A Better Biomaterial for Bone Regeneration: Type II Collagen

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ABSTRACT

Type II collagen is crucial to the development of embryonic skeletal system. Its functional defect in transgenic animal model results in severe chondrodysplasia.[1,2] The in vitro studies including ours showed that type II collagen promotes chondrogenesis of mesenchymal stem cells (MSCs).[3~6] Our recent studies have demonstrated that type II collagen also regulated MSC adipogenesis and the early stages of osteogenesis.[7] Type II collagen-coated surface enhanced osteogenesis-induced MSC calcium deposition, and increased protein expression levels of RUNX2. This mineralization-enhancing effect of type II collagen was diminished by MEK inhibitors. On the other hand, integrin α2β1 presents as the predominant receptor that responses to type II collagen in the osteogenic-induced MSCs. While implanted in the fracture bone of rats, the type II collagen-HA/TCP (nano-particle) scaffold group showed higher SFI scoring of foot-stepping and better locomotion as comparing to that of control group or type I collagen-HA/TCP group did. Collectively, type II collagen serves as an important modulator during early osteogenic differentiation of BMSCs, and enhance bone defect repair through an endochondral ossification-like process. The information advances our understanding about the cartilaginous ECM-BMSC interaction and provides perspective strategies for faster bone regeneration.

1 INTRODUCTION

The bone is an excessively hard tissue for providing support and protection from physical stress of the body. Bone tissue is a hardened connective tissue, which is composed of cells and specified extracellular matrices (ECMs). Different from other connective tissue, bone matrix is mineralized. Osteogenesis, the growth of new bone, is a part of the normal healing process and involves recruiting and activating mesenchymal stem cells or osteoblasts in bone. This can be a slow process, particularly in the elderly. In case that the bone fails to regenerate itself, bone loss occurs as a result of fractures or diseases. To accelerate the process of osteogenesis can enhance the healing of bone tissue after trauma, orthopedic operation or dental procedures.

One of surgical attempts to accelerate large bone defect repair is subchondral drilling. The subchondral drilling breaching leads to bleeding which brings in MSCs and important differentiation-inducing factors from the bone marrow. Thus, it induces the regular wound healing in the defect site. Bone implantation may be necessary in the event of reduction or damage of the bone owing to the trauma or the pathological change. Implants are commonly used in clinic to replace or reinforce injured or diseased hard bones. Besides, there have been a lot of materials and substances to promote bone repair and to replace or regenerate the defect site. These studies have been undertaken in an effort to address the problem of activating bone formation at a site which in need of bone replacement.

Materials such as type I collagen, calcium phosphate, and hydroxyapatite are wildly used as surgical implants and bone grafts in the orthopedic and dental fields. Among these materials, hydroxyapatite and tri-calcium phosphate has been extensively applied because of their superior biocompatibility and biodegradable property. However, as bone grafts have been a subject of intensive investigation, efforts have been made to develop new composites that can further promote the efficacy of bone implants. Previous studies have showed that type I collagen is able to trigger the osteogenic differentiation of bone marrow stem cells and osteoblasts through ERK1/2 signaling pathway.

As is known, MSCs and osteoblasts have great affinity toward type I collagen. Upon binding to type I collagen, ERK1/2 signaling pathway is activated to trigger the osteogenic differentiation of MSC and the ALP activity expression of osteoblasts. For this reason, the existing applications to repair bone tissue are mostly ultilizing type I collagen with calcium phosphate as a bone filling material. However, little report has addressed on the modulating effect of type II collagen during osteogenesis while it is mostly considered as a cartilaginous ECM. In previous report, type II collagen has been shown to modulate MSC chondrogenesis, chondrocyte differentiation, and to exert phenotype-restoring effect on the de-differentiated

chondrocytes. In this study, type II collagen was shown to modulate both osteogenic and adipogenic differentiation in MSCs. The possible molecular mechanisms involving the acceleration effect on osteogenesis and inhibitory effect on adipogenesis of type II collagen in MSCs were elucidated.

2 MATERIALS AND METHODS

2.1 MSC isolation

Bone marrow aspirates are obtained aseptically from donor who received femoral or iliac surgery with informed consent. Bone marrow is collected from the disposed aspirates using a 10 ml syringe. The aspirates are immediately mixed with sodium-heparin, and diluted in five volumes of phosphate-buffered saline (PBS). The cell suspension is then fractioned on a percoll gradient (1.077 g/cm3 of density, Pharmacia) and centrifuged. The MSC-enriched interface fraction is collected and plated in a 10 cm dish containing 10 ml Dulbeccos Modified Eagles Medium with 1 g/ml glucose (DMEM/LG Sigma D5523), 10 % FBS, 1x P/S/F (penicillin / streptomycin / fungizone). The medium is changed every four days. When the cells reach 80% confluence, they are trypsinized and passaged into new 10 cm dishes at a cell density of 5x105 cells / dish.

2.2 Osteogenic and Adipogenic Induction of MSCs

For osteogenic differentiation of human MSCs, cells are cultured in DMEM/LG medium supplemented with 10% FBS, 50 ug/ml L-ascorbate-2-phosphate, 10^{-7} M dexamethasone and 10 mM beta-glyceralphosphate. Adipogenesis is induced in the presence of 10% FBS, 0.5 mM methyl-isobutyl-methyl-xanthine, 0.5 mM hydrocortisone, 0.2 mM indomethacin, and 10 ng/ml insulin.

2.3 Calcium deposition assay using Alizarin Red S staining

To detect calcium deposition on the cell layer of differentiated MSCs, cells are rinsed rapidly with distilled water. Then 1 ml of pH 4.2 Alizarin Red S solution is added to cover cell surface for 5 minutes followed by washing thoroughly with distilled water. The calcium deposits exhibit orange red coloration on the cell surface and are recorded microscopically.

2.4 Animal Surgery

A bone defect model in rats was established to evaluate the repairing potency of type II collagen-HA/TCP substitute. A total of 16 male mature SD rats (8wk age) with the average weight of 300g were divided into 4 groups (Control / Agarose-HA/TCP / type I collagen-HA/TCP / type II collagen-HATCP) consisting of 4 animals for each implant type. Surgery was performed with anaesthesia of IM injection of 50 mg/kg ketamine and 10 mg/kg xylazine mixture. After shaving each animal's right hind limb with iodine-alcohol disinfection, a 5 mm segmental defect at the middle of the femur shaft was created using a 0.5 mm drill. Under sterile condition, the proximal and distal segments of femur were fixated with a 1.2 mm intramedullary rod. Aliquots of 60 ul collagen-HATCP substitutes were applied at the segmental defect site with the cover of a patch of 10 mm2 gel foam. The soft tissue was closed in two layers with absorbable sutures and the wound region was disinfected. The animals were allowed to move freely without any restrictions. Animals were followed by foot print analysis at 28 and 35 days.

2.5 Statistical Analysis

Each datum point obtained from three independent experiments or an experiment of triplicate assay was presented as mean ± SEM. The statistical analysis was performed by One-way ANOVA and Duncan's Multiple Range Test.

3 RESULTS

3.1 Type II collagen enhances osteogenic differentiation of MSCs

Alizarin red S (ARS) staining were used to examine the mineralization status of the culture. With osteogenic induction, MSCs cultured on type II collagen-coated plates exhibited a significant calcium deposition level by day 12, which was much earlier than that did the cells on the control (non-coated) plates by day 16. Furthermore, MSCs cultured on type II collagen-coated plates exhibited a greater level of calcium deposition as compared to that on the non-coated plates at day 12 and day 16 (Figure 1A).

To further examine the osteogenic marker gene expression in MSCs, the protein expression of RUNX2 in MSCs cultured on either non-coated and type II collagen-coated plates with osteogenic induction for 16 days were assessed. As shown in the Figure 2, CII-coated group showed a relative higher level of RUNX2 as compared to the non-coated control group, which was normalized by beta-actin expression level of each sample.

3.2 Type II Collagen-Modulated MSC Osteogenesis Involves ERK Signalings

MSCs were cultured in osteogenic medium on either control (non-coated) plate or type II collagen-coated plate

with / without the presence of MEK inhibitor (U0126, 10 μM). After 16 days of culture, cells were fixed and stained for the evaluation of calcium deposition. Compared to that of type II collagen-coated group, MEK inhibitor blocked the mineralization-enhancing effect of type II collagen (Figure 2). It appeared that ERK1/2 signaling play crucial roles in the type II collagen-enhanced osteogenesis of MSCs.

3.3 Osteogenic and Adipogenic Induction of MSCs

α1β1, α2β1, α5β1 and αvβ3 integrins has been reported to bind type II collagen, we examined the effects of functional blocking antibodies of these integrins on the attachment of osteogenic induced MSCs toward type II collagen. MSCs were induced with osteogenic medium for 4 days and treated with functional blocking antibodies of α1β1, α2β1, α5β1 and αvβ3 integrins for 30 minutes before they were attached to the type II collagen-coated plates. After 24 hours of attachment, cells were fixed and examined for their phenotype under microscope. The data showed that after osteogenic induction, MSCs exhibited obvious shape change with an extended cytoskeleton while attached to the type I and type II collagen-coated surface as compared to that in the control (non-coated) group. With the pretreatment of α2β1 blocking antibody, the changes in phenotype of MSCs upon attaching to type II collagen-coated surface were rescued, which means the attachment to the type II collagen-coated surface was blocked. However, pre-treatment of α1β1 and α5β1 was only partially affected the cell attachment mophology, while and αvβ3 blocking antibodies showed no alteration effect on the attachment of the cells. Combined with the data stated above, it is implied that integrin α2β1 presents as the predominant receptor that responses to type II collagen in the osteogenic-induced MSCs.

3.4 The Effect of Type II Collagen-HA/TCP bone Substitute in Bone Defect Repair

To evaluate the effect if type II collagen on bone repair in combination with HA/TCP bone substitutes, a segmental bone defect model was established in rats to examine the bone regeneration potency of type II collagen-HA/TCP. Briefly, a 5-mm segmental defect was created at the middle shaft of the right hind femur in each group of SD rats. The type I collagen-HA/TCP, type II collagen-HATCP or agarose-HA/TCP substitutes were prepared and applied in the created defect site. A control group with no substitute applied in the defect site was also performed. The footprint analysis represents the walking function (SFI score) of each group of the experimental rats at day 7, 14, 21, 28, 35 (Figure 4). The data indicates that at since day 14, the walking function of rats treated with various bone substitutes were better than the control (non-treated) group. Among the treated group, the type I collagen-HA/TCP and type II collagen-HA/TCP group showed a better improvement on walking function of rats comparing to the agarose-HA/TCP group.

4 FIGURES AND LEGENDS

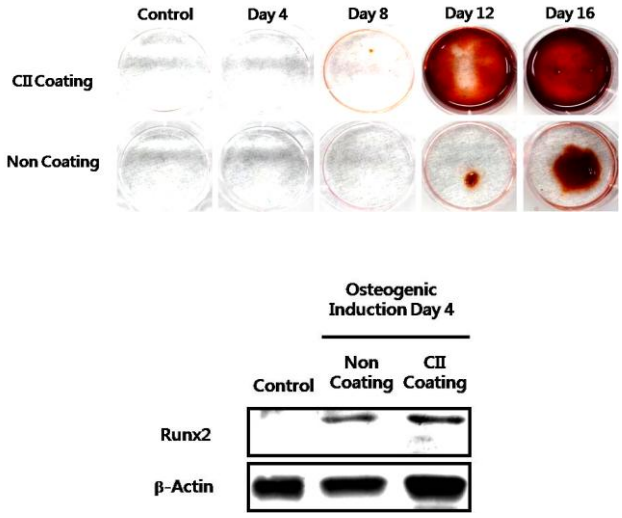

Figure 1: Calcium deposition levels and osteogenic marker gene expressions of osteogenesis-induced MSCs on type II-coated or non-coated plates: (A) MSCs were cultured on various coated culture plates in osteogenic medium for 4, 8, 12 and 16 days, fixed at each time point and subjected to alizarin S staining. The protein expression intensity of RUNX2 was normalized by β-Actin as internal control at day 4.

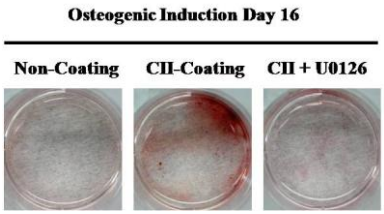

Figure 2: Alizarin red S staining of MSCs cultured on type II-coated or non-coated plates in the presence of MEK inhibitor: MSCs were cultured in osteogenic medium on non-coated or type II collagen-coated plates (CII-coated) in the presence of MEK inhibitor (U0126, 10 μM). After 16 days of osteogenic induction, cells were fixed and stained with alizarin red S for calcium deposition level evaluation.

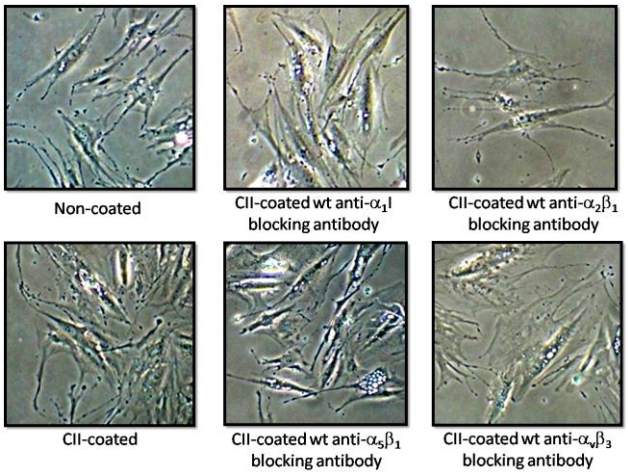

Figure 3: The attachment of osteogenic medium-induced MSCs pretreated with integrin blocking antibodies to type II collagen-coated surface. Cell morphologies were observed with MSCs attached on the control (non-coated) plates; type II collagen-coated plates; type II collagen-coated plates pretreated with α1β1 (α1I) blocking antibody; type II collagen-coated plates pretreated with α5β1 blocking antibody; type II collagen-coated plates pretreated with α2β1 blocking antibody; or type II collagen-coated plates pretreated with αvβ3 blocking antibody.

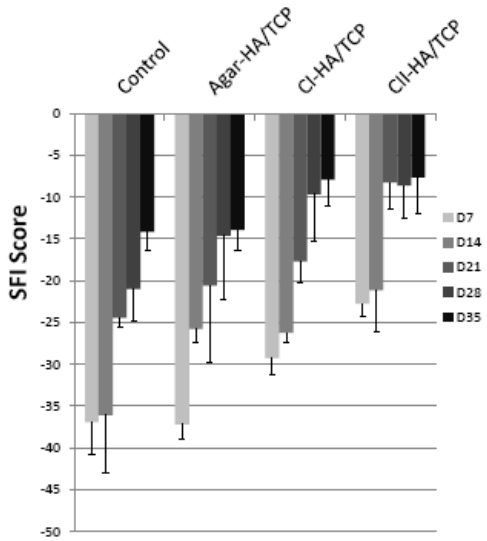

Figure 4: The SFI scoring of animals in each repairing group at 7, 14, 21, 28, 35 day after the surgery. Reference SFI Score: "0" for normal function; "-50" for complete loss of function.

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