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Research Paper

Potential proinflammatory and osteogenic effects of dicalcium silicate particles *in vitro*

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ABSTRACT

Background: Due to their biocompatibility and bioactivity, dicalcium silicate (C_2S) and hydroxyapatite (HA) are used as coating materials for prosthetic orthopedic and dental implants or as bone substitute materials to fill bone defects. However, prostheses and bone substitutes can release particles that trigger an immune response in the recipient. The immunological effects of C_2S particles have not yet been studied.

Objective: The aim of this study was to determine the cytotoxic effects of C_2S particles on primary human monocytes, a human monocyte cell line (THP-1) and an osteoblast-like cell line (MG-63). The proinflammatory effects of C_2S particles on THP-1 were also detected. Moreover, the osteogenic effects of C_2S and HA on MG-63 cells were investigated.

Methods: Characterization of C_2S and HA was performed using scanning electron microscopy (SEM), energy dispersive analysis (EDS), X-ray diffraction (XRD), Brunner–Emmett–Teller (BET) measurements and laser diffraction. The cytotoxic effect of C_2S on primary human monocytes as well as THP-1 and MG-63 cells was measured using Trypan blue assays, Cell Counting Kit-8 (CCK-8) assays and flow cytometry to detect apoptosis. THP-1 human monocytes with or without lipopolysaccharide (LPS) stimulation were exposed to C_2S and HA for 6 and 24 h. Thereafter, the mRNA expression and protein concentrations of MMP-2, MMP-9, TIMP-2, TIMP-1 and TNF-α were evaluated using real-time PCR and ELISA, respectively. RANKL and OPG mRNA expression levels in MG-63 cells were examined using real-time PCR.

Results: No significant cytotoxicity was recorded when cells were directly cultured with C_2S/HA particles. After THP-1 cells were cultured with C_2S/HA for 24 h, MMP-2, MMP-9 and TNF-α expression increased, whereas TIMP-2 and TIMP-1 expression decreased. Compared with HA, C_2S slightly increased MMP-9 expression and slightly decreased TIMP-1 expression. The MMP: TIMP ratio increased in the C_2S and HA groups; however, HA significantly increased the MMP-9: TIMP-1 ratio compared with C_2S. Compared with HA, C_2S caused less TNF-α production. C_2S/HA did not modify the expression of proinflammatory mediators in LPS-stimulated cells. Furthermore, C_2S/HA significantly increased OPG expression and

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slightly increased RANKL expression in MG-63 cells. C_2S and HA decreased the RANKL: OPG ratio.

Conclusion: Our *in vitro* data suggest that C_2S is relatively safe when directly cultured with cells. In addition, C_2S may exert proinflammatory effects; however, compared with HA, C_2S had fewer proinflammatory effects on THP-1. C_2S and HA did not alter the LPS-induced production of proinflammatory mediators and had similar osteogenic effects on MG-63 cells.

1. Introduction

The role of silicon in the formation of new bone was established in the early 1970s. Silicon was found to be involved in the early stages of bone calcification (Schwarz and Milne, 1972). Silica-based materials, such as dicalcium silicate (Ca_2SiO_4, C_2S), release silicon ions, which have important roles in skeletal development and repair (de Aza et al., 2013a). C_2S possesses excellent bioactivity when used as a coating material for titanium alloy substrates (Liu et al., 2002, 2005). The *in vitro* and *in vivo* bioactivities and biocompatibilities of such implants increase when they are coated with α-tricalcium phosphate (αTCP) doped with C_2S (Velasquez et al., 2013). In addition, αTCP doped with C_2S promotes the differentiation of human mesenchymal stem cells (hMSCs) into osteoblasts (de Aza et al., 2013a; Meseguer-Olmo et al., 2012) and supports human adipose stem cell adhesion and spreading (De Aza et al., 2014). αTCP cements modified with β-C_2S exhibit the best properties *in vitro* and *in vivo* (Correa et al., 2014; de Aza et al., 2013). Furthermore, electrospun poly (L-lactic acid) (PLLA) scaffolds containing C_2S were shown to markedly promote the proliferation and osteogenic differentiation of MC3T3-E1 cells (Dong et al., 2014). Tissue engineering scaffolds containing C_2S significantly enhanced the proliferation of MG-63 cells by stimulating the transcription of transforming growth factor-β1 (TGF-β1) and bone morphogenetic protein-7 (BMP-7) (Wang et al., 2013). This research shows that C_2S has the potential to be used as a bone substitute. In dentistry, C_2S cement has adequate biological properties and can be used as a root-end filling material and a pulp capping material, as it exhibits good bioactivity and biocompatibility *in vitro* studies Wu et al., 2014a; Chen et al., 2009; Gandolfi, 2010; Chen et al., 2011. In addition, C_2S cement exhibits high apatite-forming activity and low degradation in acidic environments when used as a root-end filling material (Chiang and Ding, 2013) Regarding its cytotoxicity, C_2S cement is significantly superior to the traditional root-end filler, mineral trioxide aggregate (MTA) (Chiang and Ding, 2010). C_2S cement is also a model system for drug release (Gou et al., 2005). These findings support the broad use of C_2S in future orthopedic and dentistry applications.

Numerous coating materials and bone substitute biomaterials in orthopedic and dental surgery possess potential proinflammatory effects. For example, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA) is considered relatively safe due to its bioactivity and biocompatibility (Jones and Hench, 2004). However, particle release from prostheses can cause aseptic inflammation depending on its amount and frequency. The interactions between HA particles and human monocytes lead to the production of inflammatory mediators, such as tumor necrosis factor alpha (TNF-α) and metalloproteinases (Laquerriere et al., 2003, 2004; Buache et al., 2012). These inflammatory mediators trigger an immune response. The released particles also trigger the release of immune mononuclear cells, leading to phagocytosis and the activation of associated immune signaling pathways. Bone substitute materials, including biphasic calcium phosphate (Curran et al., 2005), titanium alloys, polymethylmethacrylate (PMMA) and aluminum, have been shown to induce different proinflammatory mediators (Kaufman et al., 2008). These proinflammatory effects have been studied using mixed cell populations, such as peripheral blood mononuclear cells, which include both lymphocytes and monocytes. However, the cytotoxicity of C_2S particles in cell culture remains unclear because most previous studies have only focused on the safety of C_2S coatings, biomaterials doped with C_2S and C_2S cements. Direct contact between cells and particles may cause a different effect. Furthermore, the potential proinflammatory effects of C_2S particles have never been examined in immune cells. In the present study, we investigated the safety of C_2S particles when directly cultured with immune and osteoblast-like cells. Second, we evaluated whether the particles induced the release of proinflammatory mediators and whether these effects were different from those caused by HA particles. Finally, we compared the abilities of C_2S and HA to promote osteogenic effects.

2. Materials and methods

2.1. Characterization of C_2S and HA particles

The laboratory of the Shanghai Institute of Ceramics, Chinese Academy of Sciences, synthesized 99% pure C_2S and HA particles by the sol–gel process. Particle morphologies were determined using scanning electron microscopy (SEM; Nova Nano SEM 430, FEI, Finland) combined with energy-dispersive X-ray spectroscopy (EDS; DX-4 system, EDAX, USA). Crystal structures were analyzed using X-ray diffraction (XRD; Geigerflex, Rigaku Co, Japan), which measured the diffraction pattern in the 2θ range from 10° to 80° using monochromatic Cu Kα radiation. Particle size ranges were estimated by the laser diffraction method using a laser particle sizer (Analysette22, Fritsch, Idar-Oberstein, Germany). Tetrasodium diphosphate and a surfactant were used in addition to an ultrasonic bath with vigorous stirring (80 rpm and 100 rpm cycling pump) to disperse the particles. The measurable size distribution of particles was approximately 0.1–150 μm in 64