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Multifunctional role of osteopontin in directing intrafibrillar mineralization of collagen and activation of osteoclasts

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ABSTRACT

Mineralized collagen composites are of interest because they have the potential to provide a bone-like scaffold that stimulates the natural processes of resorption and remodeling. Working towards this goal, our group has previously shown that the nanostructure of bone can be reproduced using a polymer-induced liquid-precursor (PILP) process, which enables intrafibrillar mineralization of collagen with hydroxyapatite to be achieved. This prior work used polyaspartic acid (pASP), a simple mimic for acidic non-collagenous proteins, to generate nanodroplets/nanoparticles of an amorphous mineral precursor which can infiltrate the interstices of type-I collagen fibrils. In this study we show that osteopontin (OPN) can similarly serve as a process-directing agent for the intrafibrillar mineralization of collagen, even though OPN is generally considered a mineralization inhibitor. We also found that inclusion of OPN in the mineralization process promotes the interaction of mouse marrow-derived osteoclasts with PILP-remineralized bone that was previously demineralized, as measured by actin ring formation. While osteoclast activation occurred when pASP was used as the process-directing agent, using OPN resulted in a dramatic effect on osteoclast activation, presumably because of the inherent arginine–glycine–aspartate acid ligands of OPN. By capitalizing on the multifunctionality of OPN, these studies may lead the way to producing biomimetic bone substitutes with the capability of tailorable bioresorption rates.

1. Introduction

Many researchers are investigating biodegradable polymeric and composite-based biomaterials for potential use as bone grafts, where these synthetic materials are resorbed either through hydrolytic or enzymatic mechanisms. Alternatively, designing biomaterials using a biomimetic approach increases the potential for resorption through osteoclastic resorption; that is, the natural process by which bone is removed by osteoclast cells during remodeling. In this case, the entire bone does not degrade, but instead small canals are resorbed through the bone by osteoclasts, followed closely by osteoblasts that deposit new bone; thus the surrounding bone retains its structural and mechanical integrity throughout the remodeling process. Therefore, for the long-term goal of preparing a bioresorbable load-bearing bone substitute, it could be advantageous for the material to have a more bone-like resorption response that parallels the natural remodeling process. Our approach towards this goal is to emulate the natural processes involved in bone formation in order to develop nanostructured hydroxyapatite (HA)/collagen composites with a bone-like architecture that can serve as such bioresorbable materials.

In our previous work, we have used the polymer-induced liquid-precursor (PILP) mineralization process to generate intrafibrillar mineralization of collagen. In this process, negatively charged acidic polymers (considered simple mimics to the non-collagenous proteins, or NCPs, associated with bone) are added to supersaturated salt solutions in order to sequester ions or clusters that phase separate into droplets of an amorphous mineral precursor that can infiltrate the interstices of the collagen fibrils, leading to a high degree of intrafibrillar mineralization. In contrast, the conventional mineralization reaction without polymer results in heterogeneous nucleation of spherulitic clusters of HA on the surface of the collagen [1–10]. The HA crystals which form within the interior of the collagen fibrils in the PILP process are even oriented in the same way as they are in biogenic bone, aligned with their [001] direction parallel to the long axis of the collagen fibril, which is apparently dictated by the collagen itself [2,11–13]. This creates an

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interpenetrating nanostructure which closely resembles that of bone. Using this process, we have been able to mineralize a variety of collagen-based scaffolds, including reconstituted type-I collagen films prepared in-house [10], or commercially prepared porous collagen scaffolds [2,9], bovine and turkey tendon [7], demineralized manatee [8] and bovine bone [14], as well as artificial dentin lesions [15]. With the proper reaction conditions, a high degree of mineralization can be achieved, with compositions matching bone (60–70 wt.% mineral). Lausch et al. [13] have shown that this process can be used to remineralize the collagen tissues connecting bones to teeth, and with spatial control apparently dictated by other components retained in the native tissues.

Our attention here now turns to whether or not a real non-collagenous protein, such as OPN, can yield mineralization results similar to those achieved with polyaspartic acid (pASP). If OPN can also induce a PILP-type mineralization, it would support our hypothesis that this may be one important role of the NCPs in bone mineralization. Given that OPN has a domain with 8–10 consecutive aspartic acid residues, along with many phosphorylated residues [16], and the acidic NCPs (such as OPN and bone sialoprotein) tend to have a flexible and intrinsically disordered structures (non-globular, without a well-defined tertiary structure) [17–20], it might be reasonable to expect similar ion interactions with these polyanionic NCPs as seen with pASP. Furthering this possibility, other work has shown OPN to form an amorphous hydrated calcium phosphate/phosphopeptide complex in supersaturated solutions [21], which might be analogous to the PILP phase. This work showed that the calcium phosphate (CaP) nanoclusters are comprised of a spherical core of an amorphous hydrated CaP surrounded by a dense shell of sequestering phosphopeptide [21].

In addition to the putative role these NCPs play in the bone mineralization process, they can also contribute to the mechanical properties [22–25], as well as cell signaling. Thus, if a PILP-type mineralization of collagen can be directed by OPN, the OPN will become incorporated throughout the biomimetic bone substitute material, potentially providing biomechanical linkages as well as cell attachment sites. Osteopontin is known to contain arginine–glycine–aspartate acid (RGD) sequences, which serve as integrin ligands, and integrin signaling is vital for both osteoclasts bone resorption and osteoblasts function.

From the biomaterials perspective, where such biomimetic HA/collagen composites might be used as bone grafts, we are interested in determining if their bone-like nanostructure might allow for osteoclast resorption for a natural remodeling process. Kikuchi et al. [26] have shown that nanostructured collagen–hydroxyapatite composites (prepared by a non-biomimetic method) can be resorbed by phagocytosis by osteoclast-like cells, so it was anticipated that our biomimetic nanostructured composites should also be amenable to osteoclast resorption.

Here, we report on two sets of experiments with the goals of: (i) determining if OPN can act as a process-directing agent to generate intrafibrillar mineralization of type-I collagen scaffolds; and (ii) determining if incorporation of OPN into bone-like collagen scaffolds influences osteoclast activation. For the second study, dense bone slices were demineralized and then remineralized back with an OPN-mediated PILP process in order to prepare dense solid substrates that allow osteoclasts to form a sealing zone, which can be readily tracked with a fluorescent marker to determine if they have become activated. These studies demonstrate that OPN can act as a PILP process-directing agent, and that incorporation of the OPN into the matrix promotes osteoclast activation, in contrast to a matrix mineralized by conventional mineralization processes. This may represent a step towards the goal of the construction of fully biocompatible and bioresorbable load-bearing bone substitutes.

2. Materials and methods

2.1. Bovine OPN purification

Three hundred milligrams of a bovine milk-derived calcium salt osteopontin mix was purified from bovine milk as previously described [21,27]. To remove the calcium salt, the peptide mixture was dissolved in 5 ml of deionized (DI) water and placed in a dialysis tube with a porosity of 10,000 molecular weight cut-off. The osteopontin solution was dialyzed against 1 l of 1 mM Na_2H_2EDTA (disodium salt of ethylene diamine tetraacetic acid) and 1.5 mM NaN_3 (sodium azide) at 4 °C for 1 day. The EDTA solution was then replaced by 1 l of DI water and dialysis was continued for 8 h at 4 °C (3×). The remaining osteopontin was then collected, freeze-dried and stored at −20 °C until use. As described in detail elsewhere [21], the final mixture of peptides isolated contains 10 wt.% intact OPN (33.9 kDa) and 57 wt.% OPN with a molecular weight of 19.8 kDa, attributed to extensive degradation by the main milk proteinase plasmin. The remainder consists of smaller peptides.

2.2. Mineralization of collagen sponge

Commercially available collagen sponges (Ace Surgical Supply, Inc.) composed of reconstituted bovine type-I collagen were used as substrates for the mineralization experiments. The mineralization solution was prepared by mixing equal volumes of 9 mM $CaCl_2{\cdot}2H_2O$ (Sigma, St. Louis, MO) and 4.2 mM K_2HPO_4 (Sigma, St. Louis, MO) solutions, chosen based on the results of our prior studies. To maintain the pH of the mineralization solution at 7.4, calcium and phosphate solutions were made in Tris-buffered saline (TBS) containing 0.9% (w/v) NaCl and 0.02% (w/v) sodium azide (Sigma, St. Louis, MO). OPN-mix was added at a concentration of 200 μg ml^{-1} to 20 ml of calcium solution before mixing an equal volume of the phosphate counterion solution. An additional control group was made where the mineralization solution contained no polymeric additive. The sponges were incubated in the mineralization solution under vacuum conditions for 30 min to remove any air bubbles trapped within the porous matrix. After degassing the sample, the mineralization reaction was kept in an oven at 37 °C to emulate physiological conditions. At 4 days, the mineralized samples were removed from the solution, copiously washed with DI water, lyophilized and stored at −20 °C until use.

2.3. Demineralization/remineralization of bone specimens

Bovine bone slices approximately 100 μm thick were cut from the diaphysis of a bovine femur using a wet saw. Specimens reserved as native bone controls were briefly rinsed with DI water following sectioning. Other specimens were demineralized in a 0.5 M EDTA solution (made in TBS, pH adjusted to 8.0 with NaOH) for 3 days. Specimens were then rinsed in DI water for 24 h. Upon removal from the rinse, specimens were either immediately remineralized or lyophilized and stored at −20 °C.

Demineralized bone specimens were remineralized with calcium phosphate solutions to form HA as described above. Three groups of remineralized samples were made, differentiated by the type of negatively charged polymer used in the PILP process: (i) no polymer additive (conventional remineralization); (ii) polyaspartic acid–sodium salt, with an M_w of 27 kDa (Alamanda Polymers) at a concentration of 100 μg ml^{-1}; and (iii) osteopontin at a concentration of 100 μg ml^{-1}.