Cardiotrophin-1 stimulates the neural differentiation of human umbilical cord blood-derived mesenchymal stem cells and survival of differentiated cells through PI3K/Akt-dependent signaling pathways

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Abstract Cardiotrophin-1 (CT1) plays an important role in the differentiation, development, and survival of neural stem cells. In this study, we analyzed its effects on the stimulation of human umbilical cord blood-derived mesenchymal stem cells in terms of their potential to differentiate into neuron-like cells, their survival characteristics, and the molecular mechanisms involved. The treatment of cells with neural induction medium (NIM) and CT1 generated more cells that were neuron-like and produced stronger expression of neural-lineage markers than cells treated with NIM and without CT1. Bcl-2 and Akt phosphorylation (p-Akt) expression levels increased significantly in cells treated with both NIM and CT1. This treatment also effectively blocked cell death following neural induction and decreased Bax, Bak and cleaved-caspase 3 expression compared with cells treated with NIM without CT1. In addition, the inhibition of phosphatidylinositol 3-kinase (PI3K) abrogated p-Akt and Bcl-2 expression. Thus, PI3K/Akt contribute to CT1-stimulated neural differentiation and to the survival of differentiated cells.

Keywords Human umbilical cord blood mesenchymal stem cells · Cardiotrophin 1 · Neural differentiation · Apoptosis · PI3K/Akt

Introduction

Cardiotrophin-1 (CT1) is a cytokine that was originally identified based on its ability to induce cardiac myocyte hypertrophy (Pennica et al. 1995). It is structurally and functionally related to a group of factors called interleukin-6 cytokine (IL-6) family (Hunt and White 2016), also called neurotrophic cytokines, which includes IL-6, interleukin-11 (IL-11), leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF) (Thier et al. 1999).

The neurotrophic effects of CNTF are better characterized than CT1. CNTF promotes the survival of sensory, autonomic, hippocampal, striatal, cerebellar, and motor neurons, making CNTF a candidate molecule for the treatment of neurodegenerative disorders (Sendtner et al. 1994). However, CNTF lacks a conventional signal peptide and cannot be secreted by mammalian cells (Sendtner et al. 1994). At the same time, the protective effect on dopaminergic neurons is not promoted by CNTF.

CT1 plays a key role in neural tissue development and has survival-promoting, anti-apoptotic effects in neural injury or death (Pennica et al. 1995; Lopez-Yoldi et al. 2015; Wang et al. 2013; Garcia-Cenador et al. 2013). Studies have indicated that CT1 exhibits impressive neuroprotective effects and delay the procession of motor neuron degenerative disorders by prolonging the median neuronal survival time, improving motor function and promoting regeneration

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in neonatal rat motor neurons in mouse models of amyotrophic lateral sclerosis, progressive motor neuropathy and spinal muscular atrophy and in adult rats with spinal cord injuries. Pennica and coworkers found that CT1 induces a phenotypic switch in sympathetic neurons and promotes the survival of dopaminergic neurons from the central nervous system and ciliary neurons from the periphery (Pennica et al. 1995).

In contrast, both in humans and in mice, CT1 is expressed in the postnatal and adult central nervous system (CNS) (Wang et al. 2013; Calabro et al. 2009; Liu et al. 2016). CT1 has been reported to act as a trophic factor for a few neurons, such as sensory, cholinergic, dopaminergic, motor and cortical neurons (Lopez-Yoldi et al. 2015). Therefore, CT1 has survival-promoting effects in the nervous system that are not shared by other members of the IL-6 family (Latchman 2000). It may be of use as a novel neuroprotective agent (Pennica et al. 1996).

In this study, we investigated the effects of CT1 on the neural potentiality of hUCB-MSCs following neural induction. We then studied the survival of differentiated neuro-like cells by CCK8 and TUNEL. Moreover the molecular mechanisms involved in the CT1-regulated neural differentiation and survival were explored.

Materials and methods

Neural induction and human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs)

Human umbilical cord blood samples were obtained with consent from the mothers and were separated and maintained according to a previous study we have reported (Peng et al. 2016).

Based on our previous study, we observed that hUCB-MSCs could be successfully transduced by recombinant adenovirus encoding green fluorescent protein (Ad-EGFP) and Ad-CT1-EGFP (Sainuo, Beijing, China). The transduction efficiency was highest when the cells were exposed to the transducing media for 72 h at a multiplicity of infection (MOI) of 100. Moreover, hUCB-MSCs transduced with Ad-CT1-EGFP could sustainably express CT1 (date not shown). 72 h after adenovirus transfection, cells were collected for further assay.

The cultured MSCs were detached enzymatically and mechanically and plated in 6 well plates at 1×10^6 cells ml^{-1} . Then cells were transduced with the adenoviral vector (Ad-EGFP or Ad-CT1-EGFP) at MOI 100. 72 h after adenovirus transduction, cells were collected for neuronal induction. For neuronal induction, cells were washed with phosphate-buffered saline (PBS) and incubated with neuronal differentiation medium: HG-DMEM (HyClone, Logan, UT, USA) plus 200 mM glutamic acid (Solarbio, Beijing, China) and 1 μM all-trans-retinoic acid (Sigma, St. Louis, MO, USA). After induction, the cells were maintained in NIM with or without CT1 for up to 4 days. Cells were treated with PI3K/Akt inhibitor LY294002 (Cell Signaling Technology, Danvers, MA, USA).

Immunofluorescence staining

After the differentiation period time, cells were fixed for 20 min with 4% paraformaldehyde at room temperature. The fixed cells were permeabilized with 0.1% TX-100 in TBS and blocked for 30 min at room temperature with 5% BSA and incubated with primary antibodies including rabbit anti-Nestin (Abcam, Cambridge, U.K.), rabbit anti- β III-tubulin (Abcam), and rabbit anti-NeuN (Abcam) overnight at 4 °C. After three rinses in PBS, cells were incubated in the dark with secondary antibodies Cy3-conjugated goat anti-rabbit IgG (1:200, Bioss, Beijing, China) for 1 h at room temperature. After three rinses in PBS, the nuclei were stained with DAPI (Solarbio, Beijing, China). Slides were observed under fluorescent microscope (Leica, Wetzlar, Germany) and images were taken. To quantify the number of positive cells for each antibody, at least ten microscopic fields per well were counted randomly and reported as percentage relative to whole DAPI-stained nuclei.

Cell viability assay by Cell Counting Kit-8 (CCK-8)

Cell viability assays were performed using Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). CCK-8 was performed according to the manufacturer's instructions. Cells were plated in 96 well plates at 6.25×10^2 , 1.25×10^3 , 2.5×10^3 , 5×10^3 , 1×10^4 cells ml^{-1} , respectively, to get a standard curve relating cell density and optical density values. Cells of passages 3 at 5×10^3 cells ml^{-1} were

plated to develop growth curves. 5×10^3 cells ml^{-1} were seeded into 96-well plates. At the endpoint, 10 μl CCK-8 was added for further 2 h at 37 °C, the absorbance at 450 nm was obtained using a plate reader (Thermo, Waltham, MA, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling analysis (TUNEL)

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit (Roche, Indianapolis, IN, USA). TUNEL was performed according to the manufacturer's instructions. Briefly, slides were washed in PBS and then fixed for 60 min with 4% paraformaldehyde at room temperature. The fixed cells were permeabilized with 0.1% TX-100 in 0.1% sodium citrate for 2 min on ice and then washed in PBS again. 500 μl of TUNEL reaction mixture was added with plastic cover slips and incubated protected from light in a moisture chamber at 37 °C for 60 min. After three rinses in PBS, the nuclei were stained with DAPI. The slides were viewed and photographed with fluorescence microscope.

Western blot analysis

At passage 3, total proteins were harvested from the cultured cells using a total protein extraction kit (Applygen, Beijing, China) following the manufacturer's protocol. The protein concentration of the cells was measured with a BCA protein assay kit (Beyotime, Beijing, China). Cell lysate samples were separated by 12% SDS-PAGE and then transferred to a PVDF membrane. After blocking the membrane with 5% skim milk in Tris-buffered saline Tween-20 (TBST) for 1 h, the membranes were incubated with the following primary antibodies for 2 h at room temperature: Akt (1:1000), p-Akt (1:2000), Bax (1:1000), Bak (1:1000), Bcl-2 (1:1000), caspase 3 (1:1000), cleaved-caspase 3 (1:1000) (Cell Signaling Technology, Danvers, MA, USA) and β -actin (Beyotime, Beijing, China). The membranes were washed in TBST and incubated with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Baltimore, MD, USA) for 2 h, washed, and developed with electrogenerated chemiluminescence (ECL) western blotting detection reagent (7SeaPharmTech,

Shanghai, China). Proteins were visualized using X-OMAT film (Kodak, Rochester, NY, USA). The western blot band intensities were measured with ImageJ software.

Statistical analysis

Data were analyzed using SPSS 17.0. The variables are expressed as mean $\pm$ SD. Differences between multiple groups were evaluated by one-way ANOVA. All experiments were performed at least three times. $P < 0.05$ was considered as statistically significant.

Results

Neural differentiation

To investigate the effect of CT1 for promoting *in vitro* differentiation of hUCB-MSCs into neural cells, we observed cell morphology when the MSCs were treated with NIM, with or without CT1. Cells responsive to the neurogenic stimulants together with CT1 progressively assumed neuronal morphological characteristics over the first 6 h. The cytoplasm in the flat hUCB-MSCs retracted toward the nucleus, resembling a typical neuronal soma, and the process became thinner because of continuous shrinkage of the cell body. The morphological changes further progressed in the next 4 days to yield network-like structures and many long processes. However, these morphological changes were weak for the cells treated with NIM and without CT1, and only a few cells showed pyramidal or spherical cell bodies with multiple processes (Fig. 1).

In addition to their neural morphological differentiation, we further investigated the effects of CT1 by performing immunofluorescence. To identify cells at different stages of maturation, we evaluated the expression levels of neural proteins that were induced by MSCs. Immunofluorescence staining showed expression of Nestin in these presumptive neurons which increased dramatically after 6 h of induction with CT1. Cells were further induced into typical neuronal soma, which expressed β III-tubulin and NeuN. In addition, the expression of β III-tubulin and NeuN significantly increased at 4 days. Interestingly, we found that these immunoreactivities were weak for those treated with NIM without CT1 (Fig. 2).

Cell survival of the differentiated neurons

We examined whether CT1 could enhance the survival of hUCB-MSCs during neural differentiation by performing CCK8 for the cells treated with NIM and with or without CT1. Significant cell death was observed at 6 h and 4 days after induction with NIM but without CT1 addition. However, the cell death rate was decreased in cells treated with NIM and CT1 (Fig. 3).

Apoptotic phenomena and molecular analysis of the differentiating neurons

The cells treated with NIM and CT1 were more resistant to cell death than cells treated with NIM without CT1 (Fig. 3), which indicates that CT1 may act as an inhibitor of apoptotic cell death during neural differentiation. To gain further insight into this, we evaluated the rate of apoptosis in differentiating MSCs treated with NIM, with or without CT1. Apoptosis was confirmed by immunofluorescent detection of TUNEL (Fig. 4), which is an indicator of cells undergoing apoptosis. CT1 treatment also effectively reduced TUNEL-positive cells, suggesting that CT1 inhibits apoptotic cell death following neural differentiation.

Fig. 2 Expression of neural markers in presumptive neurons derived from human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). **a** Immunofluorescence analysis of hUCB-MSCs after 6 h and 4 days of incubation in neural induction medium (NIM) with or without Cardiotrophin-1 (CT1). The cells were fixed and stained with anti-rabbit antibodies against the neural lineage markers Nestin, β III-tubulin, and NeuN. The cells treated with NIM and CT1 exhibited stronger labeling for these markers than did cells treated with NIM without CT1. *Scale bar* = 100 μ m. **b** Percentages of cells showing specific immunolabeling determined by counting at least 1500 cells in five or six different microscopic fields for each marker in a minimum of three different experiments. *Scale bar* = 100 μ m

We also conducted a molecular study to analyze the expression of genes involved in CT1-mediated cell survival during neural differentiation. As shown in Fig. 5, a western blot analysis demonstrated that the expression of Bak and Bax, which are both proapoptotic molecules, were strongly increased. Moreover, cleavage of caspase 3 was observed for the cells treated with NIM without CT1. However, Akt phosphorylation and Bcl-2 expression were stronger in cells treated with NIM and CT1 than in cells treated with NIM without CT1, which suggests that Akt phosphorylation and Bcl-2 up-

Fig. 1 Phase-contrast images of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) after 6 h and 4 days of incubation in neural induction medium (NIM), with or without Cardiotrophin-1 (CT1). The differentiating MSCs assumed a neuron-like morphology rapidly and then acquired

the mature neural morphological features. However, these features were weak in the cells treated with NIM but without CT1. The morphologies are representative of at least three independent experiments. *Scale bar* = 100 μ m

Fig. 3 Cell viability of the presumed neurons derived from human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). The cell viability of hUCB-MSCs was analyzed by CCK8 after 6 h and 4 days of incubation in NIM with or without CT1. ($n = 3$, $P < 0.05$)

regulation are involved in the CT1-stimulated survival of differentiated neuron-like cells.

To define the specific involvement of these signals, we treated the cells with the pharmacological inhibitor of PI3K LY294002. Treatment of cells that had been differentiated with NIM and CT1 with 20 μ M LY294002 almost completely inhibited p-Akt and Bcl-2 expression. This inhibited simultaneously decreased cell viability. Thus, CT1 appears to block apoptosis signals through PI3K-dependent Bcl-2 up-regulation and therefore enhances cell survival during neural differentiation (Fig. 5).

Discussion

Base on our previous study, we observed that transplantation of neural stem cells (NSCs) overexpressing CT1 leads to increased neuron counts in CA1 areas indicating that CT1 promotes survival of NSCs and facilitates repair of lost neurons (Shu et al. 2011). However, the effects of CT1 on the neural differentiation of hUCB-MSCs and survival of differentiated cells have not been examined. In this study, we demonstrated that the treatment of hUCB-MSCs with NIM and CT1 enhanced their neural characteristics and increased the survival of the differentiated neuron-like cells.

To examine the effect of CT1 on neuronal differentiation, hUCB-MSCs were stimulated with NIM and CT1. In previous studies, chemicals or neurotrophic factor have been introduced to achieve neuronal differentiation (Burdon et al. 2011; Lu et al. 2004). Each method has unique disadvantages, such as low rate of cell survival and differentiation. To initiate neuronal differentiation, we used NIM with CT1. Interestingly, our results show that hUCB-MSCs could be differentiated into cells expressing Nestin, a specific marker of NSCs and neural progenitors. At the same time, our results also showed that hUCB-MSCs-derived Nestin⁺ cells have the potential for neural differentiation under appropriate conditions. Following induction for 4 days after confirmation of Nestin⁺ cells, we found that differentiated cells expressed β III-tubulin and glial fibrillary acidic protein (GFAP), suggesting that hUCB-MSCs can be efficiently differentiated into neurons. These results suggest that CT1 is not sufficient as an inducer of neuronal differentiation, but it is an efficient enhancer for the ongoing neuronal differentiation process.

hUCB-MSCs could be differentiated into various neural cells. The neurons can integrate into the injured spinal cord and form synaptic connection with host neurons and promote functional recovery. However, the low rate of cell survival limits the therapeutic efficacy. Therefore, the restriction of neuronal death is critical. The question of why and how neurons die is not fully understood, but neurons, like other cell types, need trophic support for their survival. CT1 has been reported to act as a trophic factor for a few neurons, such as sensory, cholinergic, dopaminergic, motor and cortical neurons (Lopez-Yoldi et al. 2015). Several studies have pointed out that CT1 might play a key role in neural tissue development and also in the protection of the mature nervous system against a variety of injuries and dysfunctions (Lopez-Yoldi et al. 2015). Furthermore, adenovirus-mediated gene transfer of CT-1 or the administration of CT-1 delayed neurogenic muscular atrophy and progressive neuromuscular deficiency in an experimental model of amyotrophic lateral sclerosis (Lopez-Yoldi et al. 2015; Bordet et al. 2001; Mitsumoto et al. 2001). Our finds showed that hUCB-MSCs differentiated with NIM and CT1 survived better than the cells differentiated with NIM without CT1. CT1 is regulating apoptosis by up-regulating the expression of anti-apoptotic proteins Bcl-2 and down-regulating the expression of pro-

Fig. 4 Apoptosis of the presumed neurons derived from human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs). **a, b** Apoptosis was analyzed by TUNEL staining assay carried out on hUCB-MSCs after 6 h and 4 days of incubation in neural induction medium (NIM) with or without Cardiotrophin-1 (CT1). The cells treated with NIM without CT1 exhibited stronger

labeling for TUNEL than did cells treated with NIM and with CT1. The stains are representative of at least three independent experiments. *Red* TUNEL; *blue* DAPI. The percentage of cells with condensed nuclei was determined by counting at least 1000 cells in five different microscopic fields in three different experiments ($n = 3$, $P < 0.05$). (Color figure online)

apoptotic proteins Bax and Bak, and inhibiting cleaved-caspase3. Thus, CT1 may act as an inhibitor of apoptotic cell death during neural differentiation.

PI3K was first identified as a regulator of the neurotrophin-mediated survival response in NGF-dependent PC12 cells (Yao and Cooper 1995). It is well documented (Shu et al. 2016; Le Belle 2011; Urnukhsaikhon et al. 2015), that PI3K/Akt pathway is necessary for the regulation of neural differentiation and the survival of differentiated cells. In addition, several studies have recently reported that Akt was

activated and some trophic factors such as nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1) and brain derived neurotrophic factor (BDNF) were produced to improve the survival and differentiation from NSCs to neurons (Brazel et al. 2014). Moreover, a previous study has shown that PI3K is activated after CT1 treatment in motor neurons (Dolcet et al. 2001) and in cytokine-dependent developing sensory neurons (Middleton et al. 2000). Thus, the PI3K/Akt pathway appears to be a general mediator of cytokine-induced neuronal survival and

Fig. 5 Cardiotrophin-1 (CT1) activates the PI3K/Akt pathway. **a–c** Western blot analysis of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) after 6 h and 4 days incubation in neural induction medium (NIM) with or without CT1. Western blot analysis demonstrated that the expression of Bak, Bax and cleavage of caspase 3 were strongly increased. However, Akt phosphorylation and Bcl-2 expression were stronger in cells treated with NIM and CT1 than in cells treated with NIM without CT1. **d, e** The cells were treated with NIM and CT1 for 4 days and then treated with 20 μ M of the

PI3K inhibitor LY294002 for 6 h to examine the levels of protein expression and phosphorylation by Western blot analysis. Treatment of cells that had been differentiated with NIM and CT1 with 20 μ M LY294002 almost completely inhibited p-Akt and Bcl-2 expression. **f** Percentages of viable cells treated with NIM and CT1 for 4 days and then treated with or without 20 μ M of the PI3K inhibitor LY294002. This inhibited simultaneously decreased cell viability. The blots are representative of at least two independent experiments ($n = 3$, $P < 0.05$)

differentiation (Urnukhsaikh et al. 2015). We speculate that the PI3K/Akt pathways is also important in CT1 mediated neural differentiation of hUCB-MSCs and survival of differentiated cells. Our results provided strong evidence to support this hypothesis and found that the levels of p-Akt and Bcl-2 expression were increased significantly in cells differentiated with NIM and CT1. Inhibition of PI3K with LY294002 in the differentiated cells effectively blocked CT1-enhanced p-Akt and Bcl-2 up-regulation. These results are in agreement with other early studies suggesting that PI3K kinase activity is responsible for as much as 80% of the neurotrophin-regulated cell survival (Kaplan and Miller 2000), indicating that PI3K is the major survival-promoting protein for neurons (Dolcet et al. 2001).

Recent data indicate that two of these signaling pathways, PI3K/Akt and MARK/ERK, are the major effectors of neurotrophin-activated survival (Tzeng et al.

2015; Yuan and Yankner 2000; Kaplan and Miller 2000). Calabro et al. (2009) reported that PI3K/Akt and MARK/ERK could cooperate in CT1 mediated pro-survival effects. The main function of the PI3K/Akt pathway is to maintain cell survival during neural differentiation; whereas the role of the MAPK/ERK pathway is probably to promote the maturation of differentiated MSC (Tzeng et al. 2015). Inspired by these findings, we speculated that cross-talk may occur between the PI3K/Akt and MAPK/ERK pathway in CT1 mediated the neural differentiation of hUCB-MSCs and survival of differentiated cells. Further studies are needed to understand the function of this interesting molecule.

Conclusions

The current study demonstrates that induced hUCB-MSCs treated with CT1 displayed greater neuronal differentiation and cell survival. It is likely that PI3K/

Akt contribute to these effects. Thus, CT1 is essential for neural survival during development and is highly effective in protecting neurons against apoptosis being worth exploring as a potential therapy in a variety of neuronal disorders (Quiros et al. 2016).

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