Cytotherapy, 2015; 17: 1213–1219

International Society for Cellular Therapy
ISCT®

BMP4 inhibits myogenic differentiation of bone marrow–derived mesenchymal stromal cells in mdx mice

CAO JIQING[1,2,*], LI YAQIN[1,2,*], LIANG YINGYIN[1,2], CHEN FEI[3], ZHANG HUILI[1,2], ZHU YULING[1,2], YANG JUAN[4], FENG SHANWEI[1] & ZHANG CHENG[1,2]

[1]Department of Neurology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China, [2]Guangdong Key Laboratory for the Diagnosis and Treatment of Major Neurological Disease, Guangzhou, China, [3]Department of Institute of Gynecology and Obstetrics, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, and [4]Department of Neurology, Zhujiang Hospital of Southern Medical University, Guangzhou, China

Abstract

Background aims. Bone marrow–derived mesenchymal stromal cells (BMSCs) are a promising therapeutic option for treating Duchenne muscular dystrophy (DMD). Myogenic differentiation occurs in the skeletal muscle of the mdx mouse (a mouse model of DMD) after BMSC transplantation. The transcription factor bone morphogenic protein 4 (BMP4) plays a crucial role in growth regulation, differentiation and survival of many cell types, including BMSCs. We treated BMSCs with BMP4 or the BMP antagonist noggin to examine the effects of BMP signaling on the myogenic potential of BMSCs in mdx mice. *Methods.* We added BMP4 or noggin to cultured BMSCs under myogenic differentiation conditions. We then injected BMP4- or noggin-treated BMSCs into the muscles of mdx mice to determine their myogenic potential. *Results.* We found that the expression levels of desmin and myosin heavy chain decreased after treating BMSCs with BMP4, whereas the expression levels of phosphorylated Smad, a downstream target of BMP4, were higher in these BMSCs than in the controls. Mdx mouse muscles injected with BMSCs pretreated with BMP4 showed decreased dystrophin expression and increased phosphorylated Smad levels compared with muscles injected with non-treated BMSCs. The opposite effects were seen after pretreatment with noggin, as expected. *Conclusions.* Our results identified BMP/Smad signaling as an essential negative regulator of promyogenic BMSC activity; inhibition of this pathway improved the efficiency of BMSC myogenic differentiation, which suggests that this pathway might serve as a target to regulate BMSC function for better myogenic differentiation during treatment of DMD and degenerative skeletal muscle diseases.

Key Words: *BMPs, BMSCs, Duchenne muscular dystrophy, dystrophin, mdx mice, myogenic differentiation, Smad*

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked inherited muscular disorder characterized by the absence of dystrophin [1]. Without dystrophin, the muscles undergo repetitive degeneration and regeneration cycles leading to muscle fibrosis and weakness [2]. Various attempts have been made to overcome DMD, including steroid treatment [3,4], gene therapy [5,6] and stem cell therapy [7]. Stem cell–based therapy provides a promising treatment option for DMD because healthy stem cells can restore dystrophin expression *in vitro* and *in vivo*. Many studies have demonstrated that mesenchymal stromal cells induce dystrophin protein production in mdx mice [8], dogs [9] and humans [10]. On the basis of previous studies, no high-yield myogenic cells were obtained in mdx mouse skeletal muscle after bone marrow–derived mesenchymal stromal cell (BMSC) transplantation [11]. However, because defective muscle regeneration cannot counteract repetitive degeneration, the myogenic differentiation capacity of BMSCs has unique therapeutic potential.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF–β) superfamily, and they regulate cell commitment and differentiation through intracellular proteins called Smads [12,13]. Initially, BMP4 was shown to play critical roles in osteoblast differentiation [14]. More recently, it was revealed that BMP4 also modulates myogenic differentiation in BMSCs. Grajales *et al.*

*These authors contributed equally to this work.

Correspondence: **Zhang Cheng,** PhD, Department of Neurology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou 510080, PR China. E-mail: chengzhang100@hotmail.com

(Received 23 February 2015; accepted 18 June 2015)

http://dx.doi.org/10.1016/j.jcyt.2015.06.010

[15] reported that BMP4 enhanced cardiac myogenic development and suppressed skeletal myogenesis, leading to loss of "stemness" in BMSCs. Another study showed that retinoic acid enhanced skeletal myogenesis in stem cells by inhibition of BMP signaling [16]. Noggin, a BMP antagonist, is crucial for proper differentiation of muscle progenitor cells, and it acts through inhibition of local BMP signaling during embryonic muscle differentiation [12,17]. Thus, we hypothesized that inhibition of BMP/Smad signaling may promote the myogenic differentiation of BMSCs *in vitro* and increase restored dystrophin in muscles of mdx mice. Therefore, the objective of the present study was to determine the effects of BMP4 on the myogenic differentiation potential of BMSCs *in vivo* and *in vitro* and on the molecular mechanism of BMP4 function in this context.

Methods

Bone marrow isolation and culture

Bone marrow was obtained from 6- to 8-week-old C57 mice. Mice were euthanized by means of cervical dislocation, and bone marrow was slowly flushed out the tibia and femur into lymphocyte separation medium, in which it was proportionally diluted with phosphate-buffered saline (PBS). After density gradient centrifugation, mononuclear cells were collected and centrifuged for 4 min at 1000*g* twice, after dilution in PBS. The cells were then cultured in low-glucose Dulbecco's modified Eagle's medium (LG-DMEM, Gibco, Invitrogen) containing 10% fetal bovine serum (FBS, Gibco, Invitrogen) and 1% penicillin/streptomycin and were subsequently incubated at 37°C in 5% CO_2. After 3 days, the medium was changed, and the non-adherent cells were discarded. The adherent cells were trypsinized with 0.25% trypsin–ethylene diamine tetra-acetic acid (Gibco, Invitrogen) when well-developed colonies reached near-confluency, and they were seeded into fresh plates for further expansion.

Characterization of BMSCs

To determine the purity of BMSCs, flow cytometric analysis of BMSC cell surface marker expression was performed. Cells that were 80% to 90% confluent were washed in PBS and centrifuged at 1000*g* for 4 min after trypsinization. Cells were then re-suspended in equal volumes of ice-cold PBS and 100% ethanol and incubated on ice for 30 min. Cells were incubated in PBS containing CD29, CD34, CD44, CD45 and CD105 primary antibodies (Cell Signaling Technology) for 30 min at 37°C. After incubation, cells were washed with PBS containing 1% bovine serum albumin (BSA) 3 times and then were centrifuged at 12,000*g* for 5 min at 4°C between each wash. A Becton Dickinson FACS Scan was used for fluorescence-activated cell-sorting (FACS) analysis. Cells were then tested for the ability to differentiate into adipogenic and osteogenic lineages through the use of oil red O staining and alizarin red staining, as described previously [18].

Induction of myogenic differentiation

BMSCs that reached sub-confluence were treated with 5-azacytidine (5-Aza, Sigma) for 24 h; cells were then assigned to three treatments groups, and their media was changed as follows: group 1 (control), DMEM supplemented with 2% horse serum (HS, Gibco, Invitrogen); group 2, DMEM supplemented with 10 ng/mL recombinant human BMP4 (Invitrogen) and 2% HS; group 3, DMEM supplemented with 100 ng/mL recombinant human noggin (Invitrogen) and 2% HS. The culture media was changed every 2 days. The expression of target proteins was analyzed after 14 days and 21 days of culture.

Animal injection

Twenty-two mdx mice (6–8 weeks old) were divided into four groups (with four mice in the control group, and six mice each in the other three treatment groups): group 1 (control), radiotherapy only; group 2, transplantation with BMSCs pre-treated with 5-Aza; group 3, transplantation with BMSCs pre-treated with 5-Aza + BMP4 (3 days); group 4, transplantation with BMSCs pretreated with 5-Aza + noggin (3 days). All mice were irradiated (total body with 6 Gy) from a 60 Co source. Three days after irradiation, 3×10^6 BMSCs were infused through the tail vein per mouse. Muscle tissue was harvested 12 weeks after injection. The mice were housed in a specific pathogen-free animal facility at the Laboratory Animal Center of Sun-Yet University (Guang Zhou, China), which was purchased from The Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). All animal experiments were performed in accordance with the Sun-Yet University Guidelines for Animal Care.

Immunofluorescent staining

Cells were fixed in 4% paraformaldehyde and blocked with 5% BSA; they were then incubated with the following primary antibodies: rabbit polyclonal anti-desmin (1:200; Abcam), rabbit polyclonal anti-myosin heavy chain (MHC) (1:50; Santa Cruz) and rabbit polyclonal anti-Smad or anti–phosphor-Smad