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Liposomal clodronate inhibition of osteoclastogenesis and osteoinduction by submicrostructured beta-tricalcium phosphate

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ABSTRACT

Bone graft substitutes such as calcium phosphates are subject to the innate inflammatory reaction, which may bear important consequences for bone regeneration. We speculate that the surface architecture of osteoinductive β-tricalcium phosphate (TCP) stimulates the differentiation of invading monocyte/macrophages into osteoclasts, and that these cells may be essential to ectopic bone formation. To test this, porous TCP cubes with either submicron-scale surface architecture known to induce ectopic bone formation (TCPs, positive control) or micron-scale, non-osteoinductive surface architecture (TCPb, negative control) were subcutaneously implanted on the backs of FVB strain mice for 12 weeks. Additional TCPs samples received local, weekly injections of liposome-encapsulated clodronate (TCPs + LipClod) to deplete invading monocyte/macrophages. TCPs induced osteoclast formation, evident by positive tartrate resistant acid phosphatase (TRAP) cytochemical staining and negative macrophage membrane marker F4/80 immunostaining. No TRAP positive cells were found in TCPb or TCPs + LipClod, only F4/80 positive macrophages and foreign body giant cells. TCPs stimulated subcutaneous bone formation in all implants, while no bone could be found in TCPb or TCPs + LipClod. In agreement, expression of bone and osteoclast gene markers was upregulated in TCPs versus both TCPb and TCPs + LipClod, which were equivalent. In summary, submicron-scale surface structure of TCP induced osteoclastogenesis and ectopic bone formation in a process that is blocked by monocyte/macrophage depletion.

1. Introduction

Following the evolving insight into biomaterial design [1], particular emphasis has been devoted to understanding how physical properties of CaP may influence their bone forming performance. For instance, implant geometry [2], 3D surface concavities [3,4], and interconnected porous structure [5] have all been shown to influence bone formation. Most recently, material surface architecture on the submicron- and micron-scale has been shown to be particularly important to the osteoinductivity of a small subset of CaP through an unknown biological mechanism [6,7]. It is at this CaP-tissue interface where proteins and ions are absorbed and exchanged, as a function of the material surface reactivity and physico-chemistry [8]. On a cellular level, it is at this interface where invading monocyte/macrophages interact with the material surface, mediating inflammation and tissue repair during the host response to a foreign body [9]. Accordingly, specific interactions with the host response may be essential for the functional performance of bone graft substitutes to even further stimulate bone tissue regeneration in bony defects.

Though material properties can be adjusted to mitigate the innate inflammatory reaction, inflammation is at least to some extent unavoidable after implanting CaP due to the normal host response to a foreign body [10]. It has been speculated that in the case of osteoinductive CaP, invading tissue macrophages signaled by the innate inflammatory reaction may play a role in osteogenesis because of their dense and persistent presence surrounding an osteoinductive implant [11,12]. On the other hand, an adverse host response can also obstruct bone formation: in our previous work, chronic inflammation due to the addition of a polymeric carrier completely abrogated ectopic bone formation by osteoinductive

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beta-tricalcium phosphate (TCP) although the carrier dissolved relatively quickly [13]. Indeed, macrophages, the principal cell responsible for clearing a foreign body by phagocytosis, have been shown to express a distinct family of cytokines depending on their activation state in response to material properties such as surface chemistry, topography, and bioactivity [14,15].

During the innate inflammatory reaction, invading macrophages secrete cytokines that can also spur the fusion and specialization of bone-resorbing osteoclasts from their monocyte/macrophage precursors. Pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 activate T-cell expression of soluble RANKL (receptor activator of NF-κB ligand) the essential osteoclast differentiation factor, as well as upregulate its membrane-bound receptor RANK on the surface of osteoclast precursors, thus inducing osteoclastogenesis [16–19]. On the other hand, other secreted cytokines such as IL-4 and IL-13 stimulate stromal cell expression of OPG (osteoprotegerin), the natural decoy receptor to RANKL, thus antagonizing osteoclast differentiation [20]. In this way, inflammation and osteoclastogenesis may be linked and dependent on the precise cytokine cascade and a biomaterial substrate supporting pre-osteoclast fusion and differentiation. Osteoclasts have also been implicated with the functionality of osteoinductive CaP, with reports that osteoclasts form prior to ectopic bone formation [21] and that their inhibition may stunt osteoinduction [22,23].

As it pertains to osteogenesis, macrophage-mediated inflammation has been associated with pathological heterotopic ossification (HO) that results in marrow-containing bone neogenesis in the muscle tissue triggered by injury. However, when liposome-encapsulated bisphosphonate was locally administered to selectively deplete tissue macrophages in a transgenic mouse model of HO, osteogenesis was significantly blocked. This effect was attributed to the elimination of macrophage-secreted BMP4 at the injury site [24]. In an experimental mouse model of osteoarthritis, macrophage depletion by liposomal bisphosphonate resulted in the reduction of osteophytes (heterotopic bony nodules), which was attributed to reduced macrophage expression of osteogenic TGFβ, BMP2, and BMP4. And as it pertains to the natural regenerative capacity of bone, when macrophages were depleted using liposomal clodronate (a bisphosphonate) in a long bone fracture model, bone formation in the fracture callus was fully inhibited [25], shown elsewhere to be likely mediated by macrophage-expressed TNF-α and IL-6 [26]. These studies and others like them emphasize the apparent importance of macrophages and phagocyte relatives to both aberrant and reparative bone formation.

Considering cell–material interactions, both bone cells and macrophages have been shown to be highly sensitive to surface architecture of CaP. In our previous research investigating two TCP ceramics with different sized surface features, both ectopic bone formation and the presence of actively resorbing osteoclast-like multinucleated cells were strongly promoted on *submicron-scale* TCP surface features. On the other hand, no ectopic bone and scarce non-resorbing multinucleated cells were found on the TCP implants with *micron-scale* surface features [27].

Following on these findings, we asked whether these multinucleated cells were differentiated osteoclasts or merely fused macrophages, i.e. foreign body giant cells. Because their presence and resorptive activity appeared to be linked to osteoinduction, we hypothesized that they may play a directive role in ectopic bone formation by forming differently on the two different topographies during the host response. To investigate these questions, we implanted the same two TCP ceramics with equivalent chemistry but different surface structure – serving as positive and negative controls – in a recently validated mouse model of subcutaneous osteoinduction [28] and analyzed the ectopic bone formation and the phenotype of formed multinucleated cells using (immuno-) histological and gene expression analysis. To address the role that these multinucleated cells play in osteoinduction, we applied liposome-encapsulated clodronate (LipClod) to selectively deplete invading phagocytic monocyte/macrophages [29] – the mononuclear precursors of both osteoclasts and foreign body giant cells – and then evaluated ectopic bone formation.

2. Materials and methods

2.1. Preparation and characterization of porous TCP cubes

TCP powders were synthesized as previously described [13]. Briefly, calcium hydroxide and phosphoric acid (both from Fluka) were mixed at a Ca/P ratio of 1.50. TCP powders with small (TCPs) or big grains (TCPb) in the final ceramics were prepared by controlling the reaction rates. The powders were foamed with diluted H_2O_2 (1%) (Merck) at 60 °C then dried at room temperature to get porous green bodies. The dry green bodies were subsequently sintered at 1050 °C or 1100 °C for 8 h to achieve small and big grains for TCPs and TCPb, all respectively.

Porous cubes (4 × 4 × 4 mm) were machined from the ceramic bodies using a wet saw and then ultrasonically cleaned in successive baths of acetone, ethanol, and distilled water, and dried. Prior to implantation, TCP cubes were heat sterilized at 160 °C for 2 h. Crystal chemistry of the materials was analyzed by X-ray diffraction (Rigaku Miniflex II) scanning the range $2\theta = 25–45°$ (step size = 0.01°, rate = 1° min^{-1}) and confirmed to be β-TCP as previously described [13].

The TCP ceramics were characterized to confirm that they were composed of different surface (micro)structure but similar macrostructure as previously reported [27]. Surface microstructure was characterized by scanning electron microscopy (SEM) (JEOL JSM-5600) after sputter coating with gold for 90 s (JEOL JFC 1300) and >50 surface grains and micropores were measured in the using Image J image analysis software (NIH, USA). To measure the surface profile (i.e., surface roughness), SEM stereo-micrographs of the same location taken at two different tilt angles (2500×, ±5°) were digitally reconstructed into three-dimensional surfaces for automated profile analysis using MeX v5.1 software (Alicona Imaging, Austria). Additionally, porosity and total pore area were determined by mercury intrusion testing (Table 1) (Micromeritics, USA).

In summary, the synthesis of TCPs and TCPb resulted in submicron-scale and micron-scale surface grains, micropores, and roughness, respectively. The ceramics possessed similar total porosity but different total pore area owing to the smaller surface features of TCPs.

2.2. Subcutaneous implantation in FVB mice

Ethical approval for animal experimentation was obtained from the local ethical committee (CREEA). The animals were housed in certified premises at the Experimental Therapeutic Unit at the Faculty of Medicine, University of Nantes, France. Animals were stabled in cages with food and water *ad libidum* with artificial day/night cycle of 12 h and regulated temperature of 20 ± 1 °C.

Five-week-old male FVB strain mice ($n = 14$) were received from Charles River Laboratory (France) and allowed to equilibrate to their new surroundings for one week. Prior to surgery, the mice were placed under general anesthesia using isoflurane gas (2.5% in air, 2.5 L/min, Forene). Analgesic (Buprenorphine 60 μL/kg, Buprécare, MedVet) was subcutaneously injected at the time of surgery and 1 day later. Backs of animals were shaved and disinfected with sterile gauzes soaked with iodine solution and covered with a surgical sheet. Subcutaneous dorsal pockets were created using a scalpel and blunt nosed forceps, each allowing for the insertion of a single TCP cube. Two subcutaneous pockets were created on the left side of the back, in which TCPs positive control and TCPb negative control samples were inserted. An additional TCPs cube receiving liposomal clodronate treatment was implanted in one pocket on the right side of the back in order to avoid potential contamination of the injected liposomes into the pockets containing the controls. Skin incisions were tightly closed with degradable sutures (Vicryl 4-0, Ethicon).

Immediately following surgery, sterile liposomal clodronate (100 μL) (Clodronate Liposomes Foundation, The Netherlands) was injected into one pocket containing TCPs per animal. The same volume of sterile saline was injected into the pockets containing the TCPs positive control and TCPb negative control. This same injection regiment was repeated once a week and then animals were sacrificed after

Table 1
Physical characterization of TCP.

Physical parameters	TCPs	TCPb
Average grain diameter (μm)	0.95 ± 0.27	3.66 ± 1.05
Average pore diameter (μm)	0.63 ± 0.33	1.78 ± 0.85
Average peak-to-valley roughness, R_a (μm)	0.126 ± 0.003	1.287 ± 0.011
Root-mean-square peak-to-valley roughness, R_{RMS} (μm)	0.158 ± 0.003	1.597 ± 0.011
Porosity (%)	69.6	72.0
Total pore area (m^2/g)	1.477	0.769