Enhancing bone regeneration by combining mesenchymal stem cell sheets with β-TCP/COL-I scaffolds

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Abstract: The combination of bone marrow-derived mesenchymal stem cells (BMSCs) and biological scaffolds has been demonstrated to be a promising strategy for bone regeneration. However, this method does not result in satisfactory bone regeneration, because the BMSCs are dispersed in the biological scaffolds. The current study developed a new bone regeneration system, which combines synthetic porous three-dimensional scaffolds of β-TCP/COL-I composite with cultured osteogenic sheets of BMSCs. Activity of alkaline phosphatase (ALP), a marker of bone regeneration, was assayed in vitro using enzyme-linked immunosorbent assays and quantitative real-time polymerase chain reaction. In vivo bone regeneration was assayed in male nude mice. The study samples were BMSC sheet, scaffold/scattered BMSCs, scaffold/BMSC sheet, and scaffold alone. The samples were implanted dorsally in the mice. In vitro analysis showed that β-TCP/COL-I scaffold combined with BMSC sheets significantly upregulated both gene expression and protein levels of ALP, osteocalcin, and osteopontin. Histological and micro-computed tomography showed that the only implants that demonstrated new bone formation after 4 weeks were scaffold/BMSC sheet implants. These results underscore the crucial requirement of a synergistic effect of β-TCP/COL-I scaffolds and BMSC sheets. This could be a promising novel strategy for bone tissue engineering.

Key Words: mesenchymal stem cells, β-TCP/COL-I scaffold, bone regeneration, ELISA, quantitative real-time polymerase chain reaction


INTRODUCTION

Tissue regeneration using stem cells is one of the most promising techniques used to reestablish the integrity of injured tissues.1–4 The regeneration of bone defects resulting from trauma, infection, tumors, or congenital malformation still remains a clinical challenge.5–7 Autogenous bone grafting is still considered the golden standard for bone regeneration and remains the most commonly used surgical technique at present.8,9 However, the limitations of the method, such as the insufficiency of the bone that can be harvested and potential donor site morbidity, persist.8,9 Allografts can be used to overcome these disadvantages, but there is a potential risk of disease transmission or immunological rejection of allografts.

Biomaterials developed over the past several years include demineralized bone matrices, bioactive surfaces for implant components, and more recently, recombinant bone morphogenetic proteins (BMPs).10 Evolution of concepts such as “biological chamber” and “diamond concept” prompted the scientific community to consider the need for a more complex interaction between scaffolds (matrix), cells (mesenchymal cells), and signaling molecules (growth factors) in order to induce efficient bone regeneration to fill small or large bone defects.8,10

Bone tissue engineering has been shown to be an effective and attractive approach for promoting bone regeneration in various types and sizes of bone defects, in experimental and clinical settings.2–4,10,11 Suitable scaffold materials used as carriers for cells are at the forefront of bone tissue regeneration technology at the present time.11

Bone marrow-derived mesenchymal stem cells (BMSCs) are the ideal cell source for bone tissue engineering applications, because of their great ability for self-renewal and multipotential differentiation capacity, especially differentiation
into the osteogenic lineage. Therefore, BMSC-based tissue engineering is the most researched transplant technology in the area of bone tissue regeneration. The traditional BMSC harvesting method using trypsin digestion and EDTA degrades the extracellular matrix (ECM) and growth factors in cells, which are vital to the bone formation microenvironment. Moreover, using cell suspension would result in the loss of 30–40% cells seeded onto the scaffold. Cell sheet technology provides an effective strategy for using BMSCs, not only to minimize the destruction of the ECM, but also to improve cell retention at the transplant site and attachment to the scaffold. BMSC sheets wrapped around a scaffold showed more pronounced capacity for bone regeneration than did cells dispersed in the scaffold.

Several biocompatible materials including hydroxyapatite, β-tricalcium phosphate (β-TCP), collagen, and gelatin have been used as scaffolds in bone tissue engineering. Among them, β-TCP and collagen are the commonly used bone substitute materials because of the similarity of their molecular composition to that of bone, as well as excellent biodegradability and osteoconductivity. An ideal scaffold should also provide excellent three-dimensional (3D) microstructure that promotes the migration of cells in the local microenvironment toward and into the scaffold. In a previous study, we developed a β-TCP/COL-I composite scaffold with extensive porosity (approximately 95%) and appropriate pore sizes (approximately 100 mm). The microstructure of the composite with small particles of β-TCP and interconnected porosity is believed to provide an ideal initial platform for cellular viability and osteogenic differentiation, enhancing nutrient exchange and vascularization of the new bone tissue.

We hypothesized that β-TCP/COL-I scaffolds coated with BMSC sheets will exhibit superior osteogenic performance in vitro and in vivo. The objective of this study was to confirm this hypothesis by evaluating the osteogenic protein and gene expression in vitro as well as bone regeneration outcome in vivo, thus establishing a novel bone regeneration procedure.

MATERIALS AND METHODS

Materials used for the synthesis of the β-TCP/COL-I scaffold

Porous β-TCP/COL-I composite scaffolds were prepared as described earlier. Calcium chloride (CaCl₂) and tri-sodium phosphate (Na₃PO₄; both from Nanjing Chemical, Nanjing, China) were used as starting materials for the synthesis of β-TCP. Polyethylene glycol (PEG; Shanghai Chemical, Shanghai, China) was used as an additive for forming amorphous calcium phosphate (Ca₅(PO₄)₂) precursor. Type-I-collagen (Sigma-Aldrich, Saint Louis, MO) was used for the collagen matrix, and an aqueous solution of glutaraldehyde (GA; Shanghai Pharmaceuticals, Shanghai, China) was used as an agent to crosslink collagen fibrils. All other reagents and solvents used were of analytical grade.

Synthesis of β-TCP/COL-I scaffold

Briefly, CaCl₂ and PEG were dissolved in deionized water to form 0.1M CaCl₂ solution with a PEG/Ca molar ratio of 4. Na₃PO₄ solution (0.133M) was added to the CaCl₂ solution to a final Ca/P molar ratio of 1.5. The reaction was allowed to proceed for 30 min at pH 9 and 5°C. The precipitates were washed several times to remove Cl⁻ and Na⁺, and then dried in a freezing dryer for 48 h. After calcining the precipitates at 900°C for 3 h β-TCP powder was obtained.

Collagen was dispersed in dilute hydrochloric acid solution (pH 1–4) at 20°C. The TCP powders were then slowly stirred into type I collagen (Sigma-Aldrich, Saint Louis, MO) suspension at 20°C to a weight ratio of β-TCP to collagen of 1:2. After ensuring the formation of uniform suspension, the crosslinking reagent GA (C₅H₈O₂) was added to the mixture. The mixture was then molded into plastic tubes and frozen for 10 min using liquid nitrogen. The frozen mixture was lyophilized to obtain porous β-TCP/collagen composite. The porous composites were soaked for 4 days at 20°C in deionized water, changing the water daily, to remove unreacted GA. The soaked porous composites were lyophilized again to form the samples used in this study.

The microstructure of the β-TCP/COL-I scaffold was examined using a field-emission scanning electron microscope (FE-SEM SU70; HITACHI, Tokyo, Japan).

BMSCs, BMSC sheet culture and preparation of composite material

BMSCs were isolated as described earlier. Briefly, 2-week old male Sprague-Dawley rates were purchased from the Laboratory Animal Center of the First Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China). The animals were used for experiments after obtaining approval from the Ethics Committee of the Laboratory Animal Center. The rats were sacrificed by cervical dislocation, and the tibias and femurs were collected. The bone marrow was flushed out with 10 mL low-glucose Dulbecco’s modified Eagle’s medium (Gibco/Fisher Scientific, Hampton, NH) supplemented with 10% fetal bovine serum (SclenCell, Carlsbad, CA) and 1% penicillin and streptomycin (100 U/ml, Gibco/Fisher Scientific). The cell suspension was maintained in an incubator in a humidified atmosphere of 5% CO₂ at 37°C (Fisher Scientific). The medium was refreshed every 2 days. When the cultures grew to 70–80% confluency, BMSCs were digested with 0.25% w/v trypsin/0.02% EDTA (Gibco/Fisher Scientific) and sub-cultured. All experiments with BMSCs were conducted using cultures at passage 3.

To prepare an osteogenic matrix cell sheet, BMSCs were plated at a density of 1 × 10⁵ cells/cm² in a 6-cm cell culture dish (Thermo Fisher Scientific, Hampton, NH) and cultured with osteogenic medium containing 10 nM dexamethasone, 50 µg/mL ascorbic acid phosphate, and 10 mM β-glycerophosphate (all chemicals were from Sigma-Aldrich). The medium was refreshed every 2 days. After culturing for 8 days, the cell sheets were harvested mechanically. These BMSC sheets were wrapped around β-TCP/COL-I scaffold cylinders (8-mm diameter and 2-mm thickness). Sheets with or without scaffold were cultured in 1 mL osteogenic medium for 24 h, to facilitate the reattachment of BMSCs to the surface of the scaffold or culture dish, respectively. For control, three BMSC sheets were fixed with 4% paraformaldehyde and stained with hematoxylin and eosin stain.
Dispersed BMSCs were cultured under the same laboratory conditions and seeded on β-TCP/COL-I scaffolds for comparison with the sheet protocol and further characterization of cell-scaffold interactions. The β-TCP/COL-I scaffold combined with BMSCs was fixed with 2.5% GA for 2 h, followed by serial dehydration for 15 min in each of a series of ethanol baths (30, 50, 70, 85, 90, and 100%). Finally, specimens were air dried for 60 min and gold-sputtered for 60 s at 10 A (E-1010; Hitachi, Tokyo, Japan). Cell morphology was observed using SEM (Hitachi S-3400, at 2.00 kV; Hitachi Japan). Overall four experimental groups were created:

- **Group 1**: BMSC-sheet alone without β-TCP/COL-I scaffold
- **Group 2**: BMSC-sheet in combination with a β-TCP/COL-I scaffold
- **Group 3**: scattered BMSCs in combination with the β-TCP/COL-I scaffold
- **Group 4**: β-TCP/COL-I scaffold alone

**ALP activity assay and ELISA analysis**
After treatment with 0.1% Triton X detergent (Sigma-Aldrich) and centrifugation, the supernatant was collected for further intracellular protein measurements. The osteogenic differentiation capacity of experimental groups 1–3 was evaluated using ALP assay (Alkaline Phosphatase Assay Kit; Wako Chemicals, Richmond, VA). The protein expression levels of osteocalcin (OCN) and osteopontin (OPN) were measured using an ELISA kit (Fisher Scientific). OCN and OPN are two of the major noncollagenous matrix proteins in bone and are associated with pre-osteoblastic cell stages when matrix is synthesized. The results were normalized relative to the amount of total protein measured by a BCA Protein Assay Kit (Fisher Scientific).

**Quantitative real-time polymerase chain reaction (qRT-PCR) analysis**
The expression levels of osteogenic differentiation genes of BMSCs were measured using qRT-PCR. Total RNA was isolated using RNAiso plus (Takara, Kusatsu, Japan) according to the manufacturer’s instructions. For reverse transcription of messenger ribonucleic acid (mRNA), random-primed complementary deoxyribonucleic acid (cDNA) was synthesized from total RNA using a PrimeScript RT reagent kit to the manufacturer’s instructions. For reverse transcription, BMSCs were measured using Premix Ex Taq™ (Takara). The obtained cDNA was then amplified through PCR (Takara). The obtained cDNA was then amplified through PCR (Takara). The obtained cDNA was then amplified through PCR (Takara). Finally, specimens were air dried for 60 min and gold-sputtered for 60 s at 10 A (E-1010; Hitachi, Tokyo, Japan). The obtained cDNA was then amplified through PCR (Takara). The obtained cDNA was then amplified through PCR (Takara). The obtained cDNA was then amplified through PCR (Takara). Overall four experimental groups were created:

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**Comparison of osteogenic performance in vivo**
Six 5-week old male nude mice were provided by the First Affiliated Hospital of Medical School of Zhejiang University, Hangzhou, China. The animal experiments were performed after approval by the Ethics Committee of the Laboratory Animal Center of the First Affiliated Hospital. The mice were anaesthetized with 4% chloral hydrate (0.7 mL/100 g; Sigma-Aldrich) for the implantation of the experimental specimens. Mice were implanted with materials from experimental groups 1–4. Specimens from experimental group 4 served as control. Specimens were implanted subcutaneously in the back of nude mice (four implants, one of each experimental sample per mouse). The mice were then housed under standard conditions at room temperature with food and water ad libitum. After 4 weeks, all the animals were sacrificed and implanted specimens recovered. The harvested specimens were fixed in 4% paraformaldehyde (Wako) for 24 h, for radiographic and histological evaluations. The morphology of the specimens was assessed using a micro-CT system (Inveon, Siemens, Munich, Germany). The tissues were then embedded in paraffin and sliced into 5-μm thick sections for Masson’s trichrome staining. The stained sections were observed under a light microscope (BX41, Olympus Tokyo, Japan).

**Statistical analysis**
The characterization of the β-TCP/COL-1 scaffold and the BMSCs sheet, the histological, histomorphometrical and the micro-computerized tomography (Micro-CT) analysis was performed visually. The data from the evaluation of the osteogenic differentiation of BMSCs on the scaffold (ALP, OCN, OPN) were normally distributed (Kolmogorov-Smirnov-Lilliefors test). Parametrical tests (one-way analysis of variance, Scheffe’s post hoc test) were conducted for each time setting (10, 14, 21, and 28 days) using GraphPad Prism 6.0.2 software. A level of confidence of 95% (p < 0.05) was chosen. All values are expressed as mean ± standard deviation.

**RESULTS**

**Characterization of the β-TCP/COL-1 scaffold**
The morphology of the β-TCP/COL-1 scaffold was evaluated using SEM (Figure 1). As expected, the β-TCP/COL-1 scaffold exhibited a 3D interconnected porous structure, with high porosity (approximately 95%) and large pore size (approximately 100 μm). Collagen fibrils served as a “skeleton structure” with β-TCP particles dispersed uniformly on the surface of the fibril network. As shown in Figure 1(B), BMSCs infiltrated the β-TCP/COL-1 scaffold, and were also connected to one another through lamellar structures. These findings demonstrated the biocompatibility of the scaffold used in this study.

**Characterization of the BMSCs sheet**
BMSCs seeded at high density and cultured with osteogenic medium for 8 d grew confluent and their morphology changed from long spindle shape into polygonal or square shape, indicating osteoblastic differentiation [Figure 2(A)]. Mechanically harvested BMSC sheets were semitransparent membranes [Figure 2(B)], and were thick and robust enough to be handled mechanically for subsequent...
procedures. Histologically, the BMSC sheets were composed of multiple layers of cells and abundant extracellular matrix [Figure 2(C)].

Evaluation of the osteogenic differentiation of BMSCs on scaffold
The osteogenic differentiation of BMSC sheets (group 1), β-TCP/COL-I scaffold combined with the BMSC sheets (group 2) as well as β-TCP/COL-I scaffold combined with scattered BMSCs (group 3), was evaluated after osteogenic induction for 10, 14, 21, and 28 days in culture. The activity of ALP, considered as the bone regeneration biomarker in the early stages of osteogenesis, was assayed in cell lysates. After 14 and 21 days, ALP activity was significantly higher in experimental group 2 compared to group 3 (p < 0.01; Figure 3(A)). The expression levels in the group 1 reached a peak after 21 days, while there was no significant difference in the expression levels between group 1 and group 2 (p > 0.05). ALP expression level in group 3 remained the lowest out of all groups at all tested times.

As shown in Figure 3(B,C), the expression levels of OCN and OPN after 21 days were significantly higher in group 1 and group 2 than in group 3 (p < 0.01). group 2 exhibited sustained expression of osteogenic protein until 28 days of culture. OCN and OPN mRNA expression levels, which are indicative of the osteogenic activity, are shown in Figure 3(D,E). The expression level of OCN gene in group 1 and group 2 was upregulated significantly after 14 and 28 day relative to that in group 3 (p ≤ 0.05). OPN expression also demonstrated a similar trend. Overall, group 2 displayed the most significant osteogenic activity compared to the other two groups.

Histological examination and histomorphometrical analysis
Figure 4 shows representative histological photographs of the implanted specimens four weeks after implantation. As can be seen in these photographs, new bone formation occurred only in the experimental group 2, whereas there was minimal or no bone formation in the other three groups. In the center of the implants of group 2, osteoid with typical osteocytes embedded in dense matrix was surrounded by aligned osteoblasts, indicating intramembranous bone formation [Figure 4(B,B1)]. Histological images of group 1 consisted of fibroblast-like spindle-shaped cells and vessels filled with red blood cells [Figure 4(A,A1)]. Organized collagen bundles with osteocyte-like cells could be found in samples of group 3 [Figure 4(C,C1)]. In group 4, a few macrophages were dispersed in the fibrous tissue [Figure 4(D,D1)].
Micro-CT analysis
To evaluate new bone formation, micro-CT imaging was carried out at 4 weeks after surgery [Figure 5(A–D)]. The images revealed that new bone formation occurred only in group 2. We observed large areas of high density in all group 2 specimens [Figure 5(B)], indicating substantial new bone formation. These results were consistent with the results of the histological examination.

DISCUSSION
In the current study, β-TCP/COL-I scaffold combined with BMSC sheets exhibited higher and more prolonged expression of osteogenic genes and proteins \textit{in vitro}. This was also the only experimental group that showed sufficient new bone formation \textit{in vivo}. Therefore, we could accept our hypotheses that β-TCP/COL-I scaffolds wrapped in BMSC sheets provide superior osteogenic performance \textit{in vitro} and \textit{in vivo} compared to the other three experimental groups.

The β-TCP/COL-I scaffold consisted of inorganic crystals and organic matrix, in order to chemically and structurally mimic the native extracellular matrix of the bone. Collagen fibrils comprise polypeptide chains with interchain covalent and hydrogen bonds between the carboxyl groups. Commercially available collagens provide a relatively dense structure. After lyophilization, the disassembled collagen fibrils can become a porous material with an interconnected pore structure. When a crosslinking agent is added, it changes structure with larger pore size and pore walls. When β-TCP particles are added into the acidic collagen suspension, some of the β-TCP particles will dissolve in the acidic collagen suspension. Lowering the temperature of the suspension for the purpose of freeze-drying leads to the supersaturation of Ca\textsuperscript{2+} and PO\textsubscript{4}\textsuperscript{3–} and subsequent reprecipitation of other calcium phosphate salts. The general macroscopic and microscopic features (e.g., pore sizes, scaffold structure, deposition of the β-TCP particles on the
collagen fibrils, etc.) of the scaffold developed in this study were identical to those reported in an earlier study.23

The scaffolds used in bone tissue engineering need to be porous, with interconnected pores for nutrient exchange and cell proliferation.30 The β-TCP/COL-I scaffold used in the present study showed satisfactory biodegradability, as well as remarkable biocompatibility as demonstrated by the attachment of BMSCs to its bioactive surface and their proliferation and differentiation. The 3D structure with high porosity and large interconnected pores facilitated the ingrowth of local cells and sufficient supply of nutrients and oxygen owing to neovascularization (Figure 5).17 Additionally, a few macrophages were dispersed in the fibrous tissue [Figure 4(D,D1)], which might be attributed to the biodegradation and osteoimmunomodulatory properties of the scaffold. To summarize, the main scaffold components, in addition to providing physical framework, acted as biological activity regulators, elevating the expression levels of certain osteogenic biomarkers and stimulating osteoblastic differentiation of BMSCs.31,32 There is evidence in literature indicating that β-TCP can act as an osteogenic material and facilitate bone repair in an animal model as well as in clinical usage.6,23 Various biomaterials have been developed to enhance the osteogenic differentiation of BMSCs in vitro.14,35 However, in vivo bone regeneration, especially in ectopic sites, with these systems has been inefficient and unsatisfactory, because of the random dispersal of the cells in the biological scaffold.14,18,36 Cell sheet technology, developed recently, could be a good solution for bone tissue engineering and, therefore, continues to attract significant research interest.

Cell sheet technology could not only greatly improve the utilization and biological activity of BMSCs,36 but also provides abundant ECM, which is responsible for transmitting a wealth of biological signals that regulate bone formation. Fluorescence immunohistochemistry results showed that growth factors secreted by BMSCs were partially immobilized in the ECM, suggesting that BMSC sheets act as a slow-release system.27 Furthermore, cell sheets underwent significant contraction when harvested from culture dishes and the shrinkage-generated stress translated into a biomechanical force, which was thought to specifically regulate the polarization of BMSCs and activate their differentiation program.17,37 Several reports describe how the ideal properties of cell sheets improved the tissue regenerative microenvironment in the bone. Percutaneous injection of cell sheets seemed to be an attractive, minimally invasive option, which was applied for the treatment of cases with compromised bone healing.38,39 However, BMSC sheets played only a secondary role in promoting bone formation. Ma et al.40 fabricated a scaffold-free bone construct using cell sheet technology, providing an alternative strategy for bone repair. Cell sheets could be shaped to meet diverse clinical needs. Despite the many advantages of cell sheet technology, shortcomings of scaffold-free tissue-engineering methods, such as the inability to restore large-size bone defects and poor mechanical properties at early stage healing are significant.40–42

In recent literature, depending on the area to be regenerated the combination of biodegradable scaffolds providing the stability and BMSC cell sheets providing the biology seem to be the way forward, regardless whether bone,
cartilage or nerve tissue is repaired.\textsuperscript{2,43–47} In this aspect, the current study confirms recent findings and supports the use of scaffold over scaffold-less regenerative attempts, as scaffolds seem to be able to retain transplanted cells in damaged tissue.\textsuperscript{46} However, the use of a scaffold highly depends on the transplanted region as it is also known to induce redundant immunoreactions during the degradation processes.\textsuperscript{46} Additionally, as shown in this study, the combination of BMSC sheets instead of scattered BMSC seem to have a significant impact on the effectiveness of the regenerative attempt.

Interestingly, in the current study, in vivo results showed that optimal bone formation occurred only when β-TCP/COL-I scaffold and BMSC sheets were combined. Possible explanations include good vascularization due to the macro- and micro-porous structure of the scaffold facilitating the recruitment of endogenous endothelial cells and establishment of blood vessels.\textsuperscript{55} Second, BMSC sheets were reported to express a high level of vascular endothelial growth factor,\textsuperscript{27,49} an endothelial-specific growth factor, suggesting that BMSC sheets had the potential for angiogenesis as well as osteogenesis. The vascularization mediated by the scaffold and sheets facilitated the exchange of oxygen and nutrients. Growth factors from the newly formed vessels enhanced bone formation in the scaffold/sheet group.\textsuperscript{50,51} Additionally, the biodegradability of the β-TCP/COL-I scaffolds, demonstrated by the presence of macrophages, could have facilitated further differentiation of BMSCs, as macrophages are known to be effector cells in the innate immune response.\textsuperscript{52} Osteoimmunomodulatory response elicited by biomaterials could switch the phenotype of macrophages to the M2 extreme.\textsuperscript{53} The stimulated macrophages upregulate the expression of BMP-2 to activate the BMP-2 signaling pathway of BMSCs in the sheets, increasing their osteogenic potential.\textsuperscript{54}

The current study provides researchers with a basic, but refine able procedure of how to regenerate tissue. Attempts have already been made to improve the scaffolds with, for example, nano-particles to enhance osteogenic metabolic activity and thus tissue regeneration,\textsuperscript{55} or adding active molecules to the BMSC sheets (e.g., autologous platelet-rich plasma),\textsuperscript{1} or gene transfection (e.g., adenoviruses harboring the human [h]LEP gene).\textsuperscript{56}

**CONCLUSIONS**

The current study demonstrated that β-TCP/COL-I scaffolds combined with BMSC sheets provide a strong bone regeneration potential in vitro and in vivo. This enhanced bone
regeneration capacity is probably due to the synergistic effect of the porous structure of the scaffold facilitating vascularization and tissue in-growth and the osteogenic properties of the cells of the scaffold containing abundant ECM. In summary, the interaction between β-TCP/COL-I scaffolds and BMSC sheets seems to lead to a favorable osteogenic differentiation and bone growth.

CONFLICT OF INTEREST

None.

REFERENCES

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