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Cholesterol myristate suppresses the apoptosis of mesenchymal stem cells via upregulation of inhibitor of differentiation

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ABSTRACT

To identify small molecules that suppress the apoptosis of mesenchymal stem cells (MSCs) is promising for stem cell therapy. We recently showed that bone morphogenetic protein 4 (BMP4) signalling involves the effect of cholesterol myristate on the proliferation of MSCs. The present study evaluated the effects of cholesterol myristate on the apoptosis of MSCs and the inhibitor of differentiation (Id1), target gene of BMP4 signalling. MSCs transfected by the Id1 promoter reporter construct, cholesterol myristate increases the activity of Id1 promoter. However, structurally related steroids such as cholesterol, β-sitosterol and cholesten-3-one, lack of the myristate, did not affect the activity of Id1 promoter, suggesting that myristate is essential for this effect. This effect depends on BMP signalling. Apoptosis analysis indicated that cholesterol myristate inhibited the apoptosis of MSCs induced by serum-free. Cholesterol myristate increases the expression of Id1 and its target gene bcl-x/l in MSCs treated with serum-free. Moreover, noggin, a BMP antagonist, reduced the anti-apoptotic effects of cholesterol myristate. Thus, this study aims to provide evidence that cholesterol myristate suppresses the apoptosis of MSCs via upregulation of Id1. These findings can be applied for improving MSCs survival in stem-cell transplantation, bone-marrow transplantation, treatment of bone diseases such as osteoporosis and chemotherapy.

1. Introduction

Mesenchymal stem cells (MSCs) in adult bone marrow are capable of self-renewal and differentiation into all mesodermal cell types and neuro-ectodermal cells, such as osteoblasts, chondrocytes, myoblasts, stromal cells, adipocytes, neurons, astrocytes, and so on [1–5]. These abilities make the MSCs an excellent seed cell of cell transplantation. Cell transplantation is a promising strategy for the treatment of neurotraumatic injuries, stroke, and neurodegenerative and muscle degenerative diseases. Many kinds of cells, including embryonic stem cells and tissue stem cells, have been considered as candidates for transplantation therapy. MSCs have great potential as therapeutic agents because they are easy to isolate and can be expanded from patients without serious ethical or technical problems. However, stem-cell therapy is hampered by MSC apoptosis induced by an extremely harsh microenvironment [6,7]. Thus, to identify small molecules that suppress the apoptosis of MSCs is promising for stem-cell therapy. Current research in this field focusses on the anti-apoptotic effect of lovastatin [8] and lysophosphatidic acid [9]. It is also found that berberine, which is a natural isoquinoline alkaloid in traditional Chinese medicine, protects MSCs from hypoxia-induced apoptosis [10].

Cholesterol myristate is a natural steroid present in traditional Chinese medicine with a long history of use. We recently identified that cholesterol myristate is the active compound that increases proliferation of MSCs [11,12]. Recent studies on the signal pathway have shown that autocrine bone morphogenetic protein 4 (BMP4) signalling involves effect of cholesterol myristate on proliferation of MSCs [13]. However, the effects of cholesterol myristate on the apoptosis of MSCs are not clear.

Inhibitor of differentiation or inhibitor of DNA binding (Id), a target of BMP signalling [14], plays a crucial role in the protection of cells against apoptosis [15]. Id proteins have a helix–loop–helix (HLH) dimerisation domain, but lack the basic region responsible for DNA binding. By sequestering ubiquitously expressed bHLH transcription factors, they inhibit the transcription induced by bHLH transcription factors. Id proteins can promote endothelial survival during embryonic development [16]. *In vitro* studies

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also demonstrated that over-expression of Id1 reduced the rate of human endothelial cell apoptosis [17]. In a bleomycin model of lung injury in mice, Id1 plays a crucial role in promoting endothelial survival in the adult lung on injury [18]. Therefore, Id1 may provide novel anti-apoptosis targets for the development of new anti-apoptosis drugs.

In the present study, MSCs transfected by Id1 promoter reporter construct, the effects of cholesterol myristate and structurally related steroids such as cholesterol, β-sitosterol and cholesten-3-one on the activity of Id1 promoter were evaluated. We used the *in vitro* model of serum deprivation to investigate the effects of cholesterol myristate in MSCs apoptosis. In addition, we attempted to elucidate the mechanism of its actions. Our present study demonstrated that cholesterol myristate protects MSCs from apoptosis induced by serum-free and that cholesterol myristate exerts its anti-apoptotic effects by activating transcription factor Id1. These findings can be applied for improving MSCs survival in stem-cell transplantation, bone-marrow transplantation, treatment of bone diseases such as osteoporosis and chemotherapy.

2. Experimental

2.1. Animals and materials

Sprague–Dawley (SD) rats of 4 weeks of age were obtained from the animal centre of Guangzhou University of Traditional Chinese Medicine. All animals received humane care in accordance with the guidelines set by the Care of Experimental Animals Committee of Guangzhou University of Traditional Chinese Medicine. Dulbecco's modified Eagle's medium (DMEM, Catalogue # 31600034) and foetal bovine serum (FBS, Catalogue # 16000-044) were purchased from Gibco (Grand Island, NY, USA); MTT (Catalogue # M2128) and propidium iodide (PI, Catalogue # P4170) were purchased from Sigma (St. Louis, MO, USA); recombinant BMP4 (Catalogue # 5020-BP-010) and noggin (Catalogue # 1967-NG-025) were purchased from R&D Systems (Minneapolis, MN, USA); Id1 antibody (Catalogue # BA2163), CD44 antibody (Catalogue # BA0321) and terminal deoxynucleotidyl transferase-mediated deoxy uridine triphosphate (dUTP) nick end labelling (TUNEL) detection kit were purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China); chemicals such as dimethyl sulphoxide (DMSO) and other reagents were also obtained from Sigma; cholesterol myristate, β-sitosterol, cholesterol and (+)-cholesten-3-one were purchased from Tokyo Chemical Industry Co., Ltd. (TCI; Tokyo, Japan).

2.2. Culture of MSCs [19]

Bone marrow was obtained from the femur and tibia of rat (all experimental procedures were approved by the Care of Experimental Animals Committee of Guangzhou University of Traditional Chinese Medicine). The marrow samples were diluted with DMEM (low glucose, LG) containing 10% FBS. MSCs were prepared by gradient centrifugation at $900 \times g$ for 30 min on Percoll of a density of $1.073\,g\,ml^{-1}$. The cells were washed, counted and plated at $1 \times 10^6\,cm^{-2}$ on Petri dishes in DMEM-LG supplemented with 10% FBS. The medium was replaced and the unattached cells were removed every 3 days. MSCs formed as confluent layers for 9 days were detached by treatment with 0.25% trypsin and passaged into cultural flasks at $1 \times 10^4\,cm^{-2}$. MSCs at passage 3 were evaluated for cultured cell homogeneity using detection of CD44 by flow cytometry; the cells were 95% homogeneous and were used for the described experiments. MSCs were verified on the basis of their abilities to differentiate into osteocytes, adipocytes and chondrocytes, as described by Pittenger et al. [20].

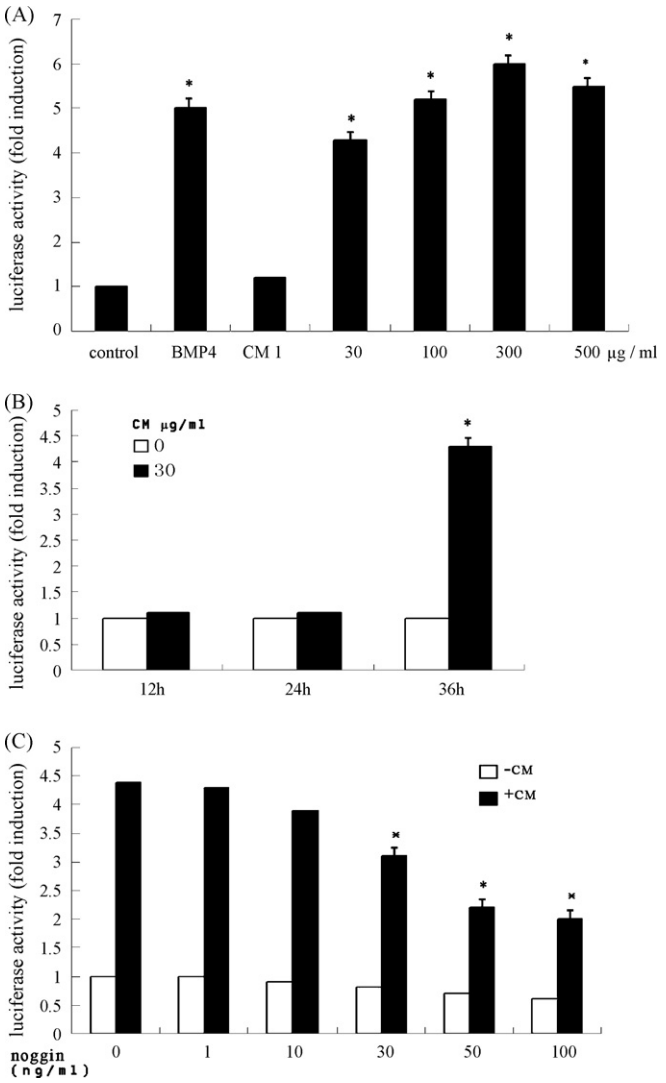

Fig. 1. Cholesterol myristate increases Id1 promoter activity. (A) Dose–response of cholesterol myristate on the Id1 promoter activity, MSCs were transfected with Id1 promoter reporter construct and then either left unstimulated or stimulated with indicated concentrations of cholesterol myristate or BMP4 for 36 h. (B) Time course of cholesterol myristate on the Id1 promoter activity, MSCs were transfected with Id1 promoter reporter construct and then either left unstimulated or stimulated with indicated time of cholesterol myristate ($30\,\mu g\,ml^{-1}$). (C) Noggin inhibited the cholesterol myristate-induced the Id1 promoter activity. MSCs were transfected with Id1 promoter reporter construct and stimulated with indicated concentrations of noggin ($0–100\,ng\,ml^{-1}$) in the absence or presence of cholesterol myristate ($30\,\mu g\,ml^{-1}$). Luciferase activity was determined in cell lysates and normalised to Renilla activity. Results are representative of three independent experiments. *Abbreviations*: BMP4, bone morphogenetic protein 4; CM, cholesterol myristate.

2.3. Plasmids, cell transfection and assay for luciferase activity

To construct the Id1 promoter-Luc vector, 1.742 kb (−1765/+88) of the 5′-flanking region of the rat Id1 gene was amplified by polymerase chain reaction (PCR) by using the forward primer, 5′-CCC AAGCTT TCTCTGAGACCCGAACTCCTAGCTC-3′ and the reverse primer, 3′-CCC AAGCTT GATCCTGAGGACAGGCGGAGAG-5′. The PCR product and pGL3-basic vector were digested with NheI and HindIII (TAKARA) and ligated together to generate the Id1 promoter-Luc vector. The plasmid for pRL-TK was co-transfected to normalise the variations in transfection efficiency. The pGL3-basic vector and the pRL-TK plasmid were kindly provided to our laboratory by Dr. Huang Qilai and Dr. Chen Yuan (State