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Comparison of *BAX and Bcl-2* Expression During Human Embryonic Stem Cell Differentiation into Cardiomyocytes and Doxorubicin-induced Apoptosis

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ABSTRACT

Although the cell differentiation is an inseparable part of development in multicellular organisms, the regulating molecular pathway of it still is not fully defined. In the other hand, apoptosis is a fundamental physiological process which plays an essential role in a variety of biological events during development. Moreover, recent studies have found that apoptosis shows several common features with differentiation but that how cells distinguish apoptosis and differentiation from each other is not clear. In this study two critical members of the Bcl-2 family, BAX (an apoptosis promoter) and Bcl-2 (an apoptosis inhibitor) ratio was investigated in both apoptosis and differentiation processes. BAX/ Bcl-2 ratio is one of the important characteristics of the apoptosis process but its role during differentiation is still unknown.

Keywords: Mitochondrial mediated apoptosis; BAX /Bcl-2 ratio; Cardiomyocyte differentiation; Chemically induced hESC

INTRODUCTION

Human embryonic stem cells (hESCs) have been established as a unique system for production of derivatives of all three primary germ layers, including beating cardiomyocytes [1]. *In vitro* production of hES cell-derived cardiomyocytes has permitted early cardiac development analysis during differentiation. On the other hand, the highly regulated process of programmed cell death ,apoptosis, is an inseparable part of development [2] coupled with both *in vitro* and *in vivo* differentiation. Recently, several studies have indicated that both apoptosis and differentiation processes show several features in common [3-7]. Apoptosis, which is generally characterized by a series of distinct morphological features and energy-dependent biochemical mechanisms [8-9], occurs in multicellular organisms in response to different environmental and internal stimuli. Regulation of apoptosis is critical for normal cell turnover, proper development, embryogenesis, chemical-induced cell death, and *etc*. There are at least two main pathways which lead to apoptosis, including extrinsic death-receptor dependent (extrinsic) apoptosis and mitochondrial mediated apoptosis (intrinsic). Bcl-2 family is the most important family of proteins involved in the regulation of apoptotic cell death. Intracellular membrane of mitochondria is the place that these proteins operate and organize intrinsic apoptosis [10, [11]. This family includes the apoptosis promoter members such as BAX and the apoptosis inhibitor members such as Bcl-2 [12,13]. Cell death stimuli cause certain Bcl-2 family proteins localization changes. For instance, upon apoptotic cell death stimuli BAX redistributes from cytosolic fragment to the outer membrane of mitochondria to promote its permeabilization and subsequent release of cytochrome *c*. Cytosolic accumulation of cytochrome *c* , in turn, causes apoptosome complex formation and caspase cascade activation [14].

BCL-2 protein resides on the outer membrane of mitochondria and promotes cellular survival due to its critical role in the inhibition of pro-apoptotic proteins. Bcl-2 operates as an attenuator of apoptosis in contrast to BAX with apoptosis promoter properties. In fact, the complex

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network of interactions between Bcl-2 family proteins determines the fate of the cell to live or die [15]. The goal of this study is the investigation of the expression of pro-apoptotic *BAX* and anti-apoptotic *Bcl-2* genes changes during cardiomyocytes differentiation. To determine the expression of *BAX* and *Bcl-2* genes, quantitative Real-Time Polymerase Chain Reaction (RT-PCR) method was applied and next to that, *BAX/Bcl-2* ratio was calculated by using the relative mRNA level during differentiation and apoptosis processes.

MATERIAL AND METHODS

Cell Culture

For aggregates formation, 2×10^5 viable hESCs per milliliter (line RH5) were cultured in 60 mm bacterial plates which were nonadhesive (Sigma-Aldrich) in 5 ml of hESC feeder free media, including Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12) (Gibco), 20% Knockout Serum Replacement (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM b-mercaptoethanol (Sigma-Aldrich), 1% nonessential amino acids (Gibco), 1%insulin-transferrin-selenite (Gibco) and 100 ng ml^{-1} basic fibroblast growth factor (bFGF) (Royan Institute for Stem Cell Biology and Technology). Cells were incubated under standard conditions (37 °C, 5% CO_2 and saturated humidity).

Cardiac Differentiation in Static Suspension Culture

To produce human embryonic stem cell-derived cardiomyocytes (hESC-CM) in Static Suspension Culture, 5-day old hESC aggregates with the size of 175 ± 25 µm were transferred to RPMI 1640 medium (Gibco) supplemented with 2% B27 without retinoic acid (Gibco), 2 mM L-glutamine, 0.1 mM b-mercaptoethanol, 1% nonessential amino acid, differentiation medium (DM), treated with 12 µM of the glycogen synthase kinase 3-β inhibitor CHIR9902 for one day. On day two, spheroids were washed with Dulbecco's phosphate-buffered saline (DPBS) and medium was changed to DM without CHIR9902 for one day. On day three, medium was changed to DM containing 5 µM IWP2 (Tocris Bioscience) as a WNT antagonist, 5 µM SB431542 (Sigma-Aldrich) as an inhibitor of transforming growth factor-b super family type I activin receptor-like kinase receptors, and 5-µM purmorphamine (Stemgent) as a sonic hedgehog agonist and spheres were maintained for two days in this medium. On day five, DM was added to spheres after they had been washed with DPBS, and medium was refreshed two days in between. The fifth day beating began and reached its highest on the tenth day [16]. Samples were collected during differentiation before aggregates started beating.

Apoptosis Induction of hESCs

For apoptosis induction doxorubicin (Ebendoxo, EBEWE Pharma Ges) was used. Due to the different degrees of sensitivity of cells to doxorubicin, hESCs were treated with different concentrations of doxorubicin and 24 hours after treatment, hESCs viability determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich) through a spectrophotometric microplate reader. And then, IC50 value was calculated. Next to that, 5-day old hESC aggregates, were treated with desired doxorubicin concentration and samples were collected within 24 h of induction.

RNA Isolation and RT-PCR

The total RNA of sample cells was extracted using the TRIzol reagent (Invitrogen, USA). RNA samples were treated with recombinant DNase I (Invitrogen) for 15 minutes at room temperature to remove possible genomic DNA contamination. Then 2 µg of total RNA was used to perform cDNA synthesis with oligo(dT) 20 and random hexamer primers according to the manufacturer's instructions (Takara) and finally RT-PCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems, CA).The expression of the genes of interest was normalized according to GAPDH expression.

Flow Cytometry

To indicate the rate of cell death, annexin V/PI staining was applied during both processes of differentiation and apoptosis using annexin-V-FLUOS Staining Kit (Roche, USA) according to the manufacturer's instructions. Samples were analyzed using the Flow Cytometer (FACSCalibur; BD Biosciences) and flowing software, version 2.5.1 (BD Biosciences).

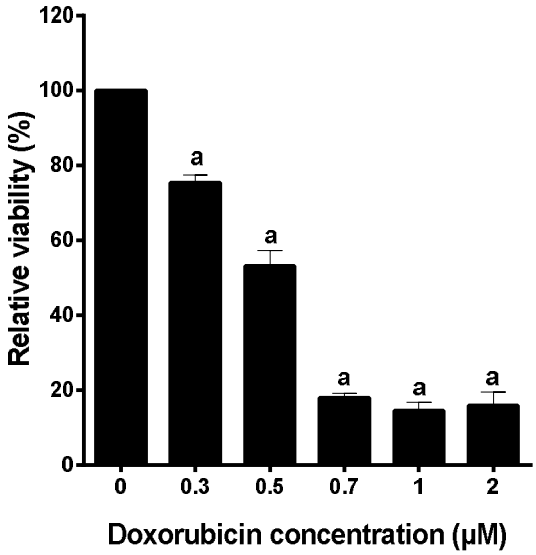

Fig. 1. Apoptosis induction. To determine apoptogenic concentration of doxorubicin MTT assay was done in the presence of doxorubicin at a variety of concentrations. Each column represents the mean ± SD from three experiments. a: $p < 0.05$ between the first column, without any treatments, and the other columns.

Immunostaining and Imaging

To determine cardiomyocytes differentiation efficiency, beating spheroids were washed and maintained 5 min in DPBS and then trypsinized (with 0.05% trypsin-EDTA, Gibco) to achieve single cells. In the next step, singled cells were seeded in 4 well gelatin-coated plate in DM. After 2 days, the attached cells were washed with DPBS, fixed with 4% (wt/vol) paraformaldehyde for 20 min at 4 °C, washed once with PBS/0.1% Tween 20, then permeabilization was performed with 0.5% Triton X-100 in DPBS for 30 min at room temperature. Blocking was performed with 5% (vol/vol) goat serum or bovine serum albumin for 1 h. Primary antibodies diluted in blocking buffer (1:100) were added to the cells and incubated at 4 °C over night. Then cells were washed three times with PBS/0.1% Tween 20, each for 5 min. Then diluted secondary antibodies in blocking buffer (1:500) were added to cells and incubated for 45 min at room temperature and finally cells were washed three times with PBS/0.1% Tween 20.

Cell Viability and IC50 Value in Doxorubicin-treated hESC

RH5 hESCs were treated with different concentrations of doxorubicin ranging between 0.3-2 µM for 24 h, and then MTT assay was used to measure cell viability. MTT assay showed that increased concentrations of doxorubicin increased cell death in a concentration dependent manner and IC50 value was 0.5 µM. To induce effective apoptosis 1 µM doxorubicin was chosen according to MTT assay results (Fig. 1).

Apoptosis Detection During hESC Differentiation

To determine the apoptotic population during differentiation process, annexin V/PI staining was applied during differentiation and doxorubicin-induced apoptosis ,as a positive control of apoptosis. Annexin V/PI staining indicated that, in both control and differentiating cells the apoptotic population were about 15%, but in doxorubicin-treated cells, apoptotic cell percentage increased significantly within 24 h of apoptosis induction (Fig. 2).

Validation of Cardiomyocyte Differentiation

To determine the efficiency of differentiation, beating spheroids were counted on day 14 of differentiation and approximately 100% of spheroids were beating (Data not shown). Furthermore, day 14, beating spheroids were

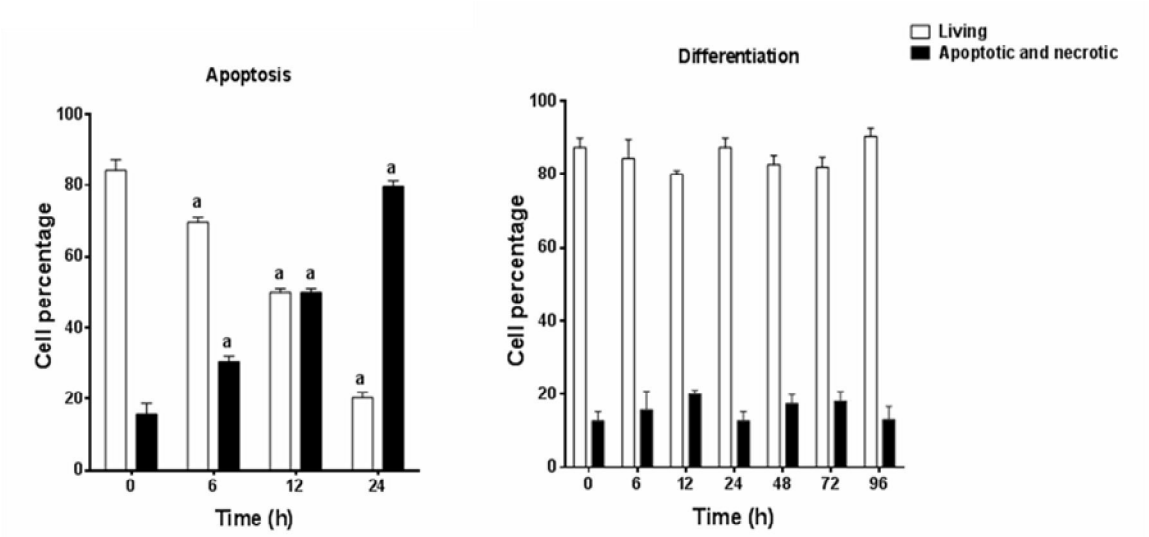

Fig. 2. Apoptotic cells detection using annexin V/PI staining. To determine the apoptosis ratio during differentiation and apoptosis process, annexin V/PI staining was done during 12, 24, 48, 72, and 96 h of differentiation and 12, 24 h of apoptosis. Data are means ± S.D. (error bars) of triplicate experiments. A: $p < 0.05$ between the first column, without any treatments, and the other columns.

collected and dissociated into the single cells for cardiac differentiation markers immunostaining, including structural protein cTNT and Actinin to show sarcomeric structures in differentiated cardiomyocytes (Fig. 3).

Expression of BAX and Bcl-2 Genes and BAX /Bcl-2 Ratio in Apoptosis and Differentiation

During apoptosis *BAX* gene expression increased significantly (2.5-fold) 12 h after 1 µM doxorubicin treatment ($p < 0.05$) (Fig. 4A). On the other hand, *Bcl-2* gene expression was dropped significantly 12 h after apoptosis induction (Fig. 4B). Our results also indicated that during apoptosis, *BAX/Bcl-2* ratio increased significantly ($p < 0.05$). As such, about 8-fold increase in the *BAX/Bcl-2* ratio was seen in doxorubicin-treated hESCs (Fig. 4C). Moreover, during differentiation of hES cell-derived cardiomyocytes a fluctuating pattern of the *BAX* gene was observed with the highest amount of 1.5-fold increase 12 and 48 h after differentiation induction ($p < 0.05$) (Fig. 4A), in the other hand, *Bcl-2* gene expression showed another fluctuating pattern with the lowest amount, 12 and 48 h after differentiation induction when the *BAX* gene expression was at the highest (Fig. 4B). Respectively, *BAX /Bcl-2* ratio significantly increased ($p < 0.05$) about 2.4-fold, 12 and 48 h after differentiation (Fig. 4C).

CONCLUSIONS

Given the importance of hESCs as an extremely attractive tool for the generation of different types of body cell for the cell therapy and other biomedical applications understanding the molecular pathway of differentiation should be taken into consideration. Furthermore, hES Cell-derived cardiomyocytes are important tools for clinical and industrial applications such as drug discovery and toxicity testing, followed by clinical applications. Therefore, understanding molecular mechanisms involved in cardiomyocytes differentiation should be considered as an important part. On the other hand, apoptosis plays a critical role in a variety of biological events during development and differentiation. Moreover, recent studies have found that apoptosis shows several common features with differentiation. The critical role of apoptosis during development, common features between apoptosis and differentiation and the high rate of cell death during differentiation suggest that apoptotic cell death and

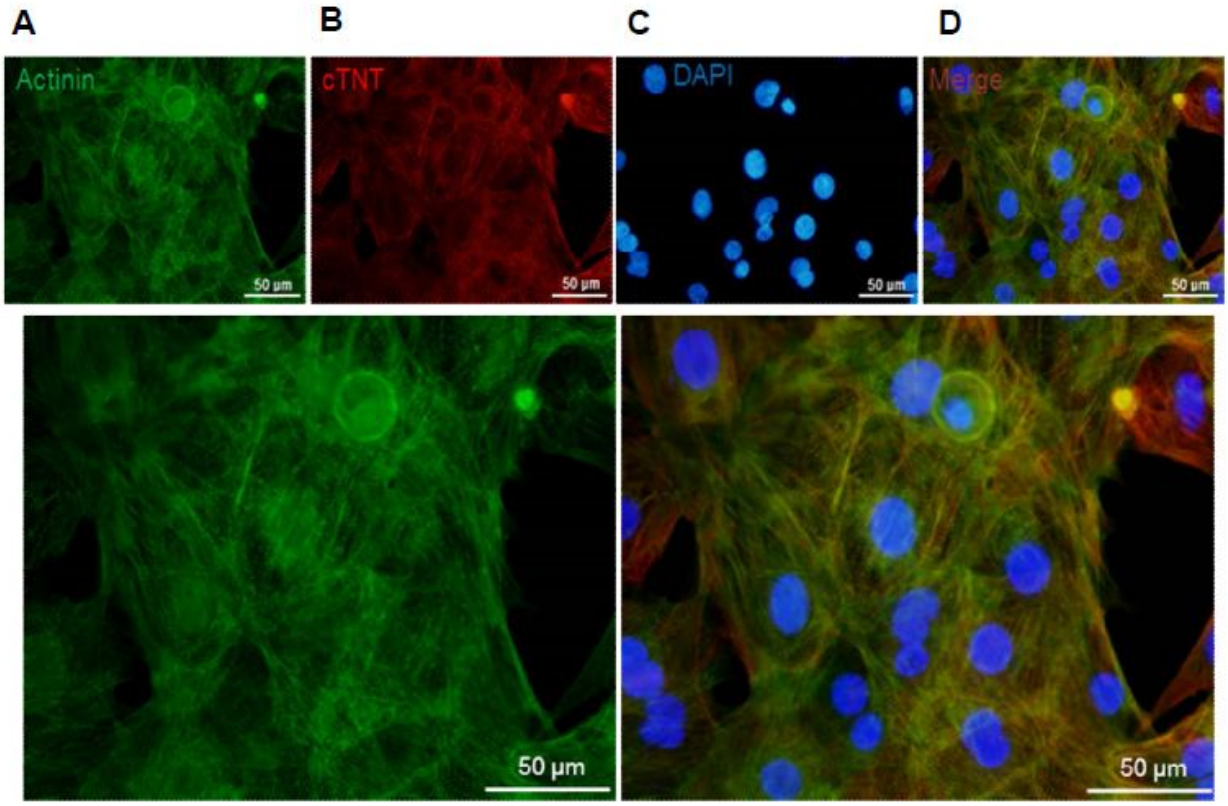

Fig. 3. Differentiation of hESC into beating cardiomyocytes. Dissociated beating spheroids were stained for cardiomyocytes-specific markers and. A. Immunostaining of dissociated hESC-derived beating spheroids at day 14 for structural protein cTNT B. Immunostaining of hESC-derived beating spheroids dissociated at day 14 for Actinin to show sarcomeric structures. C. DAPI, 4′, 6-diamidino-2-phenylindole, was used to show nucleus. D. the merge of cTNT, Actinin and DAPI.

differentiation are integral parts of the development process but how cells distinguish apoptosis and differentiation from each other is not clear yet.

In the presence of cytotoxic agents apoptotic cell death can be initiated by activation of death signaling pathways. Apoptosis induction by doxorubicin causes disturbance in mitochondrial function followed by caspase cascade activation [17]. As it mentioned one of the critical apoptosis regulators is Bcl-2 family proteins. In the present study the mRNA level of two members of this family, *BAX* (pro-apoptotic) and *Bcl-2* (anti-apoptotic) were assessed. Our data indicated that in hESCs, *Bcl-2* and *BAX* genes' expression and also *BAX /Bcl-2* ratio were changed following the apoptosis induction by doxorubicin.

To determine doxorubicin cytotoxicity, MTT assay was performed and the results demonstrated that the viability of hESCs decreased significantly in a time and dose-dependent manner. IC50 value was about 0.5 µM for 24 h incubation. Upon exposure to various death stimuli, activated BAX conformational changes and oligomerization occur, which leads to mitochondrial outer membrane permeabilization (MOMP) [12-15]. Moreover, Bcl-2 as an important apoptosis inhibitor interacts with BAX and prevents the progress of the process. When there is an extra amount of Bcl-2, cells are protected against apoptosis. On the ether hand, when there is an extra amount of BAX, cells are susceptible to apoptotic cell death. It has been reported that mitochondria-mediated apoptosis *in vivo* is involved in doxorubicin cardiotoxicity in rats and the Bcl-2/BAX ratio would decrease 4 days after doxorubicin induction due to

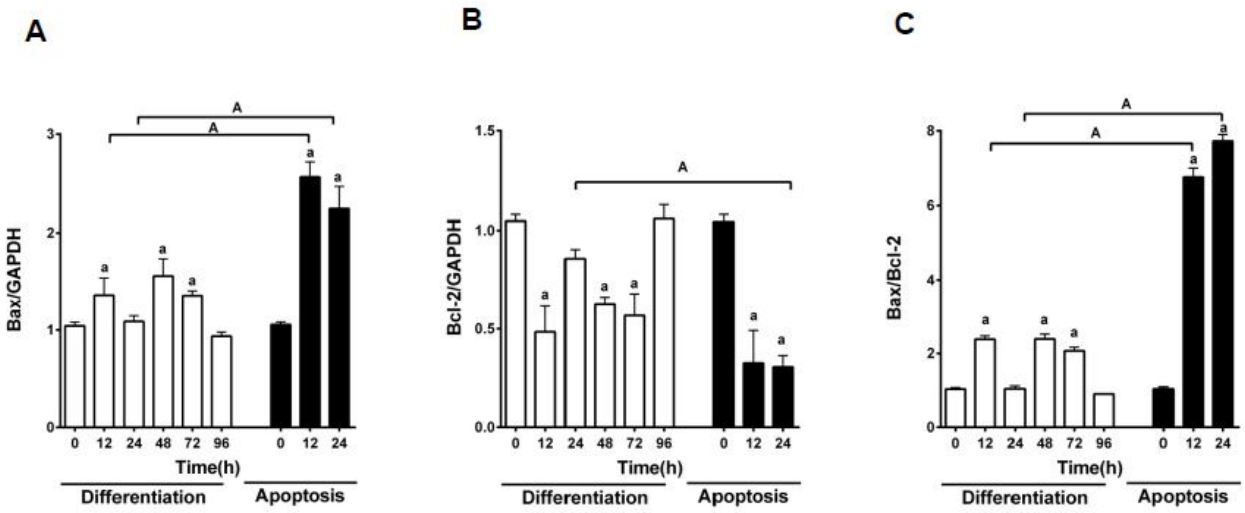

Fig. 4. Expression of *BAX, Bcl-2* genes and BAX/Bcl-2 ratio during differentiation and apoptosis. BAX expression, B. Bcl-2 expression, C. BAX/Bcl-2 ratio. Data are means ± S.D. (error bars) of triplicate experiments. a: $p < 0.05$ intragroups , A: $p < 0.05$ intergroups.

adaptation mechanisms [18].

In this study, it was shown that during differentiation *BAX* level increased, while *Bcl-2* decreased in the fluctuating pattern compared with apoptosis and also the degree of changes were low. The fluctuating pattern of *BAX /Bcl-2* ratio was observed during differentiation. There are various states of differentiation during hES cell-derived cardiomyocytes. First, hESCs differentiate into mesoderm cells then again during the process they differentiate into cardiac mesoderm and finally cardiac progenitor cells so the fluctuating pattern of *BAX, Bcl-2* and subsequently *BAX /Bcl-2* ratio might be due to these different states of differentiation.

Moreover, it has been reported that in hESCs BAX accumulated at Golgi in its activated form. It has been suggested that BAX protein causes high degrees of sensitivity of hESCs to apoptotic cell death [19]; but our data indicated that BAX function is more than its mere apoptotic role. Another recent study has shown that P53 can interact directly with BAX upon mitochondria-mediated cell death [20] which in turn leads to MOMP and caspases activation.

Taken together, our data indicated that the increased level of *BAX* and the decreased level of *Bcl-2* were required for the normal hESC differentiation into the beating cardiomyocytes and also *BAX /Bcl-2* ratio appeared to be the part of differentiation pathway, and the time of the engagement and the degree of changes can contribute to the hESCs differentiation into the beating cardiomyocytes.

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