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Synergistic interplay between the two major bone minerals, hydroxyapatite and whitlockite nanoparticles, for osteogenic differentiation of mesenchymal stem cells

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ABSTRACT

The inorganic part of human bone is mainly composed of hydroxyapatite (HAP: $Ca_{10}(PO_4)_6(OH)_2$) and whitlockite (WH: $Ca_{18}Mg_2(HPO_4)_2(PO_4)_{12}$) minerals, where the WH phase occupies up to 20–35% of total weight. These two bone minerals have different crystal structures and physicochemical properties, implying their distinguished role in bone physiology. However, until now, the biological significance of the presence of a certain ratio between HAP and WH in bone is unclear. To address this fundamental question, bone mimetic scaffolds are designed to encapsulate human mesenchymal stem cells (MSCs) for assessing their osteogenic activity depending on different ratios of HAP and WH. Interestingly, cellular growth and osteogenic differentiation are significantly promoted when MSCs are grown with a 3–1 ratio of HAP and WH nanoparticles, which is similar to bone. One of the reasons for this synergism between HAP and WH in hydrogel scaffolds is that, while WH nanoparticles can enhance osteogenic differentiation of MSCs compared to HAP, WH counterintuitively decreases the mechanical stiffness of nanocomposite hydrogels and hinders the osteogenic activity of cells. Taken together, these findings identify the optimal ratio between two major minerals in bone mimetic scaffolds to maximize the osteogenic differentiation of MSCs.

Statement of significance

Human bone minerals are composed of HAP and WH inorganic nanoparticles which have different material properties. However, the reason for the coexistence of HAP and WH in human bone is not fully identified, and HAP and WH composite biomaterial has not been utilized in the clinic. In this study, we have developed bone mimetic HAP and WH nanocomposite hydrogel scaffolds with various ratios. Importantly, we found out that HAP can promote the mechanical stiffness of the composite hydrogel scaffolds while WH can enhance the osteogenic activity of stem cells, which together induced synergism to maximize osteogenic differentiation of stem cells when mixed into 3–1 ratio that is similar to human bone.

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1. Introduction

One in three people experience bone fractures during their lifetime, in which 10% of cases develop into nonunion fractures. These fractures cannot spontaneously recover, so they require external clinical treatment [1,2]. To treat nonunion fractures, autologous bone graft transplants are considered a gold standard based on their histocompatibility, as they have similar structure and composition with skeletal tissue around the fracture [3]. However, autografts are limited in supply, require secondary surgery, and can cause morbidity at the donor site [4–6]. Therefore, there have been many attempts to develop bone mimetic materials to substitute the structure and function of autografts. In particular, hydroxyapatite (HAP: $Ca_{10}(PO_4)_6(OH)_2$) bone ceramic materials have been widely investigated and used in the clinic, since it is the major bone mineral in the human body [7–9]. In addition, metallic biomaterials such as titanium, cobalt chrome, stainless steel, and magnesium have been utilized as weight-bearing implants based on their mechanical strength and biocompatibility [10–13]. However, despite recent advances in the field of regenerative medicine, until now there has been no true bone mimetic material that recapitulates the bone niche at the nanoscale, causing a gap between implanted material and the surrounding bone tissues.

At the nanoscale, human bone is composed of collagen fibers, and bone minerals such as HAP and whitlockite (WH: $Ca_{18}Mg_2(HPO_4)_2(PO_4)_{12}$) nanoparticles (Fig. 1a) [14–19]. WH is the second most abundant bone mineral in the human body, and occupies approximately up to 25 wt% and 35 wt% of the inorganic portion of human bone and tooth, respectively (Fig. 1b) [15,16,20–26]. However, despite its significant amount in human hard tissue, WH has not been applied to the clinic, largely due to its difficulty in synthesis. The reason for this difficulty is because HAP easily precipitates from calcium ions and phosphate ions at neutral pH, as HAP is one of the most thermodynamically stable calcium phosphate compounds in physiological conditions. In addition, HAP is a nonstoichiometric compound that allows for large disruptions of its atomic structure [15,16]. Recently, we found that a pure phase of WH nanoparticles can be precipitated in acidic aqueous system where excessive amounts of Mg^{2+} ions exist. In particular, the stability of HAP becomes lower in acidic pH conditions, and Mg^{2+} ions are too small to maintain the crystal structure of HAP, thus impeding its precipitation [16,27,28]. Based on this synthetic method, our group has previously analyzed the material properties of WH, and demonstrated the superior osteogenic capability of WH compared to HAP in both *in vitro* and *in vivo* [29,30].

HAP and WH have different atomic arrangements based on their hexagonal ($P6_3/m$) and rhombohedral (R3c) crystal structures, and exhibit different material properties [15,16]. For example, while HAP has greater stability in neutral pH conditions [30,31], WH has higher stability in acidic conditions (pH < 4.2) [28,31]. As a result, WH has a higher solubility than HAP in physiological conditions, and can continuously supply ions such as Mg^{2+} or PO_4^{3-} ions that can stimulate ion channels at the membrane of stem cells, and enhance the osteogenic activity of cells [16,30,32]. In addition, while HAP has a net neutral surface charge, WH has a negatively charged surface which enables positively charged osteogenic proteins such as bone morphogenetic protein (BMP) to be adsorbed on its surface by electrostatic interactions [30,33,34]. Since HAP and WH have different material properties, it is possible that HAP and WH may have different biological roles in human skeletal tissue.

In this study, we hypothesized that HAP and WH composites may induce a synergistic effect on the osteogenic activity of cells, as human bone is composed of HAP and WH, and because composite materials often exhibit improved biocompatibility and tissue regeneration capacity than monophase materials. For example, biphasic calcium phosphate materials composed of HAP and β-tricalcium phosphate (β-TCP: $Ca_3(PO_4)_2$) have better bone formation capacity compared to HAP or β-TCP when they are used alone in both *in vitro* and *in vivo*, probably based on the synergism

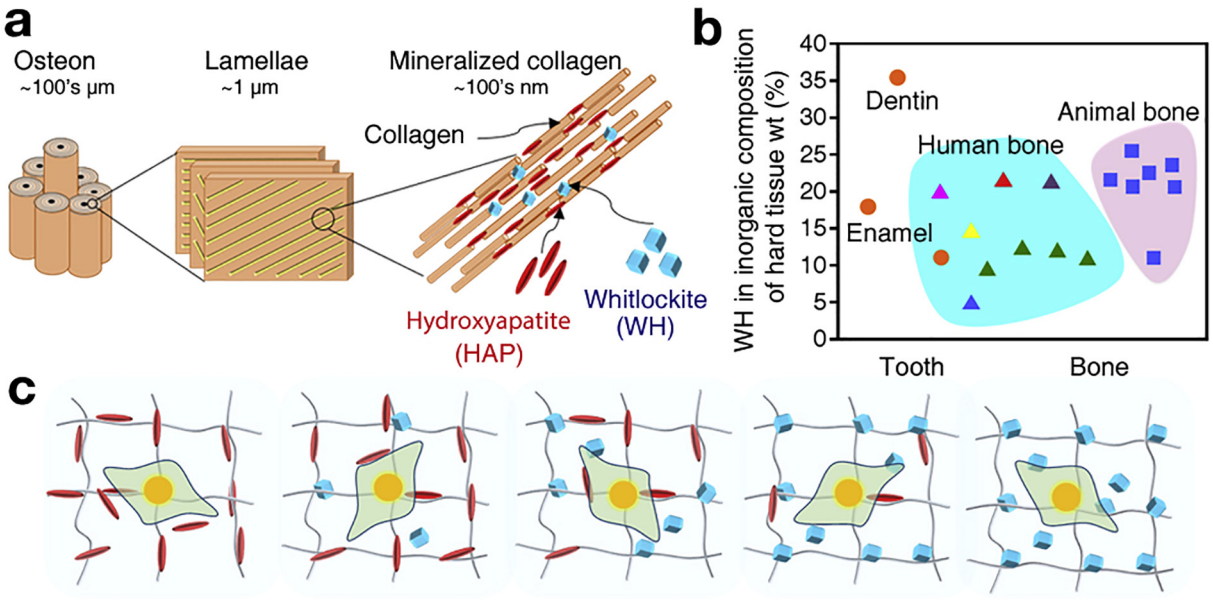

Fig. 1. The two major inorganic components of bone: hydroxyapatite (HAP: $Ca_{10}(PO_4)_6(OH)_2$) and whitlockite (WH: $Ca_{18}Mg_2(HPO_4)_2(PO_4)_{12}$) nanocrystallites. (a) Schematic of bone structure ranging from the nanometer to micrometer scales, showing that bone tissue consists of cylindrical osteon units at the microscale, which are composed of collagen nanofibers with HAP and WH bone mineral particles at the nanoscale. (b) Amount (weight percent) of WH in the inorganic part of hard tissues, calculated based on the magnesium amount. Data were obtained from previous publications, with different colors representing different references (Orange circle: Driessens et al. [16], Magenta triangle: Gabriels et al. [20], red triangle: Carlstrom et al. [26], purple triangle: Breibart et al. [21], blue triangle: Woodard et al. [22], yellow triangle: Duckworth et al. [23], green triangle: Zipkin et al. [24], blue rectangle: Long et al. [10] (c) Schematic of experiments to identify optimal conditions in the stem cell niche for osteogenic differentiation of human mesenchymal stem cells (MSCs) with regard to the different ratios of HAP and WH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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