Analysis of TdT-Mediated dUTP Nick End Labeling (TUNEL)-Positive Cells Associated with Cardiac Myogenesis in Mouse Embryo

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ABSTRACT. Caspase activation is associated with skeletal muscle differentiation in mouse embryos. We examined the relationship between cardiac myogenesis and cell death using mice hearts at embryonic days (E) 11.5–15.5 and fetal rat heart H9C2 cells. The number of TdT-mediated dUTP nick end labeling (TUNEL)-positive cells increased with fetal age and was much higher than that of single-stranded DNA (ssDNA)- and active caspase-3 (aCasp3)-positive cells. TUNEL and aCasp3 double staining resulted in 3 types of positive cells: TUNEL+/aCasp3−, TUNEL+aCasp3− and TUNEL+/aCasp3+. TUNEL+/aCasp3− cells were the most common but lacked morphological features of apoptosis, such as nuclear condensation or fragmentation. The expression of anti-apoptotic factors increased during E11.5–15.5. Furthermore, TUNEL-positive H9C2 cells without nuclear condensation or fragmentation were observed only in myotubes later in the culture period. In this study, the dynamics of TUNEL-positive cardiomyocyte was inconsistent with the activation of apoptosis cascade, and their morphological feature was clearly different from representative apoptosis. From these findings, we concluded that the increased number of TUNEL-positive cardiomyocyte, having the DNA strand breaks, would be associated with the progression of cardiac myogenesis.

KEY WORDS: cardiac myogenesis, caspase-3, cell death, mouse, TUNEL.

The heart is the first organ to form during mammalian embryogenesis [5]. Heart formation begins at approximately embryonic day (E) 8.0 in the mouse, when cardiogenic precursor cells derived from the visceral mesoderm form a linear heart tube, followed by formation of the bulboventricular loop, chamber specification, septation and valvulogenesis [23]. Cardiomyocytes gain systolic performance soon after formation of the heart tube [14]. While cardiomyocytes beat as functional myofibers, they also keep proliferating and size up the heart to facilitate blood supply for the embryo’s development. During the primary embryonic days until E10.0, cardiomyocytes proliferate actively; however, the proliferation rate starts to decline after the middle period (E12.0–14.0), and it stops by 3 weeks after birth in mice [1, 21]. Therefore, it is suggested that cell signaling must undergo some changes in this proliferation changeover; however, little is known about cardiac myogenesis at this stage of development.

Cell death plays important roles during organogenesis and homeostasis. In particular, it is known that both morphological and functional cell deaths are involved in fetal development, such as in formation of the interdigital space of the limbs and in selection of lymphocytes [16, 25]. Cell death during fetal development is a programmed cell death that is controlled by gene expression, and it is distinct from necrosis, which is a passive cell death [26]. Programmed cell death is classified into several types depending on the morphological features involved; i.e. type I and type II cell death represented apoptosis and autophagic cell death, respectively. The most common programmed cell death, apoptosis, is regulated by the caspase family proteins [24]. Caspase proteins are members of the cysteine proteases, which are translated into inactive precursors and then activated by cleavage and tetramer formation [13]. The active caspase induces apoptosis by cleaving substrate proteins [24] and causes breaks in DNA strands by activating deoxyribonuclease (DNase) [4].

Recently, it has been reported that the activation of the caspase family is associated with the differentiation of skeletal muscle, a striated muscle that is similar to cardiac muscle. It has been suggested that the caspase activation is involved in mouse myoblast differentiation, but not in apoptosis [7], and that artificial caspase activation promotes differentiation of the mouse myoblast cell line C2C12 to myotubes [17]. During the process of differentiation of myoblasts to muscle fibers via myotubes, the development of myofibers and the formation of the sarcomere are also observed. Because such structural changes also occur in cardiac myogenesis, it is suggested that there are similarities in the differentiation mechanisms between skeletal and cardiac muscles. However, the relationship between cardiac myogenesis and caspase activation or cell death remains unclear.

In the present study, we investigated the caspase activation in mice cardiogenesis and examined the relationship between cell death and cardiac myogenesis by using mouse embryo and rat cardiac myoblasts to clarify the mechanisms of cardiac myogenesis.

MATERIALS AND METHODS

Animals and collection of specimen: Eight-week-old male and female C57BL/6 (B6) mice were purchased from Japan SLC (Shizuoka, Japan) and maintained, with free access to food and water, in our facility. For embryo collection, timed mating was established by housing the female mice with the male mice overnight. At noon on the following day, the female mice were checked for the presence of vaginal plugs, which denoted pregnancy, and the embryos were recorded as being in E0.5 of development. The investigators adhered to the Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International).

Cell Culture: H9C2 cells (American Type Culture Collection; Manassas, VA, U.S.A.) were purchased (DS Pharma Biomedical, Osaka, Japan) and grown as a cell stock in Dulbecco’s Modified Eagle’s Medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; MP Biomedicals, Aurora, OH, U.S.A.), 0.06% L-glutamine (Wako, Osaka, Japan), 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Grand Island, NY, U.S.A.), under standard culture conditions (37°C, 5% CO₂, and water-saturated atmosphere). Before reaching confluence, the cells were split, plated at a lower density on a cover glass coated with collagen (Cellmatrix Type I-C; Nitta Gelatin Inc., Osaka, Japan), and cultured with 10% FBS medium for 1 day. For differentiation, the cells were then cultured in DMEM supplemented with 1% FBS and 10 nM all-trans retinoic acid (Nacalai Tesque, Kyoto, Japan). The culture medium was replaced every 2 days. Histology and gene expression were analyzed using cells extracted on days 0, 4, and 7 after differentiation.

Histological and immunohistochemical analyses of embryonic heart and H9C2 cells: The heart was removed from mice embryos of E11.5–15.5 and immediately fixed overnight with 4% paraformaldehyde at 4°C and then cut into 3-µm-thick paraffin sections. They were used for hematoyxlin-eosin staining, immunohistochemical analysis, and TdT-mediated dUTP nick end labeling (TUNEL) staining under the following procedures. For immunohistochemical analysis, the deparaffinized sections were incubated with 10 mM citric acid buffer (pH 6.0) and then 1% blocking reagent for 15 min at room temperature, TdT reaction mix (0.02 mM DIG-line phosphatase-conjugated sheep anti-digoxigenin (DIG) antibody (1:2,000; Roche) overnight at 4°C, and finally, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium salt solution (Roche) for 5 min. The sections were finally counterstained with Nuclear Fast Red. TUNEL staining for cultured H9C2 cells was performed in a similar way, except that the process was performed without protease K antigen retrieval and counterstaining. In addition, for TUNEL/ aCasp3 double staining, the aCasp3 immunohistochemical assay was performed after TUNEL staining, as described above, without counterstaining.

Histoplanimetry: For histoplanimetry, the number of positive cells was measured and analyzed by using the ImageJ software ver. 1.44 (NIH, Bethesda, MD, U.S.A.). The results were presented as the number of positive cells/mm² or the percentage of each positive cell type.

Quantitative real-time polymerase chain reaction (PCR): Total RNAs from the mice embryonic hearts were purified using the TRIzol® reagent (Life Technologies) following the manufacturer’s protocol. Purified total RNAs were treated with DNase (Nippon Gene, Tokyo, Japan), and cDNAs were synthesized using the ReverTra Ace® reverse transcriptase enzyme (Toyobo, Osaka, Japan) and oligo-dT primers (Life Technologies). Before the treatment with TRIzol® reagent, the cultured H9C2 cells were detached from a flask by treatment with 0.05% trypsin, centrifuged at 100 × g for 5 min, washed by phosphate-buffered saline after the supernatant was removed, and centrifuged again at 100 × g for 5 min. Quantitative real-time PCR analysis was performed using the Brilliant II SYBR Green QPCR Master Mix and real-time thermal cycler (MX 3000P; Agilent Technologies, Santa Clara, CA, U.S.A.) according to the manufacturer’s instructions. The mRNA expression levels of the target genes were normalized to the expression of actin, beta (Actb) for mice embryonic hearts or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for the rat H9C2 cells [9]. The summaries of the specific primer pairs used in this study are provided polyclonal antibody (1:200; Immuno-Biological Laboratories, Gunma, Japan). For immunohistochemical assay for the myosin heavy chain (MyHC), anti-MyHC mouse monoclonal antibody (1:400; Merck Millipore, Billerica, MA, U.S.A.) and the Nichirei Histofine® Mouse Stain Kit (Nichirei, Tokyo, Japan) were used. Immunofluorescence for MyHC using the cultured H9C2 cells was performed with the following procedures. The cultured H9C2 cells were fixed in acetone for 10 min at 4°C, and permeabilized in 0.1% Tween 20. The cells were incubated with the following solutions instead of those mentioned above: tetramethylrhodamine-5-(and 6)-isothiocyanate-labeled rabbit anti-mouse antibody (SouthernBiotech, Birmingham, AL, U.S.A.) as a secondary antibody, and Hoechst 33342 solution (Dojindo, Kumamoto, Japan) for nuclear staining.

TUNEL staining: The deparaffinized sections were incubated with the following: 20 µg/ml proteinase K for 15 min at room temperature, TdT reaction mix (0.02 mM DIG-dUTP, 10 U/ml TdT, pH 7.2) for 60 min at 37°C, 2× SSC for 15 min at room temperature, 1% blocking reagent (Roche, Basel, Switzerland) for 30 min at room temperature, alkaline phosphatase-conjugated sheep anti-digoxigenin (DIG) antibody (1:2,000; Roche) overnight at 4°C, and finally, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium salt solution (Roche) for 5 min. The sections were finally counterstained with Nuclear Fast Red. TUNEL staining for cultured H9C2 cells was performed in a similar way, except that the process was performed without proteinase K antigen retrieval and counterstaining. In addition, for TUNEL/ aCasp3 double staining, the aCasp3 immunohistochemical assay was performed after TUNEL staining, as described above, without counterstaining.

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Histoplanimetry: For histoplanimetry, the number of positive cells was measured and analyzed by using the ImageJ software ver. 1.44 (NIH, Bethesda, MD, U.S.A.). The results were presented as the number of positive cells/mm² or the percentage of each positive cell type.

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in Tables 1 and 2.

Statistical analysis: The results were expressed as the mean ± standard error (SE). The Kruskal-Wallis test was used for comparing over three populations or time points, and multiple comparisons were performed using Scheffé’s method when a significant difference was observed (P<0.05).

RESULTS

Development of the heart and cardiomyocytes in mice embryos: Histological analysis of mice embryonic hearts showed that the hearts had increased in size, the ventricular walls were thickened, and cell density had increased from E11.5 through E15.5 (Fig.1A-F). As a result of the immunohistochemical assay for MyHC, a myocyte marker, a positive reaction was observed in the embryonic hearts at as early as E11.5 (Fig.1G-I). In addition, the mRNA expressions of the cardiomyocyte markers troponin C, cardiac/slow skeletal (Tnnc1), myosin, heavy polypeptide 6, cardiac muscle, alpha (Myh6) and myosin, heavy polypeptide 7, cardiac muscle, beta (Myh7) were examined by real-time PCR (Fig.2). Tnnc1 and Myh7 mRNA expressions increased with fetal age, and Myh6 mRNA expression also increased from E13.5 to E15.5. In particular, Myh7 mRNA expression significantly increased from E11.5 to E15.5.

Cell death and caspase activation during cardiac myogenesis: TUNEL staining and immunohistochemical assays for ssDNA and aCasp3 were performed to examine the appearance of cell death and caspase activation during mouse heart development (Fig.3). TUNEL-, ssDNA- and aCasp3-positive cells were detected at all the embryonic ages examined, and these positive cells contained morphologically dead cells that showed nuclear condensation or fragmentation (Fig.3A, D, E, F, I). However, there were also TUNEL- and aCasp3-positive cells without morphological features of cell death (Fig. 3C, G, H). The number of TUNEL- and ssDNA-positive cells per unit cardiomyocyte area increased from E11.5 through E15.5, whereas that of aCasp3-positive cells decreased. In particular, the number of TUNEL-positive cells was much higher than that of ssDNA- and aCasp3-positive cells (Fig.3J).

Furthermore, the mRNA expressions of anti-apoptotic factors upstream of the caspase pathway such as the Bcl-2 family [B cell leukemia/lymphoma 2 (Bcl2) and Bcl2-like 2 (Bcl2l2)] and IAP family [baculoviral IAP repeat-containing 2 (Birc2), baculoviral IAP repeat-containing 3 (Birc3)], and the X-linked inhibitor of apoptosis (Xiap) were analyzed to examine caspase activation (Fig.4). Bcl-2 and Bcl-w are Bcl-2 family proteins, coded by Bcl2 and Bcl2l2, respectively. The Bcl-2 family works as anti-apoptotic proteins, which

Table 1. The mouse primer pairs used in this study

<table>
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<th>Genes (accession no.)</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size (bp)</th>
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| **Tnnc1** *(NM_009393.2)* | Forward: GGCAGAATGACTATGACGAG  
Reverse: GCCAAGGTCCAAGGACACAG | 165 |
| **Myh6** *(NM_010856.4)* | Forward: GTCAACAGCCTGACCAAGT  
Reverse: CTCCAGGTCCATGCTCT | 163 |
| **Myh7** *(NM_080728.2)* | Forward: CTTTGGAGAACCAGAATG  
Reverse: TCCACGTCTGTATATGTGA | 187 |
| **Bcl2** *(NM_009741.3)* | Forward: TCCGAGAGATGCAGCTCAG  
Reverse: ACCTCCTCCACACATGAC | 151 |
| **Bcl2l2** *(NM_0087537.1)* | Forward: AAGCTAGGGAGAAGGTTA  
Reverse: GGGTCTCAACTCCTCCA | 106 |
| **Birc2** *(NM_007465.2)* | Forward: GCCACCTAGTGTCTCCTCA  
Reverse: CAACACACTCCTGACCCTCA | 200 |
| **Birc3** *(NM_007464.3)* | Forward: GCTTACTGATGCAAAGACG  
Reverse: TTCTGTTTACAGCATTGCAC | 199 |
| **Xiap** *(NM_0069688.2)* | Forward: ACTTTGAGCCTTGAGGCA  
Reverse: AGTGACCAGATGCAGTCCAAAG | 193 |
| **Actb** *(NM_007393.3)* | Forward: TGGGATGACGGAGCTCA  
Reverse: GGCTGGATGACGGAGCTCA | 165 |

Table 2. The rat primer pairs used in this study

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<tr>
<th>Genes (accession no.)</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
</table>
| **Myl2** *(NM_00103525.1)* | Forward: CCTAACGTCCGGGCAACC  
Reverse: TGGGTGATGATGACAAACCA | 53 |
| **Gapdh** *(NM_017008.3)* | Forward: GTGATGCTGATGAGTCCAGTAG  
Reverse: CTCTGAGTGGGAGTGGG | 296 |

*, Karagiannis et al., 2010.
repress the cytochrome c release on the mitochondrial outer membrane [10, 19]. On the other hand, IAP1, IAP2 and XIAP are IAP family proteins, coded by Birc2, Birc3 and Xiap, respectively. Proteins of the IAP family directly bind to caspase proteins to prevent caspase activation and also work as apoptosis inhibitors [2]. The expressions of Bcl2, Bcl2L2, Birc3 and Xiap increased with fetal age, and that of Birc2 also increased from E11.5 through E13.5.

TUNEL and aCasp3 double staining in embryonic cardiomyocytes: To examine activation of the caspase cascade in the TUNEL-positive cells, TUNEL and aCasp3 double staining was performed. Three types of positive cells—TUNEL+/aCasp3+ (Fig.5A-C), TUNEL+/aCasp3− (Fig.5D-F) and TUNEL−/aCasp3+ (Fig.5G-I)—were detected at all fetal ages examined. TUNEL+/aCasp3+ cells were often accom-

Fig.1. Cardiogenesis in mice embryos at E11.5 (A, D, G), E13.5 (B, E, H) and E15.5 (C, F, I). Cells were stained with hematoxylin-eosin (HE; A-F), and immunohistochemical assay for the myosin heavy chain (MyHC; G-I) was performed. The heart increased in size, and the ventricular walls thickened during embryonic development. A-C: Bars=300 µm; D-I: Bars=30 µm.

Fig.2. The quantitative mRNA expression of cardiomyocyte-specific genes (A: Tnnc1; B: Myh6; C: Myh7) in mice embryonic hearts. The expressions of Tnnc1, Myh6 and Myh7 increased from E11.5 through E15.5. n≥3. *: significant differences in multiple comparisons following the Kruskal-Wallis test (Scheffé’s method, P<0.05).

Fig.3. TUNEL staining (A-C) and immunohistochemical assays for ssDNA (D-F) and aCasp3 (G-I) in cardiomyocytes. TUNEL-, ssDNA- and aCasp3-positive cells were detected at all fetal ages examined. The number of TUNEL-positive cells was much higher than that of ssDNA- and aCasp3-positive cells (J). Bars=10 µm. n=3. *, significant differences in multiple comparisons following the Kruskal-Wallis test (Scheffé’s method, P<0.05).
panied by nuclear fragmentation, whereas TUNEL+/aCasp3− cells were not. The number of TUNEL+/aCasp3− cells was the highest among these 3 types of cells, and it increased throughout the E11.5 to E15.5 ages (Fig.5J).

**DISCUSSION**

**Differentiation of mouse heart and cardiac muscle**: The heart size and the ventricular thickness grew, and the cardiomyocyte density at the ventricular wall increased with cardiogenesis in the mouse embryo from E11.5 through E15.5. Although MyHC protein expression was already detected as early as E11.5, the mRNA expression of cardiomyocyte-specific genes, including Tnnc1, Myh6 and Myh7, increased with fetal age. These results indicate that cardiomyocytes continue to differentiate throughout the E11.5–15.5 period in the mouse embryo.

**Cell death and caspase activation in mouse cardiogenesis**: A recent study clarified that activation of the caspase cascade is closely associated with differentiation of skeletal muscles, which are striated muscles similar to cardiac muscles [7]. However, immunohistochemical analysis showed that there were only a few aCasp3-positive cells, and their numbers decreased during E11.5 to E15.5. Because the caspase proteins are activated by posttranslational modification, their mRNA expression does not directly reflect their activation [13]. Therefore, we intended to confirm the inactivation of
caspase proteins by examination of the mRNA expression of anti-apoptosis factors such as the Bcl-2 family and IAP family. The mRNA expressions of these anti-apoptotic proteins increased from E11.5 through E15.5, indicating that the caspase cascade was inhibited in mouse heart, unlike in the skeletal muscle. In addition, aCasp3-positive cells showed nuclear condensation or fragmentation, and the cell death marker (ssDNA-positive cells) was also detected in cardiac muscles. Therefore, these findings suggest that caspase activation is not associated with cardiac myogenesis and that those aCasp3-positive cells in cardiogenesis are considered to be entering the cell death phase.

**TUNEL-positive cells in cardiac myogenesis:** TUNEL staining and ssDNA and aCasp3 immunohistochemical analyses showed that the number of TUNEL-positive cells increased with fetal age and was much higher than ssDNA- and aCasp3-positive cells. In addition, as a result of the TUNEL and aCasp3 double staining, 3 types of positive cells (TUNEL+/aCasp3+, TUNEL+/aCasp3−, and TUNEL−/aCasp3+) were observed. Among these 3 types of cells, TUNEL+/aCasp3− cells, i.e., TUNEL-positive cells without caspase activation, were highly detected. Furthermore, the TUNEL+/aCasp3− cells did not show the morphological features of apoptosis, such as nuclear condensation or cell shrinkage. Therefore, although these cells showed TUNEL-positive reactions, it is suggested that they were not under the process of apoptosis. TUNEL-positive cells without nuclear condensation were also detected in myosin-positive myotubes cultured from H9C2 rat cardiomyoblasts. The H9C2 cells increased the
expression of the cardiac marker Myl2 during culture with the differentiation medium, and they were thought to have differentiated to cardiomyocytes. However, H9C2 myotubes formed multinucleated syncytia, whereas cardiac muscles in vivo were joined to one another by intercalated discs but were not fused. Such a difference could be attributed to a contrast between in vivo and in vitro conditions. In either case, TUNEL-positive cells, that did not show representative feature of apoptosis, were detected in both H9C2 cells and mouse cardiogenesis, and the appearance of these cells is suggested to be associated with cardiac myogenesis.

**Relationship between non-death TUNEL-positive cells and cardiogenesis:** Recently, some researchers divided programmed cell death into 2 types; apoptosis and non-apoptotic programmed cell death (NAPCD) [22]. Interestingly, the appearance of NAPCD without activation of caspase was reported in cardiomyocytes of ischemic cardiomyopathy [12]. Furthermore, Ras-mediated NAPCD, especially autophagic cell death, contributed to spontaneous regression of neuroblastoma [11]. These reports indicate that NAPCD would be one of checkpoint system to maintain the homeostasis of terminal differentiated tissues. On the other hand, TUNEL staining is a method to detect the 3′-OH terminal end of DNA strand breaks and detects DNA damages in addition to detecting cell death [8, 15, 18]. Once a DNA strand breaks, the DNA damage response (DDR) leads to cell cycle arrest and enhancement of the DNA repair pathway, and when the level of damage is severe, it initiates apoptosis [6]. Recently, an additional role of the DDR, i.e., promotion of cellular terminal differentiation in various tissues such as skeletal muscles and neuronal cells, was reported [3, 20, 27]. To clarify the embryological meanings of the appearance of TUNEL-positive cardiomyocyte without representative feature of apoptosis, the detailed functions of these cells should be assessed, especially focused on the possibility of NAPCD or the induction of DDR.

In conclusion, the caspase cascade was suppressed in mouse embryonic cardiomyocytes, unlike in the skeletal muscle, and many TUNEL-positive cardiomyocytes were observed. We considered that the increased number of TUNEL-positive cardiomyocyte, having the DNA strand breaks, would be associated with the progression of cardiac myogenesis.

**REFERENCES**