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Research paper

Insufficient Apaf-1 expression in early stages of neural differentiation of human embryonic stem cells might protect them from apoptosis

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ABSTRACT

Recent evidence suggests that mitochondrial apoptosis regulators and executioners may regulate differentiation, without being involved in cell death. However, the involved factors and their roles in differentiation and apoptosis are still not fully determined. In the present study, we compared mitochondrial pathway of cell death during early neural differentiation from human embryonic stem cells (hESCs). Our results demonstrated that ROS generation, cytosolic cytochrome *c* release, caspases activation and rise in p53 protein level occurred upon either neural or apoptosis induction in hESCs. However, unlike apoptosis, no remarkable increase in apoptotic protease activating factor-1 (Apaf-1) level at early stages of differentiation was observed. Also the caspase-like activity of caspase-9 and caspase-3/7 were seen less than apoptosis. The results suggest that low levels of Apaf-1 as an adaptor protein might be considered as a possible regulatory barrier by which differentiating cells control cell death upon rise in ROS production and cytochrome *c* release from mitochondria. Better understanding of mechanisms via which mitochondria-mediated apoptotic pathway promote neural differentiation can result in development of novel therapeutic approaches.

1. Introduction

Apoptosis is a mechanism of programmed cell death that occurs in higher organisms to eliminate abnormal and damaged cells (Nur et al., 2004). Mainly, two extrinsic and intrinsic pathways have been defined for apoptosis. The extrinsic pathway is triggered through plasma membrane proteins of the tumor necrosis factor (TNF) receptor family, which leads to direct activation of caspases, starting with the receptor-proximal caspase 8 (Garrido et al., 2006).

The intrinsic pathway that initiates apoptosis can be triggered by a diverse array of non-receptor-mediated stimuli. The mitochondrion is a key element of intrinsic apoptosis induced by cellular stress (Green and Reed, 1998; Wang, 2001). Upon initiation of intrinsic pathway, pro-apoptotic Bcl-2 family members trigger oligomerization and activation of the death effectors Bax and Bak, which in turn permeabilize the outer mitochondrial membrane (Kluck et al., 1999). Cytochrome *c* (Cyt *c*) is then released from the intermembrane space of the mitochondrion into the cytosol, where it binds to apoptotic protease activating factor-1 (Apaf-1) in the presence of ATP/dATP to form apoptosome complex (Li et al., 1997). This multimeric Apaf-1/Cyt *c* complex recruits pro-caspase-9 via its caspase recruitment domain (CARD), promoting pro-caspase-9 efficient activation (Rodriguez and Lazebnik, 1999; Zou et al., 1999). Activated caspase-9 in turn activates the downstream effectors caspase-3 and 7 (Li et al., 1997; Liu et al., 1996), which rapidly cleave intracellular substrates.

Recent evidences have suggested that some apoptosis associated factors, including p53, caspases and calpains participate in differentiation (Aranha et al., 2010; Fernando et al., 2002; Galluzzi et al., 2012; Santos et al., 2012; Sola et al., 2012; Zheng et al., 2008). Generation of human embryonic stem cells (hESCs) and their ability to differentiate into neurons *in vitro*, have allowed scientists to study early human neuronal development (Prajumwongs et al., 2016). However, the factors that determine neural differentiation or cell death following activation of the apoptotic pathway in hESCs are still not fully understood.

In this regard, we previously investigated the role of mitochondrial apoptotic pathway and cell energy level during differentiation of mouse ESCs into cardiomyocytes and through apoptosis (Akbari-Birgani et al.,

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2014). In fact, by application of a real-time assay for Apaf-1 oligomerization based on split-luciferase complementary assay, it was shown that during differentiation of mouse ESCs to cardiomyocytes, apoptosome formation was delayed (Akbari-Birgani et al., 2014; Torkzadeh-Mahani et al., 2012).

To have a better understanding of the role of the apoptotic molecules in early neural differentiation of hESCs and to compare it with apoptosis, we attempted to monitor mitochondrial pathway of cell death, in terms of ATP content, mitochondrial complex I activity, ROS levels, cytochrome *c* release from mitochondria, caspases-3/7 and 9 activities, Apaf-1 and p53 protein expression. We showed a transient release of cytochrome *c* from mitochondria during neural induction of hESCs. Nevertheless, increased ROS levels, cytochrome *c* release and caspases activity did not lead to cell death during differentiation. Our data revealed that the mitochondrial death pathway is activated at the early neural differentiation states, but this activation is critically different from what happens during apoptosis.

2. Materials and methods

2.1. hESCs culture and neural differentiation

The hESC line, RH6 (Baharvand et al., 2006) was maintained under feeder-free conditions on Matrigel in DMEM/F12 medium (Gibco; 21331-020) supplemented with 20% knockout serum replacement (KSR, Gibco; 10828-028), 2.0 mM L-glutamine (Gibco; 25030-024), 0.1 mM β-mercaptoethanol (Sigma; M7522), 1% nonessential amino acids (Gibco; 11140-035), 1% ITS (Gibco; 41400-045), 100 units/ml penicillin, 100 µg/mL streptomycin (Gibco; 15070-063), and 100 ng/mL basic fibroblast growth factor (bFGF, Royan BioTech). The medium was changed daily and cells were passaged weekly using collagenase/dispase (1:2, v/v; both from Gibco; 17104-019/17105-041).

Neuroepithelial cells were produced based on previously published protocol with some modifications (Li et al., 2011). hESCs were passaged using Accutase (Gibco; A11105-01) on Matrigel-coated plates. Cells (about 20% confluency) were treated for 7 days with neural induction medium containing advanced DMEM/F12 (Gibco; 21331-020) and Neurobasal media (Gibco; 21103-049) (1:1), 1x N2 (Gibco; 17502-048), 1x B27 (Gibco; 17504-044), 2.0 mM L-glutamine (Gibco; 25030-024), 5.0 µg/mL bovine serum albumin (BSA, Sigma-Aldrich, A3311) and 10 ng/mL human leukemia inhibitory factor (LIF, Royan BioTech), 3.0 µM CHIR99021 (Sigma-Aldrich; 1046), and 2.0 µM SB431542 (Sigma-Aldrich; 4317).

2.2. Apoptosis induction

Due to the different degrees of sensitivity of cells to doxorubicin, different concentrations of doxorubicin (EBEWE Pharma Ges) from 0.1 to 1.0 µM, were added to undifferentiated hESCs medium for 24 h. Then hESCs viability determined using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, M5655) through a spectrophotometric microplate reader and by which IC50 value was calculated. Next, all experiments were performed in the presence of desired doxorubicin concentration. To prepare cell extract, cells harvested at 2, 6, 12 and 24 h, washed twice with cold PBS, trypsinized, pelleted by centrifugation and stored at −80 °C.

2.3. Immunofluorescence staining and flow cytometry

For immunofluorescence staining, cells were fixed in 4% w/v paraformaldehyde (Sigma-Aldrich, P6148) for 15 min, permeabilized by 0.25% Triton X-100 (Sigma-Aldrich, T8532) and blocked using 10% host serum of the secondary antibody and 1% BSA (Sigma-Aldrich, A3311). Cells were incubated overnight at 4 °C with the following primary antibodies previously diluted in blocking solution: rabbit anti-PAX6 (Thermo, PIPA525970), mouse anti-NESTIN (Millipore, MAB5326) and mouse anti N-cad (Abcam, ab6528). Cells were incubated for 45 min with the following secondary antibodies: Donkey anti-rabbit Alexa Fluor® 546 (Invitrogen, A10040) and donkey anti-mouse Alexa Fluor® 488 (Invitrogen, A21202). Nuclei were counterstained with DAPI (Sigma-Aldrich, D8417) and analyzed using a fluorescence microscope (Olympus, IX71).

For flow cytometry analysis, cells were harvested using 0.05% trypsin/EDTA (Gibco) and fixed in 4% paraformaldehyde. Intracellular antigens were labeled by incubating with rabbit anti-PAX6 (Thermo, PIPA525970) and mouse anti-NESTIN (Millipore, MAB5326) primary antibodies for 30 min at 4 °C in the dark, followed by incubation for 30–40 min with the appropriate Alexa Fluor® 488 or Alexa Fluor® 546 fluorescent secondary antibodies. After washing, flow cytometric analysis was performed by a BD-FACS Calibur Flow Cytometer. The experiments were replicated three times and the acquired data were analyzed by flowjo software.

2.4. Cell extracts preparation and protein concentration measurement

To prepare cell extract, cells harvested at 24, 48, 72, 96 and 120 h, were washed twice with cold PBS, trypsinized, pelleted by centrifugation and stored at −80 °C.

To prepare cytosolic fraction for determination of cytochrome *c* release, cells were lysed in buffer A (20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 1 mM DTT and 250 mM Sucrose) containing protease inhibitor cocktail (Sigma) and passed 10 times through a 30-gauge needle (Liu et al., 1996). Following centrifugation at 15,000*g* for 30 min at 4 °C, cytosolic extract (supernatants) were collected.

For isolation of total protein extracts, the cells were homogenized in mammalian cell lysis buffer (Mammalian Protein Prep Kit, Qiagen). Lysates were centrifuged at 14,000*g* for 10 min, and supernatants were collected to perform Western blotting analysis. Protein concentration was determined by Bradford assay.

2.5. Immunoblotting

Western blotting analysis was performed according to the standard procedures. Briefly, equal amounts of proteins were separated by SDS PAGE. After electrotransfer to PVDF membrane (Bio-Rad), the blots were blocked using 5% skim milk and incubated overnight at 4 °C with mouse anti-Cyt *c* (Abcam, ab13575), rabbit anti- Apaf-1 (Abcam, ab32372), Rabbit anti-p53 (Cell signaling, 2527), Rabbit anti-PARP (Roche, 11835238001) and mouse anti-GAPDH (Abcam, ab9484). Membranes were incubated with peroxidase-conjugated secondary antibodies, anti-rabbit IgG (Sigma-Aldrich, A2074) and anti-mouse IgG (Sigma-Aldrich, A0168) as appropriate, for 1 h at room temperature. Finally, immune-reactive bands were detected by enhanced ECL (Amersham Bioscience). For both apoptosis and differentiation western blot were repeated 3 times. Subsequently, the films were scanned with a densitometer (GS-800, Bio-Rad). Quantification of each band was performed by Image J software and the average of 3 different bands is plotted. GAPDH was used as an internal reference for each experiment to normalize protein expression compared to 0 h.

2.6. ATP measurement

Cell lysate prepared by Cell Culture Lysis Reagent (CCLR) containing 100 mM potassium phosphate, pH 7.8, 1.0 mM EDTA, 7.0 mM 2-mercaptoethanol, 1% (v/v) Triton X-100, and 10% (v/v) glycerol, was subjected to ATP measurement. Cellular ATP content was measured by the firefly luciferase assay. A standard curve was obtained by preparing a dilution series of ATP as standard solutions. Luciferin-luciferase assay was performed by a luminometer, as previously described (Ghiasi et al., 2012).