Porous Collagen-Hydroxyapatite Scaffolds With Mesenchymal Stem Cells for Bone Regeneration

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Current bone grafting materials have significant limitations for repairing maxillofacial and dentoalveolar bone deficiencies. An ideal bone tissue-engineering construct is still lacking. The purpose of the present study was first to synthesize and develop a collagen-hydroxyapatite (Col-HA) composite through controlled in situ mineralization on type I collagen fibrils with nanometer-sized apatite crystals, and then evaluate their biologic properties by culturing with mouse and human mesenchymal stem cells (MSCs). We synthesized Col-HA scaffolds with different Col:HA ratios. Mouse $C_3H_{10}T_{1/2}$ MSCs and human periodontal ligament stem cells (hPDSCs) were cultured with scaffolds for cell proliferation and biocompatibility assays. We found that the porous Col-HA composites have good biocompatibility and biomimetic properties. The Col-HA composites with ratios 80:20 and 50:50 composites supported the attachments and proliferations of mouse MSCs and hPDSCs. These findings indicate that Col-HA composite complexes have strong potentials for bone tissue regeneration.

Key Words: collagen scaffolds, hydroxyapatite, mesenchymal stem cells, tissue engineering, bone regeneration

INTRODUCTION

Combining a scaffold and living cells to form a tissue-engineering construct is an important concept for promoting the repair and regeneration of bone tissues. Mesenchymal stem cells are often used in such constructs due to their abilities to proliferate and differentiate toward bone-forming cells.[1] The design and fabrication of scaffolds, stem cell isolation and characterization, and the manipulation of stem cell differentiation and function are essential steps toward the development of a biologically viable and clinically useful construct for bone tissue regeneration and function.[2,3]

Autografts, allografts, xenografts, and alloplastic grafts have been used for bone repair and regeneration.[4] Autograft is derived from the individual for whom the graft is intended. Though it is considered the "gold standard" of bone grafting to induce bone formation and regeneration through osteogenesis, osteoinduction, and osteoconduction, the application of autografts is limited due to insufficient donor bone supply and additional surgical wounds. Allograft is the tissue taken from another individual of the same species as the host. It possesses osteoinductive property due to the presence of the natural components and growth factors. Xenografts are derived from species other than human and provide a natural architectural matrix and an excellent source of calcium and phosphate for new bone generation. Allografts and xenografts have the potential risks of disease transmission and are shunned by some patients. Alloplasts are synthetic materials that can be designed with various chemical compositions, physical forms, and different surface configurations for the repair of bone defects and enhancement of osseous in-growth. They only have osteoconductive property, thus limiting their ability in the repair of challenging defects. In the case of bone regeneration, the grafts are eventually remodeled and replaced by new bone in the host tissue.[5,6] Due to the limitations of these bone grafts materials, tissue engineering approaches involving scaffolds and living cells have been in the forefront of biomedical research with the advent of biotechnology and stem cell sciences.[7]

Alveolar bone deficiency due to tooth loss, infection, and trauma is the primary limiting factor for dental implant-supported prosthetic therapies. Though numerous bone grafting materials are commercially available, these products invariably fall into the categories of allografts, xenografts, and alloplastic grafts and have significant limitations in clinical applications. An ideal bone tissue-engineering construct is still lacking. A properly constructed scaffold-cell complex may encourage the formation and apposition of new bone by osteoconduction, osteoinduction, and osteogenesis.[8,9]

The purpose of the present study was to synthesize and develop a collagen-hydroxyapatite (Col-HA) composite through controlled in situ mineralization on type I collagen fibrils with

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nanometer-sized apatite crystals, and evaluate their biologic properties by culturing with mouse and human mesenchymal stem cells (MSCs) for cell attachment and proliferation assays and biocompatibility assays in vitro.

Materials and Methods

Synthesis of the Col-HA composites by direct precipitation in situ

Solutions of calcium salt and phosphoric acid (Ca/P = 1.66 mol) were used to synthesize HA particles and incorporate them on bovine type I collagen fibrils by a direct precipitation technique in situ. This technique was optimized to produce 3 different ratios of Col-HA composites (20%Col-80%HA; 50%Col-50%HA; and 80%Col-20% HA) as follows: bovine type I collagen (Sigma) was directly dissolved in 0.1 M phosphoric acid (H_3PO_4) and stirred at 40°C for 30 minutes, resulting in a 2.0% (w/w) collagen gel mixture in the final composites. The Ca/P 1.66 mol solution of calcium chloride dehydrate ($CaCl_2$) (EMD Chemical Inc) and phosphoric acid (H_3PO_4) (Sigma) was added drop-wise into the reagent mixture stirring at 40°C (Stire & Temp) to allow HA precipitate onto bovine type I collagen fibrils in the gel mixture. The pH of the mixture was adjusted to between 8.0 and 9.0 using NaOH solution (J.T. Backer) and hydrochloric acid (HCl) (Sigma), and then lowered to a final pH of 7.0–7.2. After aging and prefreezing at −20°C overnight, the Col-HA mixture was freeze-dried in a freeze dryer (FreeZone, Labconco 117) at −52°C under vacuum pressure of 0.052 mbar for 24 hours, followed by drying at 19°C, 0.97 mbar for 24 hours. Dehydrothermal cross-linking of the residual collagen fibrils was performed in a vacuum oven at 110°C for 24 hours. Final products of collagen-hydroxyapatite composites were cooled down to room temperature. One sample from each product was fractured to observed the internal structures using a scanning electron microscope (SEM) (Supra 40VP FESEM).

Mesenchymal stem cell attachment and proliferation in Col-HA composite scaffolds

To evaluate the biologic properties of prototype Col-HA composites as scaffolds, we performed MSC attachment and proliferation assays in vitro. Mouse MSCs ($C_3H_{10}T_{1/2}$) were seeded (2×10^5 cells/well) on 3 different ratios of Col-HA (20%Col-80%HA; 50%Col-50%HA; 80%Col-20%HA) composites in a 12-well plate and cultured in Basal Eagle Media (Invitrogen)which contained 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10% fetal bovine serum in a CO_2 incubator with 5% carbon dioxide, 95% humidified air at 37°C for 1, 3, 5, and 7 days. A commercial, pure type I collagen wound dressing material (CollaPlug, Zimmer Inc, Carlsbad, Calif) was used as a control. To observe the attachment and proliferation of mouse MSCs under the fluorescence microscope, the cells were cultured by Adi-GFP (3 μL/mL) transfection overnight.

Biocompatibility of Col-HA composite scaffolds

The 50%Col-50%HA composite was selected for further testing as it has the best mechanical properties suitable for bone grafting applications. We performed cytotoxicity assays using human periodontal ligament stem cells (hPDSCs) in vitro. We prepared and extracted solutions from 4 different test materials: 3 pure type I collagen (CollaPlug; Foundation, OTB, Tokyo, Japan; and a prototype collagen without HA) and the 50%Col-50%HA composite. Six samples of each material were prepared with each sample 5 mm in height and 8 mm in diameter. Samples were soaked in 15-mL centrifuge tubes with 10-mL phosphate buffered saline and placed into a shaking incubator (Max Q Mini 4000) at 37°C, 120 rpm for 48 hours.

Human periodontal ligaments were collected from the extracted third molars, and soaked into the Dulbecco's Modified Eagle Medium (DMEM) with 250 μg/mL gentamicin sulfate, glutamine, 5 pμg/mL amphotericin B, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10% fetal bovine serum, PDL was digested by a solution of collagenase type I (3 mg/mL) and Dispase (4 mg/mL) for 1 hour. hPDSCs were cultured by DMEM supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10% fetal bovine serum in a CO_2 incubator with 5% carbon dioxide, 95% humidified air at 37°C. Upon nearing confluence, cells are detached using 0.08% trypsin/0.04%EDTA (pH 7.2) and passage 1:3 into fresh culture dishes. The fourth passage hPDSCs (5×10^4 cells/well) were seeded in 60-mm culture dishes and cultured with 50% extracted sample solutions and 50% modified α-MEM supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10% fetal bovine serum in a CO_2 incubator for 1, 3, and 6 days. Living cells were harvested and the cell numbers were counted by a hemacytometer under a light microscope. Each sample had 6 duplicated count cell numbers and the means with SD were calculated. Analysis of variance (ANOVA) statistical analysis was applied to assess the differences of the cells proliferation between the test groups and the control group; a P value of $<.05$ was considered statistically significantly different between groups.

Results

The sponge-like plugs of prototype Col-HA composites were successfully fabricated with different collagen and HA ratios. The macroscopic and SEM views of the prototype type I collagen without HA and 3 different ratios of collagen-HA (20%Col-80%HA; 50%Col-50%HA; 80%Col-20%HA) composites are shown in Figure 1. The SEM views show the inside microstructures of the prototype pure type I collagen without HA and 3 different ratios of collagen-HA composites (Figure 1). Pure type I collagen and the 80%Col-20%HA composite showed more porosities and less densities.

Following 1, 3, 5, and 7 days of culturing mouse MSCs with Adi-GFP transfection ($C_3H_{10}T_{1/2}$ + Adi-GFP), we found that the 80%Col-20%HA and 50%Col-50%HA composites possessed the best biocompatibility and supported the attachment and proliferation of mouse MSCs (Figure 2). CollaPlug and the high HA (20%Col-80%HA) composite had unfavorable cell morphologies and quantities in comparison with 80%Col-20%HA and 50%Col-50%HA composites. These results suggested that an appropriate HA proportion in the composites facilitated cell attachment, proliferation, and differentiation.

As the 50%Col-50%HA composite appeared to promote attachment and proliferation of mouse MSCs and have

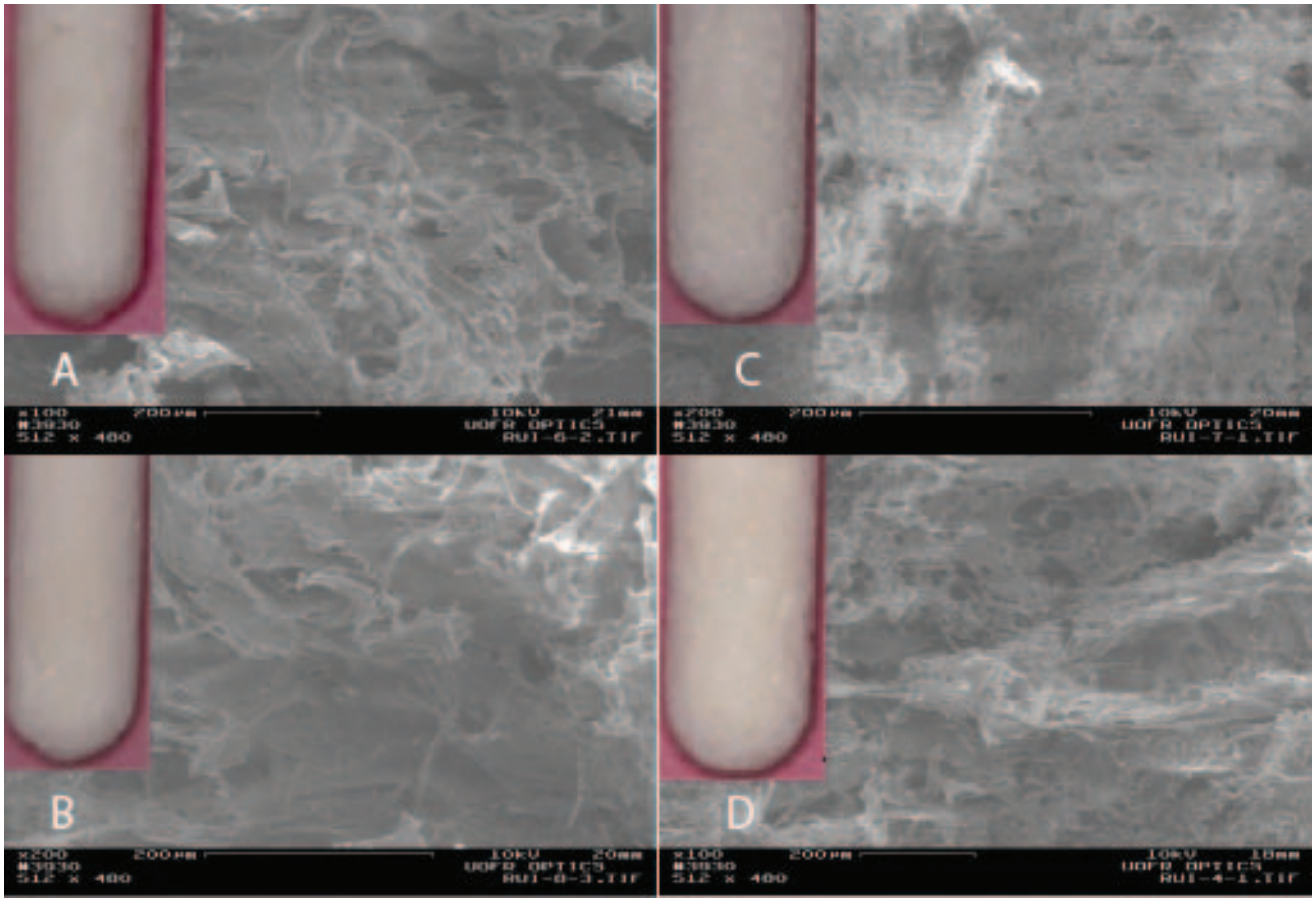

Figure 1. Macroscopic and scanning electron microscopy views of the prototype collagen-hydroxyapatite (Col-HA) composites. (a) Pure type I collagen without HA. (b) 80%Col-20%HA composite. (c) 50%Col-50%HA composite. (d) 20%Col-80%HA composite.

favorable mechanical properties in comparison to 20%Col-80%HA and pure type I collagen-based scaffolds, we used it for further testing. The hPDSCs biocompatibility assays showed that the 50%Col-50%HA composites have the same cell growth and proliferation properties as pure type I collagen materials (CollaPlug, Foundation, and a prototype collagen without HA) ($P > .05$, ANOVA; Figure 3).

Discussion

The findings of the presented study indicate that the porous sponge-like Col-HA composites have good biocompatibility and biomimetic properties and may be used as scaffolds for bone tissue regeneration. The Col-HA composites with ratios 80:20 and 50:50 supported the attachments and proliferations of mouse MSCs and hPDSCs. These findings indicate that Col-HA composite complexes have strong potentials for bone tissue regeneration.

A biocompatible scaffold is one of the basic ingredients for bone tissue regeneration.[10] The scaffold provides the framework for cell growth and differentiation at the implant sites. An ideal scaffold should facilitate cell attachment and proliferation and have a suitable biodegradation profile and good tissue biocompatibility.[11] The Col-HA composites developed in this study appear to be good candidates for scaffolds that can be used in bone tissue-engineering constructs. The major solid components of human bone are hydroxyapatite [$Ca_{10}(PO_4)_6(OH_2)$] (a natural ceramic, also found in teeth) and collagen (a natural polymer, also found in skin, cartilage, blood vessel walls, and tendons). The Col-HA composites have been shown to have the potential to mimic and replace skeletal tissues.[12]

In natural bones, carbonate substituted HA crystals are mineralized within small gaps of the collagen fibrils and have been quoted as 50 × 25 × 2–5 nm in length, width, and thickness, respectively.[13] The ideal bone graft materials should mimic the nano-textured environment to the natural bone. Nano-structured biomaterials are identified as less than 100 nm in at least one dimension. The HA crystals or particles produced by the direct precipitation method in this study are nano-meter scaled and resemble those of natural bone. This type of HA is perceived to be beneficial for bone regeneration applications, as it possesses the functional properties that facilitate cell growth and bone formation.[14–16]

The 3-dimensional (3D) scaffolds provide the necessary support for cells to adhere, proliferate, and in combination with host tissues, produce a mineralized matrix. Various 3D scaffolds have been seeded with osteoblasts or its precursor cells for tissue engineering applications.[17] In the present study, we used 3D collagen-hydroxyapatite composites and MSCs for bone regenerations. The images of the prototype type I collagen

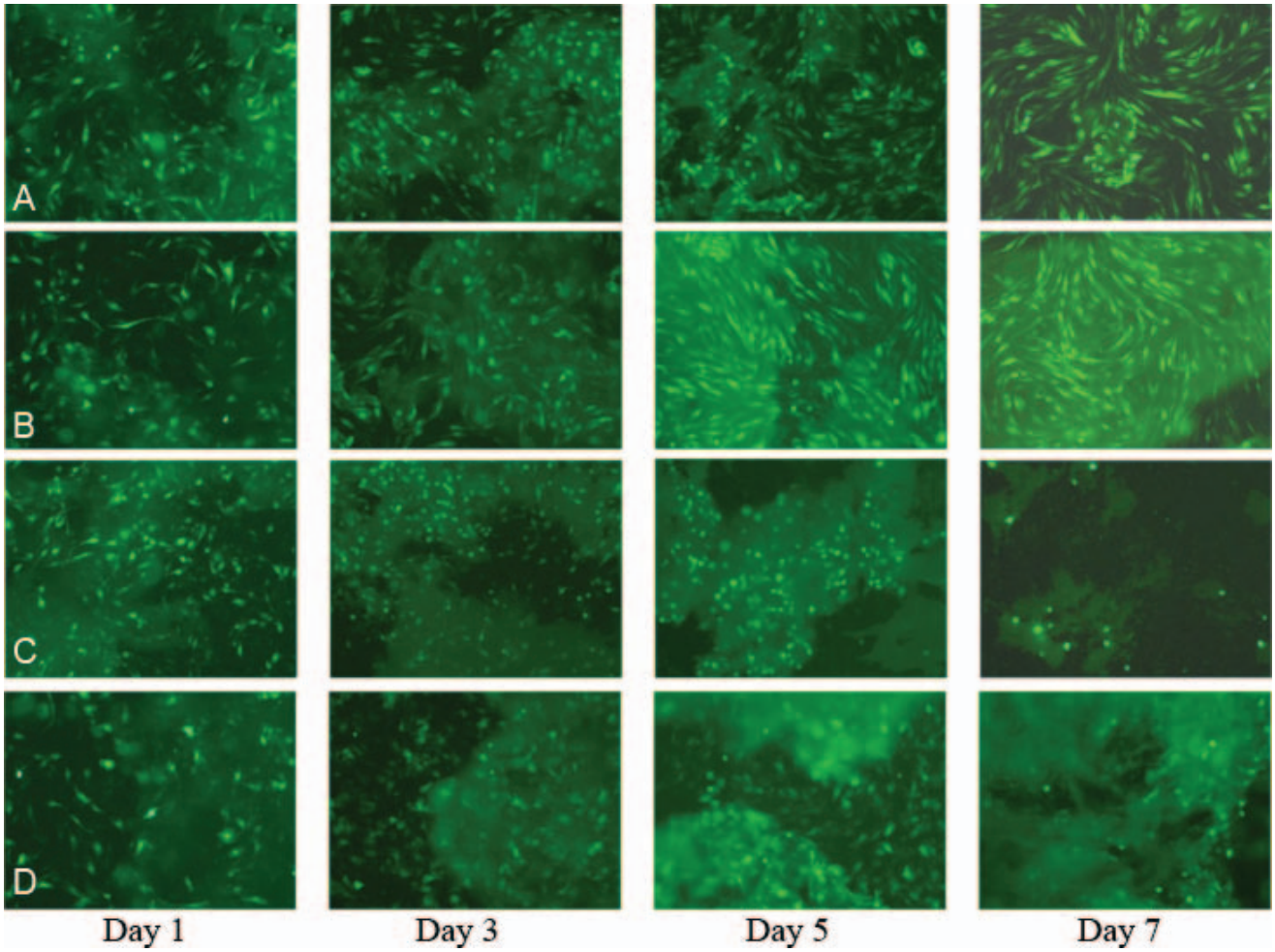

Figure 2. Prototype collagen-hydroxyapatite (Col-HA) composites support the attachments and proliferations of GFP tagged mesenchymal stem cells ($C_3H_{10}T_{1/2}$ + Adi-GFP) for 1, 3, 5, and 7days (fluorescence microscopy ×50). (a) 80%Col-20%HA. (b) 50%Col-50%HA. (c) 20%Col-80%HA. (d) Pure type I collagen (Collaplug).

without HA and 3 different ratios of Col-HA composites are sponge-like plugs with different density and rigidity. Several methods for synthesis of Col-HA composite exist.[13,18–20] We found that the methods developed in the present study using direct precipitation of HA on collagen fibrils, freeze-drying, and thermal cross-linking, consistently produced porous Col-HA composites that are suitable for stem cell and growth factor loading.

Osteogenesis is the mechanism for forming bone directly from osteoblasts. Osteoinduction means that bone graft materials are capable of inducing the transformation of mesenchymal cells into osteoblasts, and thus enhance bone growth. Osteoconduction is the process that permits bone apposition from the existing bone. Both type I collagen and hydroxyapatite are osteoconductive materials. While combined together, they were shown to promote osteogenesis by osteoblasts. Biomimetic collagen-hydroxyapatite composites were also shown to have good biocompatibility and biologic properties in animals and humans.[21–22]

MSCs possess self-renewing capacity and multilineage differentiation potential. High rate of ex vivo proliferation

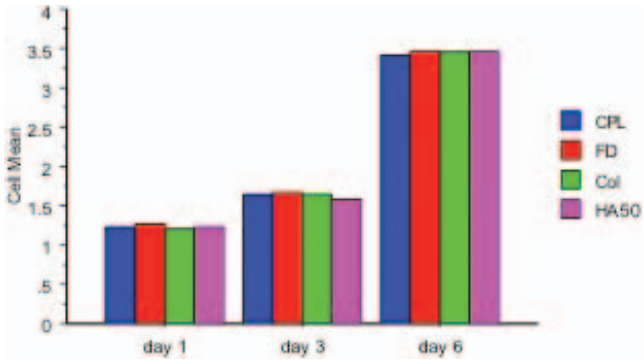

Figure 3. The results of cytotoxicity assays in vitro: the living human periodontal ligament stem cell (hPDSC) numbers were counted by a hemacytometer under a light microscope at 1, 3, and 6 days. The proliferation of hPDSCs (cell number × 10^5) on CPL (CollaPlug), FD (Foundation), collagen (a prototype collagen without HA), and hydroxyapatite (HA50) (50%Col-50%HA composite) ($P > .05$, ANOVA).

capacity makes these cells promising therapeutic candidates for many human diseases. Adult human MSCs, isolated from bone marrow or periosteum, have been shown to differentiate into a variety of mesodermal cell lineages, including osteoblasts. Multipotent human MSCs from various oral tissues including periodontium (PDSCs), dental pulp, apical papillae, and exfoliated deciduous teeth have been isolated and characterized.[23–25] The hPDSCs used in the present study are capable of differentiating into odontoblasts, adipocytes, neural cells, and osteoinductive cells. As hPDSCs have excellent biocompatibility with the Col-HA scaffolds, they may be a suitable resource of cells for maxillofacial and alveolar bone regeneration.

Conclusions

The porous Col-HA composites developed in the present study are biocompatible and can be used as scaffolds for bone tissue regeneration. The Col-HA ratio is an important factor in promoting the attachment and proliferation of mouse MSCs. The Col-HA composite complexes have strong potentials in bone tissue regeneration applications. hPDSCs may be a suitable resource of cells for maxillofacial and alveolar bone regeneration.

Abbreviations

Col: collagen
DMEM: Dulbecco's Modified Eagle Medium
HA: hydroxyapatite
hPDSCs: human periodontal stem cells
MSCs: mesenchymal stem cells
SEM: scanning electron microscope

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