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The Cu-containing TiO_2 coatings with modulatory effects on macrophage polarization and bactericidal capacity prepared by micro-arc oxidation on titanium substrates

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ABSTRACT

The implant materials with both osteogenic and anti-bacterial properties are promising for orthopedic and dental applications. Moreover, the inflammatory response induced by biomaterials has been recently recognized as one of the critical factors in determining implantation fate. A new generation of implant materials should have modulatory effects on the local inflammatory environment such that it favors osteogenesis and osteointegration instead of being bio-inert. In this study, the micro-arc oxidation (MAO) technique was employed to fabricate Cu-containing ceramic coatings on titanium substrates. The macrophages cultured on Cu-containing MAO-fabricated surfaces were polarized to M1 phenotype, evidenced by the high expression levels of inducible nitric oxide synthase (iNOS), low expression levels of arginase1 (Arg1), enhanced pro-inflammatory cytokine interleukin-6 (IL-6) release and inhibited IL-4 and IL-10 (anti-inflammatory cytokines) release. The MAO-treated surface incorporated with larger amounts of Cu (referred as Cu(h)-MAO) could modulate a favorable inflammatory microenvironment for osteoblast-like cell differentiation. Moreover, the macrophages cultured on Cu(h)-MAO surface exhibited enhanced bacteria uptake and killing rate, indicating that the Cu(h)-MAO surface promoted the bactericidal capacity of macrophages. Together, Cu could be used as a promising modulatory agent for macrophage functions. The integration of Cu in biomaterials could lead to enhanced macrophage-mediated osteogenesis and bactericidal capacity.

1. Introduction

The implantation of bone biomaterials commonly leads to an inevitable inflammatory response, which is mediated by the recruitment and activation of immune cells, particular macrophages. The macrophages can release a series of cytokines and chemokines that influence the migration, proliferation and differentiation of osteoblastic lineage cells which are well known as main functional cells for osteogenesis and bone remodeling [1]. Therefore, the inflammatory response is considered as one of the critical factors in determining implantation fate. However, this factor is commonly neglected when evaluating the osteogenic capacity of bone biomaterials. For example, Chen et al. recently reported that the cobalt incorporated β-tricalcium phosphate (CCP) could exhibit completely opposite *in vitro* osteogenic capacities when macrophages were involved or not, and the results from biomaterials/osteoblastic lineage cells/macrophages were found to be consistent with *in vivo* findings [2]. Accordingly, the paradigmatic design for the next generation of bone biomaterials should be shifted from being relatively inert to minimize the inflammatory response to being modulatory on the local inflammatory environment such that it favors osteogenesis and osteointegration [3]. Among all the immune cells, the macrophages tend to receive the most attention due to their multiple roles in regulating the biomaterial-induced inflammatory response as well as mediating the bone healing process [4]. The remarkable plasticity of macrophage phenotypes allows them to alter their physiology and functions in response to biological, biophysical and biochemical stimuli [5,6].

Copper (Cu), as one of the trace elements in the human body, is well known for its anabolic effects in bone metabolism [7,8]. Smith et al. reported that the dietary depletion of Cu caused a reduction of bone mineral density in rats [9]. There are *in vitro* evidences showing that the exogenous Cu^{2+} promoted the osteogenic differentiation of

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mesenchymal stem cells (MSCs) in osteogenic culture medium by stimulating alkaline phosphatase (ALP) activity, increasing the synthesis of collagen I, osteopontin and osteoprotegerin as well as enhancing *in vitro* mineralization [8]. In general, most of the relevant works focused on the direct roles of Cu in stimulating osteogenesis. However, relatively little is known about how Cu mediates the osteogenic and angiogenic processes by modulating the inflammatory response of macrophages. Recently, Shi et al. reported that the Cu-containing mesoporous silica nanospheres (Cu-MSNs) could modulate the inflammatory response after being phagocytized by macrophages [10]. Compared to the MSNs group without Cu, the Cu-MSNs were found to polarize the macrophages to their M1 phenotype. Meanwhile, the Cu-MSNs-treated macrophages released proper pro-inflammatory cytokines and expressed higher levels of osteogenic and angiogenic growth factor genes. The inflammatory microenvironment induced by the Cu-MSNs enhanced the osteogenic differentiation of MSCs, indicating that Cu^{2+} has beneficial modulatory effects on macrophage-mediated osteogenesis [10]. In that work, a portion of Cu-MSNs were phagocytized by macrophages, and thus the intracellularly released Cu^{2+} modulated macrophage behavior. In fact, three-dimensional (3D) biomaterials are commonly too large for the phagocytosis of macrophages as compared to the nanoparticles. Therefore, it is necessary to investigate the modulatory effects of extracellular Cu^{2+} released from 3D biomaterials on macrophage functions.

Besides being favorable for osteogenesis, the Cu-containing biomaterials are well known for their anti-bacterial properties. Here, we broadly classify the bactericidal behaviors of Cu into two action modes. One is the direct bactericidal mode, which means killing bacteria directly by Cu^{2+}. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Cu^{2+} against *Staphylococcus aureus* (*S. aureus*) were reported to be 448 and 512 ppm, respectively [11]. The mechanism of the direct bactericidal mode is probably related to the increased influx of Cu^{2+} into bacteria. This process generates reactive oxygen species (ROS), results in loss of cytoplasmic membrane integrity and inhibits the respiration and degradation of DNA [12]. By taking advantage of this bactericidal mode, Cu has been integrated into various kinds of biomaterials to endow them with anti-bacterial properties [8,11,13,14]. The other mode is an indirect one, which can be named as the macrophage-mediated bactericidal mode. It means killing the bacteria by modulating macrophage activity *via* Cu^{2+}. Cu is required by the immune system for optimal function whereas Cu deficiency impacts innate and acquired immune responses [15]. Achard et al. reported that the Cu chelator BCS (bathocuproinedisulfonic acid, disodium salt) increased intracellular survival of bacteria within macrophages after 24 h of infection, suggesting that Cu^{2+} is related to the bactericidal capacity of macrophages [16]. Stafford et al. reported that the exogenous Cu^{2+} at a concentration of 1.3 ppm promoted the bactericidal activity of Raw 264.7 macrophages against *Escherichia coli* (*E. coli*), and this effect was inhibited by the antioxidant drug such as Ebselen [17]. This suggests that Cu^{2+} is a regulator for macrophage function and may contribute to the ROS-dependent bactericidal properties of macrophages. So far, few anti-bacterial biomaterials have been developed based on the macrophage-mediated bactericidal mode. The effective concentration of Cu^{2+} related to the macrophage-mediated bactericidal mode is far lower than that related to the direct one. Considering the toxicity of Cu^{2+}, the development Cu-containing anti-bacterial biomaterials based on the indirect bactericidal mode is beneficial for the safety and biocompatibility.

In the current work, the micro-arc oxidation (MAO) technique was employed to fabricate ceramic coatings on titanium (Ti) substrates and simultaneously facilitate Cu incorporation. The modulatory effects of MAO-fabricated Cu-containing surfaces on macrophage polarization and macrophage-mediated osteogenesis were investigated by taking MAO-fabricated Cu-free surface as control. Finally, the bactericidal capacity of Raw 264.7 macrophages cultured on Cu-containing coatings was evaluated by using gram positive *S. aureus* as bacteria model.

2. Experimental procedure

2.1. Generation of material surfaces

Grade 2 commercially pure Ti discs (14.5 mm in diameter and 1 mm in thickness) were treated by micro-arc oxidation (MAO) in an electrolytic solution prepared as previously reported [18]. Briefly, 0.10 M $Na_2(EDTA)$, 0.10 M $Ca(CH_3COO)_2{\cdot}H_2O$ and 0.25 M NaOH were dissolved in distilled water and stirred for 24 h. After that, 0.02 M $Na_2SiO_3{\cdot}9H_2O$ was added into the solution. Finally, the Cu^{2+} was introduced into the electrolyte in the form of $CuSO_4{\cdot}5H_2O$ at a concentration of 0.2 or 2 mM. During MAO, an applied voltage of 250 V, pulse frequency of 50 HZ, duty cycle of 50% and duration time of 5 min were employed. The specimens treated with 0, 0.2 and 2 mM $CuSO_4{\cdot}5H_2O$ were referred as MAO, Cu(l)-MAO and Cu(h)-MAO, respectively.

2.2. Analysis of physicochemical properties

The surface morphology of the specimens was observed by field emission scanning electron microscopy (FESEM; Merlin Compact, Zeiss, Germany). The 3D surface profiling of the samples was evaluated using a 3D profiling system (MicroXAM-3D Phase Shift, ADE Co., USA). The static water contact angles of different specimens were measured by sessile drop using an optical tensiometer (JC2000C1; Powereach, China).

2.3. Cell culture models

The murine-derived RAW 264.7 macrophages (supplied by China Infrastructure of Cell Line Sources, China) were cultured in high glucose Dulbecco's modified eagle medium (HG-DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in an atmosphere of 5% CO_2 and 100% humidity. The cells were passaged at approximately 80% confluence by scraping and expanded through two passages before use.

Human osteoblastic SaOS-2 cells (supplied by China Infrastructure of Cell Line Sources, China) were incubated in normal culture medium which consists of McCoy's medium, 15% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in an atmosphere of 5% CO_2 and 100% humidity. Until 80% confluence was reached, the cells were routinely subcultured after trypsinization.

Before cell culture, all disks were sterilized by ultraviolent irradiation for 24 h. The disks were put in 24 well plates for cell seeding. The experiments were carried out in quadruplicate.

2.4. Macrophage response to material surfaces

2.4.1. Cell morphology

The macrophages were seeded on various surfaces at a density of 6×10^4 cells per well. After culturing for 1, 3 and 5 days, the cells were washed twice with Dulbecco's phosphate-buffered saline (DPBS) and fixed with 2.5% glutaraldehyde (in DPBS) overnight. Then, the cells were dehydrated by gradient ethanol solutions (20%, 40%, 60%, 80%, 90% and 100%), each for 10 min. Thereafter, the residual ethanol within cells was substituted with a series of graded tertiary butanol (25%, 50%, 75% and 100% in ethanol) each for 10 min. The treated samples were freeze dried, sputter-coated with gold and observed using SEM.

2.4.2. Cell proliferation

The proliferation of macrophages in response to various material surfaces was visualized by Calcein-AM staining and quantified by CCK-8 assay.