The effect of type II collagen on MSC osteogenic differentiation and bone defect repair

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ABSTRACT

The function of type II collagen in cartilage is well documented and its importance for long bone development has been implicated. However, the involvement of type II collagen in bone marrow derived mesenchymal stem cell (BMSC) osteogenesis has not been well investigated. This study elucidated the pivotal role of type II collagen in BMSC osteogenesis and its potential application to bone healing. Type II collagen-coated surface was found to accelerate calcium deposition, and the interaction of osteogenic medium-induced BMSCs with type II collagen-coated surface was mainly mediated through integrin $\alpha2\beta1$. Exogenous type II collagen directly activated FAK-JNK signaling and resulted in the phosphorylation of RUNX2. In a segmental defect model in rats, type II collagen-HA/TCP-implanted rats showed significant callus formation at the reunion site, and a higher SFI (sciatic function index) scoring as comparing to other groups were also observed at 7, 14, and 21 day post-surgery. Collectively, type II collagen serves as a better modulator during early osteogenic differentiation of BMSCs by facilitating RUNX2 activation through integrin $\alpha2\beta1$-FAK-JNK signaling axis, and enhance bone defect repair through an endochondral ossification-like process. These results advance our understanding about the cartilaginous ECM-BMSC interaction, and provide perspective for bone defect repair strategies.

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1. Introduction

Osteogenesis involves the differentiation of bone marrow derived mesenchymal stem cells (BMSCs), which is an important process contributing to normal growth and wound healing of the bone tissue. The lost of function of BMSCs may result in delayed fracture healing and further pathological changes of the bone tissue [1,2]. Means to enhance the osteogenic differentiation of BMSCs could contribute to the healing of bone tissue after trauma, orthopedic surgery, or dental procedure. Type II collagen, a cartilaginous ECM molecule mainly present in the cartilage and developing bone, has been implicated to play important roles in both fracture healing and long bone development [1,3]. Transgenic mice bearing partially deleted type II collagen gene showed a temporary impairment of callus remodeling and fracture healing [4]. On the other hand, mice carrying a partially deleted type II collagen gene present phenotypes of chondrodysplasia, with characteristics of dwarfism, thick limbs, and delayed mineralization of bone [5].

Furthermore, transgenic mice with inactivated type II collagen gene expressed phenotypes that lack of endochondral bones and epiphyseal growth plates [6].

Some *in vitro* studies also imply the role of type II collagen matrix in the osteogenic differentiation of BMSCs. A proper chondrogenic induction of mesenchymal stem cells before implantation resulted in a more successful bone formation, in which a persisting type II collagen expression of the implant was observed [7]. Similarly, the chondrogenic pre-induction of β-TCP/BMSC composites which exhibited a significant production of type II collagen could enhance full bone formation, even including marrow organization [8]. At the cellular level, it is suggested that skeletal progenitor cells displayed type II collagen expression is sufficient to drive ectopic ossicle formation with myelosupportive stroma and adipogenesis [9]. Furthermore, it have been demonstrated that exogenous type II collagen promotes BMSC osteogenesis and inhibits adipogenesis, providing a clue that type II collagen itself may play an important role in cell fate commitment during the early stage of BMSC differentiation [10]. These results implied that type II collagen matrix is crucial to the differentiation of BMSCs not only during the early stage of embryonic bone development but also the fracture healing process. Consequently, it is hypothesized that through an endochondral ossification-like process, type II collagen is an important modulator for osteogenesis of BMSCs.

As is known, type I collagen enhances osteogenic differentiation and facilitates cell attachment of BMSCs [11]. When osteoblasts or BMSCs bind to type I collagen, ERK1/2 signaling pathway is activated to trigger the osteogenic differentiation of the cell [12,13]. Thus type I collagen has been widely applied for bone regeneration, such as combined with hydroxyapatite or calcium phosphates as a bone filling material [14–16]. On the other hand, the signaling pathway that type II collagen modulates BMSC osteogenic differentiation, or its possible application in bone regeneration has not been well elucidated. Herein, the molecular mechanisms of type II collagen for modulating BMSC osteogenic differentiation, and its possible role in bone defect repair, were carefully evaluated in this present study.

2. Materials and methods

2.1. Ethics statement

The protocols and informed consent form for BMSC isolation were approved by the Taipei Medical University Joint Institutional Review Board (TMUH-03-08-12). The specimen donor was provided with the IRB-approved formal consent form describing sufficient information for one to make an informed decision about his/ her participation in this study. The formal consent form was signed by the subject before specimen collection.

2.2. Reagents

Antibodies against human antigens CD105, CD73, CD44, CD29, CD90w, CD34, CD45, and CD14 were purchased from BD Biosciences (San Jose, CA, USA). Antibodies against human STRO-1 and RUNX2 were obtained from R&D Systems (Minneapolis, MN, USA). Functional blocking antibodies against integrins α1β1, α2β1, α5β1, and αvβ3, and antibody against VLA-2 complex used in FACS analysis were purchased from Millipore (Billerica, MA, USA). Antibodies against pFAK397, pFAK576/577, pFAK925, pERK1/2, pJNK, ERK1/2, JNK, and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibody against RUNX2 was purchased from MBL International Corporation (Woburn, MA, USA). Antibody against phosphoserine was purchased from Abcam (Cambridge, CB4 0FW, UK). Protein A-agarose immunoprecipitation reagent was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Alexa Fluor® 594 phalloidin was from Invitrogen (Carlsbad, CA, USA). DAPI (D9542) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium low glucose (DMEM/LG), Dulbecco's Modified Eagle Medium high glucose (DMEM/HG), Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F12), fetal bovine serum and other cell culture-related supplies were from Invitrogen (Carlsbad, CA, USA). Percoll solution was from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Type II collagen of chicken sternal cartilage (c9301) and type I collagen of rat tail tendon (c3867) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Alizarin Red S (A5533) was purchased from Sigma–Aldrich (St. Louis, MO, USA). RT-PCR related reagents include TRIzol®

SuperScript® III RT system were from Invitrogen (Carlsbad, CA, USA). SYBR Green I qPCR system was obtained from Roche Applied Science (Indianapolis, IN, USA). The porous biphasic HA/TCP scaffolds (Sinbone HT, hydroxyapatite/tricalcium phosphate = 60/40 in weight %) used in the *in vitro* study were obtained from Purzer Pharmaceutical Co., Ltd. (Taipei, Taiwan). The biphasic HA/TCP bone substitutes (BoneGraft, hydroxyapatite/tricalcium phosphate = 60/40 in weight %, granule size: 63–250 um) used in the animal study were obtained from Biotech One Inc (Taipei, Taiwan).

2.3. Mesenchymal stem cell (BMSC) isolation, cultivation and storage

Bone marrow aspirates were obtained aseptically from three donors (male, 40–65 years old) with informed consent. Bone marrow specimen was collected from the disposed aspirates using a 10 ml syringe. The aspirates were immediately mixed with sodium-heparin (10000U/ml), and diluted in five volumes of phosphate-buffered saline. The cell suspension was then fractionated on a Percoll gradient (1.077 g/cm^3 of density, Pharmacia) and centrifuged at $800 \times g$ for 30 min. The BMSC-enriched interface fraction was collected and plated onto a 10 cm dish containing 10 ml Dulbecco's Modified Eagles Medium with 1 g/ml glucose (DMEM/LG), 10% FBS, $1 \times$ P/S/A (penicillin/streptomycin/fungizone). The medium was changed every four days. When the cells reached 80% confluence, they were trypsinized and passaged into new 10-cm dishes at a cell density of 5×10^5 cells/dish. The cells were sub-cultured till passage 3 (P3). P3 cells were then seeded at a cell density of 6.5×10^3 cells/cm^2 and subjected to various studies. The remaining cells were collected, resuspended in 10% DMSO in FBS to a concentration of 2×10^6 cells/ml, and then stored in liquid nitrogen for later use.

2.4. Differentiation assay

The multipotency characteristics of BMSCs toward osteogenic, chondrogenic and adipogenic differentiations were assessed. For osteogenic differentiation of human BMSCs, cells were cultured in DMEM/LG medium supplemented with 10% FBS, 50 μg/ml L-ascorbate-2-phosphate, 10^{-7} M dexamethasone and 10 mM β-glyceralphosphate for 21 days. The chondrogenic differentiation of BMSCs was achieved by high-density micromass culture in the chondrogenic medium (DMEM/F12, 5% FBS, 10^{-7} M dexamethasone, 50 μg/ml L-ascorbate-2-phosphate, and 10 ng/ml TGF-β₁) for 21 days [17,18]. Briefly, BMSCs were suspended in DMEM/F12 medium at the density of 1×10^7 cells/ml. Droplets of 10 μl cell suspension were loaded into the culture dishes to form cell aggregates on the substratum. The droplets of high density cells were allowed to stand at 37 °C for 2 h, and then the chondrogenic medium was carefully loaded into the culture dishes. For adipogenic differentiation, BMSCs were induced in DMEM/HG medium in the presence of 10% FBS, 10^{-6} M dexamethasone, 0.5 mM methyl-isobutyl-methyl-xanthine, 0.2 mM indomethacin, and 10 μg/ml insulin for 21 days.

2.5. Flow cytometry analysis

BMSCs were fixed with ethanol overnight at -20 °C. Aliquots of 5×10^5 cells were incubated separately with each of the fluorochrome-conjugated antibodies against a panel of cell surface markers, including STRO-1, CD105, CD73, CD29, CD44, CD90w, CD34, CD14 and CD45 for 45 min at 4 °C. Cells were resuspended in Con's tube (BD) containing 200 μl of phosphate-buffered saline/1% bovine serum albumin and analyzed by Flow Cytometry using the FACS *Calibur* system (BD Biosciences).

2.6. Immunofluorescence staining

To evaluate the effect of type II collagen-coated surface on BMSCs, cells were cultured on either type I collagen-coated, type II collagen-coated or non-coated plates for designated intervals before subjected to immunofluorescence staining. After fixation, cells were blocked with blocking buffer (10% BSA, 0.3% Triton X-100) for 30 min, followed by incubation with each specific primary antibody for 2 h and with fluorescence conjugated-secondary antibody for 1 h at room temperature (RT). The cells were washed 3 times (10 min each) with PBS after the incubation. Cells were further stained with 0.1 μg/ml of DAPI (blue) at RT for 30 min to visualize the nuclei. For F-actin filament staining, BMSCs with or without osteogenic induction were allowed to attach on variously coated plates for 120 min, followed by formaldehyde fixation and immunostaining with Alexa Fluor® 594 conjugated-phalloidin (red) for 20 min at RT. Cells were further stained with 0.1 μg/ml of DAPI (blue) at RT for 30 min to visualize the nuclei.

2.7. Calcium deposition assay

To detect calcium deposition, the differentiated BMSCs were fixed with 4% formaldehyde at RT for 30 min, and rinsed rapidly with distilled water. Then, 1 ml of pH 4.2 Alizarin Red S solution was added to cover cell surface for 5 min, followed by washing thoroughly with distilled water. The calcium deposits exhibited as orange red sediments on the cell surface and were recorded microscopically.

2.8. Total RNA isolation and RT-PCR

Total RNA of cells after various treatments was extracted with TRIzol® reagent and stored at -80 °C for later use. RNA (500 ng) was then reverse-transcribed in a