiments were performed in triplicate.