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Characterization of beta-tricalcium phosphate as a novel immunomodulator

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ABSTRACT

Calcium phosphate (CaP) ceramics including hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP) have been widely used for bone substitution in orthopedic, maxillofacial and dental surgery, as well as in tumor resections. CaP particles are also known to cause inflammatory responses, which are thought to be an unfavorable characteristic of prosthetic coating materials. On the other hand, the immunostimulatory effect of β-TCP induces an anti-tumor effect in xenograft tumor models in athymic mice. To date, in depth analysis of the biological effects of β-TCP has not been studied in mice.

In the present study, *in vivo* biological effects of β-TCP were investigated by subcutaneously injecting β-TCP particles into mice. This induced extensive migration of immune cells to the area surrounding the injection. In addition, we found that *in vitro* treatment with β-TCP in murine monocyte/macrophage cells (J774A.1) induced up-regulation of surface expression of CD86, and increased production of TNF-α, MIP-1α, and sICAM-1. Furthermore, conditioned medium from J774A.1 cells treated with β-TCP facilitated migration of murine splenocytes in a transwell migration assay. These findings clarify that β-TCP induces an immunostimulatory effect in mice, and suggest a potential for β-TCP as a novel adjuvant for cancer therapy.

1. Introduction

Calcium phosphate (CaP) ceramics including hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP) have been widely used for bone substitution in orthopedic, maxillofacial and dental surgery, as well as tumor resections because of their biocompatibility and bioactivity. HA also has been used to coat the metal parts of prostheses in order to improve biocompatibility. In addition, use of HA increases integration of the implant. Unfortunately, HA is known to have a tendency to fragment and generate particles (wear debris) [1], which activate leukocytes and cause inflammatory responses [2,3]. Adverse tissue responses to prosthetic wear debris have been shown to cause osteolysis and implant loosening [4]. Subsequent extensive research on CaP, and its biological effects has shown that it is influenced by physicochemical properties such as stoichiometry [5], crystallinity [6], solubility [7], surface area [8], rugosity, porosity [9], and granulometry [10].

Naito et al. reported an anti-tumor effect of β-TCP in a xenograft tumor model in athymic mice, suggesting a novel potential role for β-TCP as an adjuvant for cancer treatment [11]. We decided to confirm the anti-tumor effect of β-TCP in another experimental setting as an initial step following the study by Naito et al. β-TCP was used in combination with tumor antigen in E.G7-OVA syngeneic murine tumor model [12], and it was demonstrated that β-TCP enhanced the prophylactic anti-tumor effect caused by vaccination of tumor antigen, ovalbumin protein (Supplementary Fig. 1). When compared to a control group, a significant delay in tumor growth was observed in a group administered tumor antigen and β-TCP simultaneously. In these studies, anti-tumor tests were performed in mice tumor models; however, the biological effects of β-TCP have not been independently studied in mice.

In the present study, we investigated the *in vivo* effect of β-TCP by subcutaneous implantation in C57BL/6 (B6) mice, and the *in vitro* function of β-TCP using a murine monocyte/macrophage cell line, J774A.1. In mice, the subcutaneous injection of β-TCP induced extensive migration of immune cells to the area surrounding the injection site. Addition of β-TCP to J774A.1 cells in culture up-regulated surface expression of CD86, and increased production of tumor necrosis factor-alpha (TNF-α), macrophage inflammatory protein 1 alpha (MIP-1α), and

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soluble intercellular adhesion molecule-1 (sICAM-1). Furthermore, conditioned medium from J774A.1 cells treated with β-TCP facilitated migration of splenocytes prepared from B6 mice. These findings revealed that β-TCP exert the immunostimulating effect also in mice as in the other mammals previously reported.

Taken together, our findings shown in this article including anti-tumor effects suggest a potential of β-TCP as a novel adjuvant for cancer immunotherapy.

2. Materials and methods

2.1. Preparation of β-TCP

Particles of β-TCP were prepared by Olympus-Terumo Biomaterials (Tokyo, Japan). In brief, a fine powdered formulation of β-TCP with a 1–50 μm diameter was prepared by chemical synthesis followed by sintering at 1000 °C for 10 h. Granulometry of the β-TCP particles was analyzed by laser diffraction analyzer (SALD-2000 J, Shimadzu, Kyoto, Japan). Particles had a diameter of 5.373 ± 0.342 μm (mean ± standard deviation), and more than 90% of particles were 1–20 μm with d_{50} and d_{90} values of 4.994 μm and 16.323 μm, respectively (Supplementary Fig. 2). The β-TCP preparation was endotoxin free, and its purity was more than 99%. Fine powder of β-TCP was subjected to experiments as a suspension in phosphate buffered saline (PBS).

2.2. Animals, implantation of β-TCP, and histological analysis

Male C57BL/6 (B6) mice of 5–6 weeks old were purchased from CLEA Japan (Tokyo, Japan), and subjected to experiments after a 1–2 week acclimatization period. All mice were housed and used for experiments in accordance with standard ethical guidelines for the care and use of laboratory animals (Science Council of Japan; Guidelines for Proper Conduct of Animal Experiments, 2006), and the study was approved by the Animal Experiment Ethics Committee of Shizuoka Cancer Center. Animals were subcutaneously injected with a solution containing 100 μg of the fine β-TCP particles suspended in PBS through a 26-gauge needle into the flank. After implantation periods from 1 day to 6 months, tissue samples surrounding β-TCP including skin, subcutaneous tissue, and abdominal muscle were collected, and fixed with 10% formaldehyde. Fixed tissue was subjected to a decalcification procedure using 5% formic acid, then embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined under a microscope.

2.3. Cell culture and in vitro stimulation with β-TCP

The J774.1 mouse monocyte/macrophage cell line [13], was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in growth medium (DMEM supplemented with 10% FBS; Life Technologies, Carlsbad, CA, USA). J774A.1 cells were plated into 12-well plates at 1.9×10^5 cells/well and left overnight. On the following day, medium was changed, and cells were stimulated with 100 μg/ml β-TCP or 1 μg/ml lipopolysaccharide (LPS) from *E. coli* O111:B4 (Sigma–Aldrich, Saint Louis, MO, USA) for 24 h. After stimulation, cells and culture supernatants were harvested.

2.4. Phenotype analysis of J774A.1 cells

Phenotypic analysis of J774A.1 cells was performed by flow cytometry. All fluorescently labeled monoclonal antibodies used including anti-CD11b (clone M1/70), anti-CD86 (GL1), anti-CD80 (16-10A1), anti-CD40 (3/23), and anti-I-A/I-E mouse MHC classII (2G9) were purchased from BD Biosciences (San Jose, CA, USA), except for anti-F4/80 (Cl:A3-1; AbD Serotec, Oxford, UK). J774A.1 cells were stained with the above antibodies and analyzed by using BD Accuri C6 flow cytometer (BD Biosciences).

2.5. Analysis of cytokine, chemokine, and soluble adhesion molecule production in J774A.1 cell culture medium

Protein levels of cytokines, chemokines, and soluble adhesion molecules in supernatants were semi-quantitatively measured by using a Proteome Profiler™ Mouse Cytokine Array kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. TNF-α, MIP-1α, and sICAM-1 were quantified by commercially available ELISA kits (BD Biosciences for TNF-α, R&D Systems for MIP-1α and sICAM-1).

2.6. In vitro migration assay of splenocytes

Conditioned medium from J774A.1 cells was prepared as described in Section 2.3. Splenocytes were prepared from spleens of B6 mice. Cell migration was assayed by using a 12-well transwell chamber (Biocoat, Becton Dickinson, MA, USA) in which the upper chambers and lower wells are separated by a transparent tracked-etched polyethelene terephthalate (PET) membrane with 3 μm pores. In brief, J774A.1 conditioned medium was poured into the lower wells (1 ml/well), and 3×10^6 splenocytes suspended in 1 ml of serum free RPMI1640 medium were added to the upper chambers. After a 24 h incubation, cells that had migrated to the lower wells were collected and counted by using trypan blue and a hemocytometer. The conditioned medium from J774A.1 cells was centrifuged and filtered through 0.22 μm membrane filter (Millex-GV; Millipore, Billerica, MA, USA) to remove the β-TCP particles before use.

2.7. Statistical analysis

The results are presented as means ± standard errors. Differences between control groups and β-TCP- or LPS-treated groups were analyzed by two-way ANOVA and Student's *t*-test, and differences were considered statistically significant when the *p* value was less than 0.05.

3. Results

3.1. In vivo stimulatory effect of immune cell migration induced by β-TCP

The *in vivo* effect of β-TCP was evaluated in immunocompetent B6 mice. β-TCP particles were suspended in PBS and subcutaneously injected into the flanks of mice. Tissue samples surrounding the β-TCP injection site were excised from mice after 1 day, 3 days, 1 week, 2 weeks, 1 month, and 6 months of implantation (Fig. 1). We found that on the day following the implantation, abundant neutrophils migrated around the β-TCP particles (Fig. 1A and B). Three days after implantation, the number of neutrophils decreased, and small lymphocytes, histiocytes and fibroblasts predominated around the β-TCP material (C, D). One week after implantation, multinucleated giant cells appeared (E, F) and continued to digest β-TCP materials from two weeks to six month (G, H, I). However, no evidence was observed to suggest osteogenesis even after 1 or 6 months (Fig. 1H and I). Thus, subcutaneous implantation of β-TCP particles induced an inflammatory reaction and promoted migration of a significant number of various immune cells.

3.2. In vitro effect of β-TCP on J774A.1 mouse monocyte/macrophage cells

3.2.1. Phagocytosis of β-TCP particles by J774A.1 cells

It has been reported that β-TCP might have a stimulatory effect on macrophages [11]. To study the effects of β-TCP on macrophages, we used the J774A.1 murine monocyte/macrophage cell line which has adherent growth properties (Fig. 2A). We found that J774A.1 cells cultured in the presence of β-TCP particles had sequential morphological changes. As shown in Fig. 2B, β-TCP particles were precipitated on the bottom of plates several minutes after addition to the cultures. The