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The influence of delayed compressive stress on TGF-β1-induced chondrogenic differentiation of rat BMSCs through Smad-dependent and Smad-independent pathways

Juan Li [a], Jun Wang [a], Yuanwen Zou [b], Yifan Zhang [a], Dan Long [c], Lei Lei [a], Lijun Tan [a], Rui Ye [a], Xuejin Wang [c], Zhihe Zhao [a,*]

[a] State Key Laboratory of Oral Diseases, West China College of Stomatology, West China Hospital of Stomatology, Sichuan University, Chengdu 610041, China
[b] Laboratory of Biomechanical Engineering, Sichuan University, Chengdu 610041, China
[c] Key Laboratory of Transplant Engineering and Immunology, Ministry of Health, West China Hospital, Sichuan University, Chengdu 610041, China

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ABSTRACT

Mechanical stimuli play important roles in regulating chondrogenic differentiation, but seldom studies have focused on when and how mechanical stimuli should be initiated. We have previously shown that Col2α1 mRNA was increased by delayed dynamic compressive stress initiated at the 8th day of chondrogenic culture. The current work is to further study the possibility of using delayed mechanical stress to relay chondrogenesis initiated by exogenous TGF-β1. Mechanical stimulation was delivered from day 8 to day 14 of chondrogenic culture. It showed that delayed compressive stress not only stimulated gene expression and protein synthesis of chondrocyte-specific markers, but also stimulated the endogenous TGF-β1 gene transcription, protein expression and the subsequent activation even when exogenous TGF-β1 was discontinued. Furthermore, mechanical stress also promoted protein phosphorylation and nuclear translocation of Smad2/3, the TGF-β1 downstream effectors. Inhibition TGF-β with SB431542 significantly affected the stress-induced chondrogenic gene expression. In addition, phosphorylated-p38 and RhoB were upregulated by delayed loading in a TGF-β-related manner. Phosphorylated-ERK1/2 and Wnt7a were also increased, but in a TGF-β-independent way. It indicates that delayed compressive stress can be used as an effective substitute for TGF-β1 supplement in inducing chondrogenic differentiation.
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1. Introduction

Cartilage tissue has a poor ability to self repair. Cell-based tissue engineering is believed as a promising treatment for damaged or diseased cartilages, and bone marrow derived mesenchymal stromal cells (BMSCs) are widely considered as a prospective cell source due to their rapid self-renewing capacity and chondrogenic differential potential. Many biochemical and biophysical means have been applied to enhance chondrogenic potential of BMSCs. Members of transforming growth factor-β (TGF-β) family, which have been proved to play central roles in regulating chondrogenic differentiation, are the most often used to induce chondrogenic differentiation of BMSCs [1–3]. On the other hand, mechanical stimuli are showing great potentials in regulating cartilage development both in vivo and in vitro, with similar effects to supplementation of exogenous growth factors like TGF-β [4–7]. Mechanical forces are reported to increase gene expression of extracellular matrix (ECM) such as type II collagen (Col2) and Aggrecan, result in more proteoglycan and collagen contents, and thus enhance chondrogenesis of MSCs [4,8–10].

Many efforts have gone to maximize mechanical regulation on chondrogenesis by optimizing loading parameters, especially in magnitude and frequency. However, the temporal effects were scarcely investigated and poorly understood so far. Seldom studies have focused on when and how the mechanical stimuli should be initiated [11]. As is well known, chondrogenesis is a complicated process beginning with mesenchymal condensation and followed by subsequent stages of proliferation, prehypertrophy, hypertrophy, and terminal differentiation [12]. In most studies, however, mechanical stress was applied at the initial stage of chondrogenic culture or concurrently with chemical chondrogenic ingredients. Mouw et al. reported that the level of mechanical stimuli was

* Corresponding author. State Key Laboratory of Oral Diseases, West China College of Stomatology, West China Hospital of Stomatology, Sichuan University, 14#, 3rd Section, Renmin South Road, Chengdu 610041, China. Tel.: +86 28 85503645; fax: +86 28 85582167.
E-mail addresses: alibaba1998@sina.com.cn (J. Li), zhaozhihe1963@yahoo.com.cn (Z. Zhao).

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related to its initial time [5]. Lima et al. also reported delayed dynamic loading applied after the discontinuation of the growth factors significantly increased tissue mechanical properties relative to applied concurrently with TGF-β3 supplementation [13]. Our research further showed that gene transcription of chondrogenic markers was increased by delayed compressive stress started at the 8th day of chondrogenic culture no matter whether the chondrogenic defined medium was continued or not [6]. These studies provide a cost-effective protocol to promote chondrogenesis by sequential application of growth factors and mechanical loadings.

TGF-β signal has been established as the core intracellular signal transduction in chondrogenic differentiation [14,15]. Huang et al. reported that compressive loading promoted chondrogenic gene expression in rabbit BMSCs by inducing endogenous gene expression of TGF-β1 as well as its receptors [16,17]. Recently, Li et al. reported that mechanical loading also induced protein synthesis of TGF-β1 and TGF-β3 in human MSCs [18]. These findings suggest that mechanical loading may stimulate MSCs to differentiate into chondrogenic lineages by activating endogenous TGF-β signal transduction, and can be used as an alternative substitute for exogenous TGF-β supplement when appropriately used. To further this area of inquiry, the current work is aimed to study the possibility and underlying mechanism of using delayed mechanical stress as a substitute to relay chondrogenesis initiated and progressed by TGF-β1.

The interaction between TGF-β signaling and mechanical stimulation seems to be quite complicated and could be due to a wide range of potential mechanisms. TGF-β triggers a series of downstream pathways via a transmembrane serine/threonine kinase receptor complex [19,20]. Smads are accepted as central cytoplasmic mediators of TGF-β [21]. Smad2 and Smad3, two structurally similar but functionally distinct proteins [22], are phosphorylated and translocated into the nucleus to regulate transcription of target genes such as Sox9, Col2 and Aggrecan upon TGF-β activation [23]. In addition to the Smad pathway, TGF-β also activates non-Smad pathways, such as ERK, p38, RhoB, Wnt and PKC [24]. Mouw et al. reported that phosphorylation of the Smad2/3 proteins was increased by mechanical loading during chondrogenesis [5]. We further reported that p38 MAPK was activated by cyclic compressive force in a rapid and transient manner to mediate subsequent transcriptional regulation in chondrogenic differentiation of rat BMSCs [6]. It gives clues that cartilage tissue development in culture may be facilitated by regulating interaction between mechanical stimuli and TGF-β-related cascades. However, the mechanism through which mechanotransduction occurs in chondrogenic differentiation still remains largely elusive. Substantial investigations are needed to further clarify the mechano-biochemical transduction during chondrogenesis.

The plan or strategy of this research is summarized in the following: (1) to find out the ideal initial time point for mechanical stimuli, and focus the research on the phase when differential BMSCs began to stably exhibit with typical chondrocyte features; (2) to find a cost-effective protocol to promote chondrogenesis by sequential application of growth factors and mechanical loadings; (3) to investigate the role of endogenous TGF-β1 in chondrogenic differentiation initiated by exogenous TGF-β1 and relayed by mechanical stress; (4) to preliminarily investigate the TGF-β1-related signal network including Smad-dependent and Smad-independent pathways which might be involved in mechanotransduction of the delayed loading protocol.

2. Materials and methods

2.1. Isolation of rat BMSCs

The BMSCs were isolated from bone marrow of rat tibias and femurs based on MSCs' selectively adherence to plastic surfaces as described previously [6,25]. The isolated marrow cells were cultured in α-MEM (Gibco, USA) supplemented with 10% FBS (Gibco, USA). Non-adherent cells were removed away by changing culture medium. When at confluent, the primary cultures were detached with 0.1% trypsin-EDTA and subcultured at a density of 10,000 cells/cm^2.

2.2. Chondrogenic induction in alginate scaffolds

The passage 2 cells were suspended in a low-viscosity alginate (Sigma, USA) solution (1.5% in 0.15 M sodium chloride) at a density of 5.0×10^6 cells/ml, and then added into a 120 mM $CaCl_2$ solution to produce alginate beads. The spherical beads were formed 10 min later. After being rinsed three times with PBS, the cell beads were cultured in chondrogenic defined medium which is serum-free high-glucose DMEM (Gibco, USA) containing 10^{-7} M dexamethasone (Sigma, USA), 50 μg/ml ascorbic acid phosphate (Sigma, USA), 1% ITS-A supplement (Gibco, USA) and 1% antibiotic-antimycotic. The chondrogenic culture medium was changed every 3 days. Recombinant human TGF-β1 (Peprotech, USA) was added into the chondrogenic defined medium according to the experimental design outlined in Fig. 2. The final concentration was 10 ng/ml. Control cells were cultured in the absence of TGF-β1.

2.3. Histological and immunohistochemical staining

Samples were collected for histological and immunohistochemical staining at the 1st, 3rd, 7th, and 14th day. Briefly, alginate beads were washed twice with cold PBS, polymerized in 55 mM $CaCl_2$ solution, fixed in 4% paraformaldehyde, and then processed into paraffin wax. Cell morphological changes were examined with hematoxylin and eosin (HE) staining. Sulfate cartilage matrix proteoglycan was stained with 0.2% Toluidine Blue and 0.5% Safranin-O, and type II collagen depositions were detected with immunostaining with rabbit polyclonal antibody against type II collagen (Boster Bio-tech, China, diluted 1:200) by a 3-step indirect immunoperoxidase technique. BMSCs cultured without TGF-β1 were used as a negative control.

2.4. Glycosaminoglycan (GAG) quantification

Wet weights of the alginate beads were measured at indicated time points. Then the alginate beads ($n = 5$ for each group) were incubated overnight at 60 °C in a papain solution containing 125 μg/ml papain, 5 mM L-cystein, 5 mM EDTA and 100 mM sodium phosphate. The glycosaminoglycan (GAG) contents were examined using the 1,9-dimethylmethylene blue (DMMB) assay, and chondroitin sulfate (Sigma, USA) was used as a standard for quantification. GAG content was normalized to the wet weight of alginate beads. Results were presented as fold inductions over that of the control.

2.5. Dynamic compressive loading

We have preliminarily proved that the rat BMSCs began to exhibit typical chondrocyte features represented as GAG depositions and ECM gene expression at the 7th day of chondrogenic culture (Fig. 2). So in the loading experiment, mechanical stress was applied to the cells beginning at the 8th day of chondrogenic culture. Specimens were divided into four groups: group 1 (TGF-β1 discontinued group), group 2 (mechanical stress group), group 3 (TGF-β1 continued group), and group 4 (TGF-β1 plus stress group). In group 1 and 2, the specimens were cultured in basal chondrogenic medium without supplement of TGF-β1; in group 3 and 4, the specimens were cultured in chondrogenic medium continued being supplemented with TGF-β1. The experimental protocol was schematically outlined in Fig. 2.

Mechanical stimulation was applied to the samples via a custom-made, computer-operated pressure system. Compressive pressure was implemented by increasing gaseous tension above the supernatant media in a sealed chamber. The details of the device were described in our previous report [6]. The loading specimens (group 2 and 4) were exposed to sinusoidal dynamic compressive force at 14–36 kPa with a frequency of 0.25 Hz. A daily regimen was delivered at 1 h per day. The loading experiments were conducted for 1, 3, 5, 7 consecutive days. The samples treated with TGF-β1 for 7 days were used as controls.

2.6. Gene expression analysis

Cells were recovered by digestion buffer containing 50 mM NaCl, 55 mM sodium citrate and 2 mM EDTA. Total RNA was extracted using Trizol Reagent (Invitrogen, USA), and quantified with a spectrophotometer. About 500 ng mRNA was reverse-transcribed into cDNA with PrimeScript™ RT Enzyme Mix I, Oligo dT Primer, Random 6 mers, and 5 × PrimeScript™ Buffer (DRR037A, TaKaRa, China) according to the manufacturer's protocol. Real-Time PCR was performed using the SYBR Prime Script™ RT-PCR kit (DRR041A, TakaRa) in an ABI PRISM 7300 Real-Time PCR System. The primers for chondrocyte-specific genes were described previously [6]. The primer sets for TGF-β1 were forward 5′-CTTGCCCTCTACAACCAACA-3′ and reverse 5′-CTTGCGACCCACGTAGTAGA-3′. The PCR program was initiated by 30 s at 95 °C before 40 thermal cycles, each of 5 s at 95 °C and 31 s at 60 °C. Sterilized ddH_2O was used as generated from a dilution series of an arbitrary sample. Results were

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