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Cellular response to zinc-containing organoapatite: An in vitro study of proliferation, alkaline phosphatase activity and biomineralization

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Abstract

We present a series of experiments investigating the in vitro biological activity of zinc-containing organoapatite (ZnOA)-coated titanium meshes. ZnOA is a hydroxyapatite-based material that contains poly(L-lysine) and zinc ions and can be coated onto titanium by treating the metal surface with poly(amino acids) that allow for electrostatic bonding of the mineral to the titanium surface. Preosteoblastic mouse calyaria cells were cultured on ZnOA-coated titanium meshes in a three-dimensional (3D) bioreactor, which provides an in vitro culture environment that better simulates what cells experience in vivo, compared to traditional 2D cultures. Results of these studies show a time-dependent cascade of events leading to an earlier onset of alkaline phosphatase (ALP) expression and biomineralization of ZnOA-coated samples as compared to controls. After the observation of peak ALP levels in ZnOA-coated titanium samples, mineralized bone nodules were observed by scanning electron microscopy. Tetracycline staining confirmed that the observed mineral nodules were newly synthesized biomineral, and not due to the inorganic coating. ZnOA-coated titanium substrates represent a new class of materials for human repair that provide, mechanical stability, as well as chemical and biochemical signals to promote new bone growth.

Keywords: Biomineralization; Bioreactor; Bone repair; Bone tissue engineering

1. Introduction

Bone regeneration requires materials capable of providing mechanical and chemical signals to promote biomineralization [1–3]. Traditional inert biomaterials that illicit a minimal immune response were considered ideal for implants, however, these materials often do not match the mechanical needs of the implant site, or are subject to fibrous encapsulation over time, leading to implant failure [4,5]. Bioactive materials, such as hydroxyapatite (HA) have become increasingly useful as a new generation of biomaterials [6–10]. HA is the mineral component of bone, and has been shown to be both osteoinductive and osteoconductive, stimulating biomineralization in vitro and in vivo [11–19]. Recent studies of the mechanical properties of implant materials have shown that nanocrystalline HA increases the adhesion and proliferation of osteoblasts in culture [20,21] and that surface roughness of metallic implants, which are still needed to provide mechanical stability for load-bearing implants, can promote cell differentiation and protein synthesis [22–26].

Combining the benefits of HA and titanium implants into hybrid materials for bone repair, we have developed bioactive materials known as organoapatites (OAs) which are composed of HA and organic macromolecules [27–29]. Stable OA coatings can be formed on titanium substrates by using poly(amino acids) as an electrostatic coating on the surface of titanium to nucleate the

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formation OA crystals [30]. OA coatings are synthesized by the precipitation of HA onto pretreated titanium substrates in the presence of additional poly(amino acid), which leads to a coating that contains small amounts of the poly(amino acid) and forms a nanocrystalline, porous, and coralline morphology [30,31]. OA-coated titanium substrates have been shown to promote the adhesion and proliferation of osteoblastic cells in in vitro studies [32].

Zinc, an important trace element found in bone, is known to increase biomineralization both in vitro and in vivo [33–35]. Treatment of cells or tissues with zinc ions can affect proteins and enzymes associated with matrix vesicle-mediated biomineralization [36,37]. In this process, small extracellular vesicles, referred to as matrix vesicles, are released by the cell and serve as incubators during the initial phases of biomineralization [38–40]. Crystals of HA grow inside the vesicle, rupturing the membrane of the vesicle as the crystals grown. These new mineral crystals are then deposited onto the extracellular matrix (ECM), forming a mineralized matrix [41,42]. Matrix vesicles are believed to contain high levels of enzymes such as alkaline phosphatase, a zinc enzyme responsible for the cleavage of inorganic phosphate from organic phosphates, and zinc ions have been shown to specifically increase alkaline phosphatase (ALP) activity and biomineralization both in vitro and in vivo [43–45]. Zinc is also involved in regulating the production of collagen ECM and the expression of sodium-dependent vitamin C transporter 2 (SVCT 2), which has been shown to promote osteoblast differentiation and biomineralization [46–48].

We have recently reported on the chemical modification of OA to introduce zinc ions into these materials, leading to zinc-containing organoapatite (ZnOA) [49]. Zinc ions were added as a post-treatment to preformed OA, which avoided problems associated with zinc inhibition of HA mineralization. Unlike other zinc-containing materials for bone repair, such as zinc-containing β-tricalcium phosphate (ZnTCP) or mixtures of ZnTCP and HA [50–52] ZnOA is phase-pure HA with approximately 2% zinc content by weight, which we proposed consists of zinc ions adsorbed to the surface of the OA crystals. ZnOA coatings have a similar morphology to OA and form uniform coatings on Ti wire meshes. Zinc ions are released from ZnOA over a period of 12 days in concentration range from 10–20 μM, which falls well within the therapeutic range for in vitro zinc treatment. We describe below in vitro studies with osteoblastic cells to determine the effect of ZnOA on cell proliferation, morphology, and biomineralization.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. All cell culture media and media supplements were purchased from Invitrogen, except fetal bovine serum (FBS), which was purchased from Hyclone.

2.1. Synthesis of zinc-containing organoapatite

ZnOA was synthesized by a method previously described [49]. Briefly, Ti mesh samples (Goodfellow, Berwyn PA, 0.23 mm wire diameter, twill weave, 20% open area) were cleaned, passivated and dried prior to use. Meshes were pretreated in 1 mM poly(L-lysine) ($M_W = 50{,}000$) pH 7.4 for 22 h and 1 mM poly(L-glutamic acid) ($M_W = 52{,}000$) for 20 h. Ti samples were then transferred to a reaction flask containing 1 mM poly(L-lysine) and maintained at 37 °C. 9 mM $Ca(OH)_2$ and 9 mM H_3PO_4 were added and allowed to react to form OA, which precipitated both on the surface of the Ti mesh and in solution. Insoluble OA was collected by centrifugation and resuspended in a solution containing 4.54 mM $ZnCl_2$ at pH 7.4. Ti samples were added to the zinc-containing solution and stirred overnight to introduce zinc ions into the material. Bulk OA was added to the zinc solution to prevent the dissolution of OA from the surface of the Ti meshes.

2.2. Cell culture

Preosteoblastic MC3T3-E1 cells were obtained as a gift from Dr. Lonnie Shea. Cells were maintained in MEM-α media supplemented with 10% FBS, penicillin/streptomycin, and 50 μg/mL β-glycerolphosphate. Cells were cultured in T25 culture flasks prior to use.

2.3. Three-dimensional cell culture

A cylindrical three-dimensional (3D) rotating bioreactor was used for all in vitro studies (Synthecon Industries). The reactor vessel was 7 cm in diameter (120 mL volume), and was rotated at 20 rpm throughout the experiment. Prior to cell seeding, the bioreactor was conditioned with a bovine serum albumin to block protein adhesion and prevent cell attachment to the walls of the bioreactor, followed by MEM-α media containing FBS. To suspend titanium meshes in the 3D culture, samples were placed on round stainless-steel skewers (1 mm diameter), separated by cylindrical Teflon spacers (2 mm diameter), and inserted into the bioreactor. Three different titanium mesh coatings were used: ZnOA, OA and poly(Lys)/poly(Glu), as well as bare, uncoated titanium controls. MEM-α media used for 3D cell culture was supplemented with an additional 50 μg/mL ascorbic acid, with media exchanges occurring every 3 days. MC3T3-E1 cells were dynamically seeded into the bioreactor at a density of 1.0×10^5 cells/mL. After 4 days in culture, 50 μg/mL tetracycline was added