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Stimulation of cardiomyogenesis from mouse embryonic stem cells by nuclear translocation of cardiotrophin-1☆

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ABSTRACT

Background: Cardiotrophin-1 (CT-1) controls cardiomyogenesis of mouse embryonic stem (ES) cells.
Objectives: To investigate the signaling pathway underlying the action of CT-1 on cardiac cell differentiation.
Methods: Protein expression was analyzed by western blot technique and cardiac areas by immunohistochemistry. Calcium, reactive oxygen species (ROS) and nitric oxide (NO) were assessed by microfluorometry using fluo-4, H_2DCF, and DAF-2DA, respectively. Gene inactivation of CT-1 was achieved by siRNA technology.
Results: CT-1 as well as its receptor gp 130 were transiently upregulated during differentiation of ES cells. Exogenous CT-1 enhanced cardiomyogenesis, increased the cardiac transcription factors MEF2c, Nkx-2.5, TEAD3 and GATA4, the cardiac proteins α-actinin, MLC2a, MYH7, MLC1a, MLC2v and HCN4 as well as vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB), fibroblast growth factor-2 (FGF-2) and atrial natriuretic peptide (ANP). CT-1 downregulation by small interfering RNA (siRNA) inhibited cardiomyogenesis and decreased VEGF, PDGF-BB, FGF-2 and ANP expression. CT-1 raised intracellular calcium which was abolished by the intracellular calcium chelator BAPTA, AM and thapsigargin. Moreover, CT-1 treatment increased ROS, followed by NO generation and NOS3 activation. During ES cell differentiation CT-1 was translocated to the cell nucleus. Exogenous CT-1 induced nuclear translocation of endogenous CT-1, which was inhibited by BAPTA, the NOS inhibitor L-N^G-Nitroarginine methyl ester (L-NAME), the radical scavenger N-(2-mercaptopropionyl)-glycine (NMPG) as well as the janus kinase 2 (JAK2) inhibitor AG490 and the PI3 kinase (PI3K) inhibitor LY294002.
Conclusions: Nuclear translocation of CT-1 regulates cardiomyogenesis of ES cells and involves calcium, NO, ROS as well as CT-1 regulated signaling pathways.

1. Introduction

CT-1 is a cardio-active protein belonging to the interleukin-6 (IL-6) family which besides IL-6 includes leukemia-inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), and IL-11 [36]. CT-1 elicits pleiotrophic effects through the LIF receptor (LIFR) complex consisting of the gp130/LIFRβ heterodimer [37]. Although it plays major roles in the heart, CT-1 expression is not restricted to cardiac cells, but is also expressed in non-cardiac cells within the heart as well as in many other organs, including the brain, the liver, and the kidney [26]. It exerts a variety of different effects, from which the most important are: the support of survival, the inhibition of apoptosis and the stimulation of organ growth [45]. In the cardiac muscle cell survival is most important since adult cardiac cells are post-mitotic, and any heart injury may result in scarring and loss of heart function. In rather low, nanomolar concentrations, CT-1 has been shown to maintain proliferation and survival of embryonic and neonatal cardiac myocytes [43]. In addition CT-1 causes cardiac myocyte hypertrophy in adult cardiomyocytes as a compensatory mechanism to hypertension. The increase in cardiac cell size induced by CT-1 is due to an increase in cell length, whereas cell width remains unaffected. This was presumably due to additional assembly of sarcomeric units in series rather than in parallel, the latter being typical for α-adrenergic stimulation-mediated hypertrophy [50]. Data from our group have demonstrated that CT-1 is stimulating cardiomyogenesis of ES cells [42]. In the ES cell system CT-1 exerts its pro-cardiogenic effect not through induction of cell hypertrophy but presumably by stimulation

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of cardiac cell commitment in early and proliferation in later stages of differentiation [42]. Further data of ours and others demonstrated that the expression of CT-1 is increased under hypoxia [1,38], which occurs under conditions of cardiac infarction, when cardiac cells become necrotic and cardiac regeneration is required. In this respect it has been previously reported that elevated levels of CT-1 occur in patients with unstable angina [46], acute myocardial infarction [19,48] and heart failure [47]. Interestingly CT-1 is commonly used as a cell culture ingredient for the culture of cardiospheres containing Isl-1$^+$ cardiac progenitor cells, suggesting that CT-1 may also exert effects on the differentiation and/or proliferation of cardiac progenitor cells [34,52] within the adult heart — and thus may be involved in regenerative and repair processes [18].

The signaling cascade elicited by CT-1 downstream of gp130/LIFRβ consists of at least three pathways, i.e. the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, the extracellular receptor kinase-1/2 (ERK1/2) pathway and the phosphatidylinositol 3-OH kinase (PI3K)/Akt pathway [9]. These signaling pathways may be activated by second messengers such as intracellular calcium, ROS and NO. We have previously demonstrated that CT-1 signaling involves the generation of ROS [42]. Furthermore, endogenous CT-1 levels are upregulated under elevated redox states which occur under hypoxic conditions [1]. Moreover, CT-1 has been shown to upregulate NOS2 which may be associated with the blood pressure lowering effect observed in rats [22,25]. NO and ROS signaling has been previously shown to be closely related to calcium signaling events. Indeed it has been demonstrated in cardiac fibroblasts that cell migration initiated by CT-1 is associated to intracellular calcium signals [17]. In adult rat ventricular myocytes CT-1 induces sarcoplasmic reticulum calcium leak and arrhythmogenesis [40].

Although some knowledge about the intracellular signaling events elicited by CT-1 has been so far established, nothing is known about the impact of endogenous intracellular CT-1 expression for signaling cascades initiated upon exogenous CT-1 binding to the extracellular gp130/LIFRβ. For members of the IL-1 family it has been demonstrated that they are not only able to bind to their cognate receptors on target cells, but also can act intracellularly in the producing cell, can translocate to the nucleus and have been discussed to affect gene expression [39]. Processes affected by intracellular IL-1α include regulation of cell proliferation, migration and senescence [49]. Whether exogenous CT-1 may exert its intracellular signaling via nuclear translocation of cellular CT-1 has so far not yet been investigated. It is furthermore not known whether CT-1 regulates the expression of pro-cardiogenic growth factors, e.g. VEGF, PDGF-BB, FGF-2 and the peptide hormone ANP to stimulate cardiomyogenesis of ES cells. The present study was therefore undertaken to study the effects of CT-1 on cardiomyogenesis and regulation of cardiogenic growth factors in ES cells with special emphasis on the impact of intracellular calcium signals, ROS and NO on nuclear translocation of CT-1. Our data demonstrate that cardiomyogenesis of ES cells is regulated by CT-1 which may exert its effects via translocation into the cell nucleus.

2. Materials and methods

2.1. Materials

CT-1 was obtained from BioVendor GmbH (Heidelberg, Germany). AG490, thapsigargin, L-NAME and LY294002 were obtained from (Merck Millipore, Darmstadt, Germany). NMPG and BAPTA, AM were obtained from Sigma (Deisenhofen, Germany).

2.2. Spinner-culture technique for cultivation of embryoid bodies

To obtain three-dimensional embryoid bodies, ES cells (line CCE) were grown on mitotically inactivated feeder layers of primary murine embryonic fibroblasts in Iscove's medium (Gibco, Live Technologies, Helgerman Court, MD, USA) supplemented with 15% heat-inactivated (56 °C, 30 min) fetal calf serum (FCS) (Sigma), 2 mM glutamine (PAA, Cölbe, Germany), 100 μM 2-mercaptoethanol (Sigma), 1% (v/v) NEA non-essential amino acids stock solution (100×) (Biochrom, Berlin, Germany), 1% (v/v) MEM amino acids (50×) (Biochrom), 1 mM Na^+-pyruvate (Biochrom), 0.4% penicillin/streptomycin (100×) (Biochrom, Berlin, Germany), 2.5 μg/ml plasmocin (InvivoGen, San Diego, CA) and 1000 U/ml LIF (Chemicon, Hampshire, UK) in a humidified environment containing 5% CO_2 at 37 °C, and passaged every 2–3 days. At day 0 of differentiation adherent cells were enzymatically dissociated using 0.05% trypsin–EDTA in phosphate-buffered saline (PBS) (Gibco, Helgerman Court, MD), and seeded at a density of $3 \cdot 10^6$ cells ml^{-1} in 250 ml siliconized spinner flasks (CellSpin, Integra Biosciences, Fernwald, Germany) containing 125 ml Iscove's medium supplemented as described above but devoid of LIF and plasmocin. Following 24 h 125 ml medium was added to give a final volume of 250 ml. The spinner flask medium was stirred at 20 r.p.m. using a stirrer system (Integra Biosciences, Fernwald, Germany), 125 ml cell culture medium was exchanged every day. For evaluating the number of contractile activity approximately 30 embryoid bodies in each individual experiment were plated into 6 cm cell culture dishes. Contractile activity was evaluated by microscopic inspection. Maximum contractile activity of embryoid bodies was observed within 10 days of cell culture. Contracting and non-contracting embryoid bodies were determined irrespective or the number of contracting areas in individual embryoid bodies.

2.3. Measurement of ROS generation

Intracellular ROS levels were measured using the fluorescent dye 2′ 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Life Technologies, Darmstadt, Germany), which is a nonpolar compound that is converted into a nonfluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane impermeable and is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS. For the experiments, embryoid bodies were incubated in serum-free medium, and 20 μM H_2DCF-DA dissolved in dimethyl sulfoxide (DMSO) was added. After 30 min intracellular DCF fluorescence (corrected for background fluorescence) was evaluated in 3600 μm^2 regions of interest using an overlay mask unless otherwise indicated. For fluorescence excitation, the 488 nm band of the argon ion laser of a confocal laser scanning microscope (Leica SP2 AOBS, Leica, Bensheim, Germany) was used. Emission was recorded at an emission band of 515–550 nm.

2.4. Measurement of NO generation

NO generation was evaluated by the use of the cell permeable specific fluorescent NO indicator 4,5-diaminofluorescein diacetate (DAF-2DA) (Life Technologies). After incorporation into cells DAF-2 reacts rapidly with NO to yield the highly fluorescent compound triazolofluorescein (DAF-2T). Embryoid bodies were incubated for 45 min with 10 μM DAF-2DA dissolved in cell culture medium. Subsequently, embryoid bodies were washed with serum-free medium, and cells were incubated for further 30 min on a cell shaker. They were finally transferred to an incubation chamber mounted to the inspection table of the confocal setup, and DAF-2T fluorescence was recorded in single embryoid bodies using the 488-nm of the argon-ion laser of the confocal setup. Emission was recorded at >515 nm.

2.5. Recording of intracellular calcium concentrations

Intracellular calcium was recorded in single cells. Single cell preparations were obtained by enzymatic digestion of 3-day-old embryoid bodies for 30 min at 37 °C in PBS containing 2 mg/ml collagenase B (Boehringer, Ingelheim, Germany). Dissociated single cells were plated