International Society for Cellular Therapy ISCT

Cytotherapy (2009) Vol. 11, No. 7, 849–863

informa healthcare

Inhibition of myostatin promotes myogenic differentiation of rat bone marrow-derived mesenchymal stromal cells

Jia Geng[1*], Funing Peng[2*], Fu Xiong[3], Yanchang Shang[1], Cuiping Zhao[1], Wanyi Li[3] and Cheng Zhang[1,3]

[1]Neurology Deparment, The First Affiliated Hospital, Sun Yat-Sen University, Guang Zhou, 510080, PR China, [2]Neurology Department, Shenzhen Second People Hospital, Shen Zhen, 518035, PR China, and [3]Center for Stem Cell Biology and Tissue Engineering, Sun Yat-Sen University, Guang Zhou, 510080, PR China

Background aims

Mesenchymal stromal cells (MSC) have been thought to be attractive candidates for the treatment of Duchenne muscular dystrophy (DMD), but the rate of MSC myogenesis is very low. Thus MSC treatment for DMD is restricted. Myostatin (Mstn), a negative regulator of myogenesis, is known to be responsible for limiting skeletal muscle regeneration. We hypothesized that inhibition of Mstn by using anti-Mstn antibody (Ab) would ameliorate the myogenic differentiation of MSC in vitro *and* in vivo.

Methods

MSC were isolated from rat bone marrow. Induced rat MSC (rMSC) were treated with various concentrations of anti-Mstn Ab. The expression of myogenic differentiation antigen (MyoD), myogenin and myosin heavy chain-type α (MHC-α) were estimated by immunofluorescence analysis and reverse transcription–polymerase chain reaction (RT-PCR). Adipogenic differentiation of rMSC inhibited by anti-Mstn Ab was evaluated by Oil Red O staining. The expression of dystrophin was detected 16 weeks after anti-Mstn Ab injection and rMSC transplantation by immunofluorescence staining, RT-PCR and Western blot. Motor function, serum creatine kinase (CK) and histologic changes were also evaluated.

Results

Five-azacytidine-mediated myogenic differentiation induced significant endogenous Mstn expression. Anti-Mstn Ab improved the expression of MyoD, myogenin and MHC-α and inhibited adipocyte formation. Sixteen weeks after transplantation, the inhibition of Mstn had improved motor function and muscle mass. In accordance with the increased motor function and muscle mass, dystrophin expression had increased. Furthermore, serum CK and centrally nucleated fiber (CNF) levels decreased slightly, suggesting specific pathologic features of the dystrophic muscle were partially restored.

Conclusions

Using anti-Mstn Ab, we found that inhibition of Mstn improved myogenic differentiation of rMSC in vitro *and* in vivo. *A combination of Mstn blockade and MSC transplantation may provide a pharmacologic and cell-based strategy for the treatment of DMD.*

Keywords

bone marrow mesenchymal stem cell, Duchenne muscular dystrophy, mdx, myogenic regulatory factors, myostatin.

Introduction

Duchenne muscular dystrophy (DMD) is the most common and lethal genetic muscle disorder in children. The incidence is about 1/3500 live male births and currently there is no efficient pharmacologic treatment [1]. Patients generally cannot walk and often need a wheelchair by 12 years of age. Although improvements in multidisciplinary care have extended survival into the third and fourth decades, patients generally succumb to respiratory failure or cardiomyopathy [2,3].

Cell transplantation therapy offers hope for the treatment of DMD. Embryonic stem cells (ESC) and

*These authors contributed equally.

Correspondence to: **Cheng Zhang**, Neurology Department, The First Affiliated Hospital, Sun Yat-Sen University, Guang Zhou, 510080, PR China. E-mail: zhangch6@mail.sysu.edu.cn

DOI: 10.3109/14653240903131632

mesenchymal stromal cells (MSC) have been considered as candidates for transplantation therapy [4–6]. Compared with ESC, MSC are easy to isolate and expand rapidly without leading to major ethical and technical problems, so have great potential as therapeutic agents. However, the potential of MSC for use in cell transplantation therapy and practical application to DMD depends on controlling their differentiation into functional skeletal muscle cells with high efficiency. The chemical demethylating agent 5-azacytidine (5-AzaC) is usually used to induce MSC from both mice and humans to generate muscle cells *in vitro*. But the conversion rate of MSC to myogenic phenotype is typically low [5,6]. The fusion rate of transplanted MSC and rate of dystrophin expression are also very low (1–3%) in humans and other animals [7]. So, to be able enhance the differentiation rate after MSC transplantation is the key issue for the future.

Myostatin (Mstn), a transforming growth factor (TGF)-β superfamily member, also known as growth and differentiation factor-8 (GDF-8), plays an essential role in negatively regulating skeletal muscle growth [8]. During skeletal muscular development, Mstn expression is initially restricted to the myotome compartment of developing somites and continues to be limited to the myogenic lineage at later stages of development and in the adult. Transgenic mice that overexpress recombinant Mstn protein in the skeletal muscle have lower skeletal muscle mass and higher whole body fat mass compared with wild-type controls [9]. In general, Mstn expression is higher under conditions in which the content of muscle mass is reduced [10]. Conversely, null mutations of the Mstn gene in knockout mice and double-muscle cattle are associated with hypermuscularity and decreased fat mass [8,11,12]. Thus expression of the Mstn gene is an important modulator of body composition in experimental animals. A number of studies have been performed *in vitro* on C2C12 myoblast cells and *in vivo* during chick embryonic muscle development that have shown that Mstn prevents differentiation of muscle precursors [9,13,14]. When Artaza *et al.* [15] used 5-AzaC to differentiate C3H 10T(1/2) cells, a mesenchymal fibroblast cell line of embryonic origin, into myotubes and adipocytes, they demonstrated that C3H 10T(1/2) cells could generate endogenous Mstn in most cells, and anti-Mstn antibody (Ab) up-regulated myogenic differentiation and down-regulated adipogenesis. In addition, Mstn inhibits expression of myogenic regulatory factors (MRF), which encode transcription factors regulating muscle differentiation [16,17].

In light of the negative regulating role of Mstn during myogenesis, we hypothesized that anti-Mstn Ab would promote the commitment and differentiation of rat (r) MSC into the myogenic lineage *in vitro* and in mdx mice. To test this hypothesis, we treated rMSC and mdx mice using anti-Mstn Ab. The results of our investigation suggest that Mstn inhibition plays an important role in the differentiation of rMSC and may have therapeutic application for DMD.

Methods

rMSC isolation and culture

Bone marrow was harvested from femurs and tibias of 4-week-old male Sprague–Dawley rats [18]. Briefly, the marrow plugs were disaggregated by sequential passage through 18-gauge, 20-gauge and 22-gauge needles and these dispersed cells were centrifuged and resuspended twice in Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen) containing 10% fetal bovine serum (FBS; Gibco Invitrogen) (complete medium). After the cells were counted in a hemocytometer, nucleated cells (5×10^7) were seeded into 100-mm culture dishes. Three days later, the medium was changed and non-adherent cells discarded. Then the monolayer of adherent cells was incubated further in complete medium until the cells became nearly confluent. The adherent cells were trypsinized (0.25% trypsin-EDTA; Gibco Invitrogen), resuspended in complete medium, split at a 1:2 ratio, and seeded into fresh plates. After these once-passaged cells became nearly confluent, they were trypsinized and aliquots taken for freezing and storage or further culturing. The medium was changed every 3–4 days. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

Treatment of rMSC

Nearly 80% confluent rMSC (passage 2) were trypsinized, resuspended in complete medium, split at a 1:2 ratio, and seeded into six- or 12-well culture dishes and allowed to recover for 1 day. Then rMSC were incubated in complete medium containing 10 μM 5-AzaC for 24 h to induce differentiation, as described by Wakitani *et al.* [18], and incubated with or without 10–100 μg/mL polyclonal anti-Mstn Ab (R&D Systems Inc.) in differentiation