Apoptotic versus autophagic cell death in heart failure

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Abstract

Objective: Progressive loss of cardiomyocytes is one of the most important pathogenic characteristics of heart failure. Apoptosis may be an important mode of cell death in heart failure but it must be demonstrated by multiple criteria and not just TUNEL staining alone. Previously, we and others have demonstrated that besides apoptosis other phenomena like active gene transcription can result in TUNEL positivity. Moreover, other types of cell death that are caspase-independent could be important in heart failure. This study examined the hypothesis whether TUNEL labeling parallels caspase activation. Methods: Cardiac tissue of patients in the terminal stage of heart failure as a consequence of ischaemic cardiomyopathy (ICM) or dilated cardiomyopathy (DCM) were studied. Embryonic mice hearts were used for positive control for detection of the classical apoptosis. Results: In mice embryonic hearts we could clearly find apoptotic cell death detected by TUNEL labeling and immunohistochemistry for activated caspase-3. In heart failure, TUNEL-positive cardiomyocytes were negative for active caspase-3 but showed signs of active gene transcription (SC-35). However, autophagic cell death could be found in 0.3% of the cardiomyocytes. Autophagic cell death was demonstrated by granular cytoplasmic ubiquitin inclusions, an established marker of autophagocytosis in neurons. Interestingly, these autophagic cardiomyocytes were TUNEL and activated caspase-3 negative but were also negative for C9, a marker for necrosis. Western blot analysis confirmed that in cardiomyopathies no cleavage of caspase-3 and caspase-7 occurred. Conclusion: The present study demonstrates two fundamentally different situations of cell death in cardiac tissue. In embryonic mice, cardiomyocytes undergo caspase-dependent cell death. However, cardiomyocytes in heart failure show caspase-independent autophagic cell death rather than apoptotic cell death.

Keywords: Apoptosis; Heart failure; Cardiomyopathy; Myocytes

1. Introduction

The terms apoptosis, DNA fragmentation and TUNEL-positive labeling describe related but not necessarily congruent events. In that respect, terminally differentiated cells like neurons and cardiomyocytes can exhibit one or more of these characteristics independently. In the last decade several papers have described the appearance of apoptotic cell death in cardiomyopathies \cite{1-5} and reported a wide variation in the rate of apoptosis. The frequency of apoptotic cell death of cardiomyocytes is, however, still questionable and caution should be taken in the interpretation of the TUNEL technique used to detect apoptosis \cite{6-8}. Recently, we showed that RNA synthesis/splicing could interfere with the TUNEL technique used for the detection of apoptotic cell death \cite{9}. Moreover, Ohno and coworkers \cite{10} observed TUNEL-positive cardiomyocytes with oncotic morphology in the infarcted heart. Kanoh and co-workers \cite{11} described that cardiomyocytes in dilated cardiomyopathy were TUNEL-positive although they were not apoptotic but living cells with an increased activity of DNA synthesis/repair. Anversa et al. found that fluorescence TUNEL labeling

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combined with confocal microscopy improve the detection of apoptotic cell death in heart tissue [4,12]. Kang and Izumo performed a critical analysis of the literature and concluded that the use of the TUNEL technique alone is not sufficient to define the role of apoptosis in heart failure [13]. Different techniques have been used to resolve the role of apoptosis in heart failure but the importance of cell death is still unresolved [14]. The TUNEL technique detects DNA fragmentation, a hallmark of apoptotic cell death. Previously, we and others have discussed that fixation delay and enzymatic treatment can induce artificial DNA fragmentation in non apoptotic cells [15–18]. This could explain how aspecific TUNEL labeling is almost exclusively seen in cells showing active gene transcription whereas nuclei of non synthetic cells remain absolutely TUNEL negative [9,15]. Moreover apoptotic cells in the execution phase of cell death lose their ability for RNA synthesis/splicing [19] leading us to conclude that TUNEL labeled nuclei without signs of RNA synthesis/splicing indicate in vivo DNA cleavage. In this paper, we further colocalize the TUNEL-positive and RNA synthesis/splicing negative nuclei with other markers involved in apoptosis. This is important to understand the different cell death pathways in heart failure. Possibly, caspase-independent cell death pathways are activated in heart failure. Fiers et al. [20] stated that caspase-independent pathways of cell death could play an important role in various cell types under different physiological as well as pathological situations. Moreover, autophagic cell death in denervated skeletal muscle cells has been reported that is different from classical apoptosis [21]. Therefore, this study examines whether cardiomyocytes in cardiomyopathies undergo caspase-dependent or caspase-independent cell death. These data were compared with apoptotic cell death during cardiac development of embryonic mice.

2. Methods

2.1. Patients

Heart tissue was studied from patients in the terminal stage of heart failure suffering from ischaemic cardiomyopathy (ICM) (n=8; mean age 53 years) or dilated cardiomyopathy (DCM) (n=12; mean age 48 years). The distinction between ischaemic and dilated cardiomyopathy was based on coronary angiography. Ischaemic cardiomyopathy was diagnosed when LVEF<40 and coronary lesions were present. The tissue samples were taken from the lateral wall of the left ventricle and contained the whole thickness of the left ventricle wall. Acute ischaemic lesions were not present. Biopsies from normal transplanted hearts (n=5) were used for controls. The study conformed to the declaration of Helsinki. For histological examination, heart tissue samples were taken and fixed in 3.7% formalin. The paraffin-embedded tissues were sectioned at 5 μm thickness and mounted on slides precoated with 3-amino-propyltriethoxysilane (Sigma, Dorset, UK). To study apoptosis during cardiac development the distal outlet segments of the embryonic mice hearts (n=5, gestation 13 days) were taken.

2.2. TUNEL labeling

The sections were deparaffinized, rehydrated and incubated with 3% citric acid. Subsequently, the sections were incubated for 1 h at 37°C in a solution consisting of 25 mM Tris; 200 mM sodium cacodylate; 1.25 mg/ml bovine serum albumin; 1.25 mM CoCl₂; 10 μM dATP (Sigma); 2.5 μM fluorescein-dUTP (Amersham Pharmacia Biotech, Rainham, UK); 50 U/ml TdT (Roche Diagnostics GmbH, Mannheim, Germany) pH 6.6. The incorporated fluorescein-dUTP was detected with a sheep-anti-fluorescein peroxidase conjugated antiserum (Roche Diagnostics GmbH) (1/300 for 45 min). The labeled antibody was visualized by 3-amin-9-ethylcarbazole (AEC). For the fluorescence-based TUNEL method a TSA (tyramide signal amplification) labeling was used (Dupont, Boston, MA). Negative controls included omission of TdT from the labeling mixture. Tonsils of both human and mice were used as positive control.

2.3. Immunohistochemistry

The following monoclonal antibodies; SC-35 (Sigma), ssDNA (Alexis, Läufelfingen, Switzerland), CD31, CD68, LCA (Dako, Copenhagen, Denmark), α-SMC actin (Sigma), C9 (Novocastra, Newcastle upon Tyne, UK) and polyclonal antibodies activated caspase-3 (Pharmingen, San Diego, CA), desmin (Eurodiagnostica, Arnhem, Netherlands), ubiquitin (Dako) were used. All antibodies were diluted in PBS and detected by an indirect peroxidase antibody conjugate technique. The sections were incubated with a goat-anti-mouse peroxidase antibody (Jackson, Bar Harbor, ME) or a goat-anti-rabbit antibody (Dako) for 45 min. AEC was used as a chromogen. The sections were counterstained with hematoxilin. Omitting the primary antibody and/or substituting the antibody by an unrelated antibody at the same concentration checked the immunohistochemical reaction. Tonsils and embryonic mice hearts were used as controls.

2.4. Protein isolation and immunoblot assays

Heart tissue of ICM and DCM were homogenized in a buffer containing 0.9% (w/v) NaCl, 20 mM Tris–HCl pH 7.6, 1 mM phenylmethysulphonyl fluoride, 0.01% (w/v) leupeptin and 0.2% (v/v) Triton X-100. Subsequent SDS/
PAGE and Western blotting of cell lysates were performed according to standard procedures. Caspase-3 and caspase-7 (Pharmingen) antibody detection was accomplished by means of enhanced chemiluminescence (Amersham Pharmacia Biotech) and Lumi-imaging (Roche Diagnostics GmbH). The human U-937 cells were used for positive control. Apoptosis was induced by treatment with a combination of 10 000 U/ml human TNF and 10 μg/ml cycloheximide (CHX) for 5 h.

2.5. Co-localization, quantification and statistics

Sections stained by the TUNEL technique were colocalized with SC-35, ssDNA, or cleaved caspase-3 by a digital imaging and co-localization system as previously described [9]. The percentages of the TUNEL-labeled interstitial located fragments and autaphagic cardiomyocytes were estimated from 50 images (approximately 100 000 cells) sampled systematically at random [22,23]. Estimated percentages were given as the mean±S.E.M. and were compared using the Mann–Whitney U-test (1% significance level).

3. Results

This study examines apoptotic cell death in the myocardium of embryonic mice heart and in human heart failure (ICM, DCM) by the TUNEL technique combined with immunohistochemical markers for RNA synthesis/splicing (SC-35) and activated caspase-3.

3.1. Caspase-dependent versus caspase-independent cell death

Caspase-dependent cell death could be detected in the distal outlet segment (Fig. 1) of embryonic mice hearts at day 13 of development. In this region, apoptotic bodies were both TUNEL and activated caspase-3-positive (Fig. 1C,D). Moreover, in the germinal centers of human lymphoid tissue, TUNEL-positive nuclear fragments were present in the cytoplasm of the macrophages. Activated caspase-3-positive B cells can be detected in the same region. The activated caspase-3-positive cells showed a complete absence of RNA synthesis/splicing that fits with caspase cleavage and inactivation of splicing factor during the execution phase of apoptosis. These data demonstrate that caspase-dependent cell death can be detected in both the embryonic heart and human lymphoid tissue. Using the same techniques, we could rarely observe caspase-dependent cell death in cardiomyocytes of ICM and DCM hearts. However, autophagic cell death was detected by a granulovesicular ubiquitin staining in the cytoplasm of cardiomyocytes (Fig. 2). The majority of these ubiquitin containing cardiomyocytes lost nuclear staining, whereas a minority showed condensed nuclei. All autophagic myocytes were negative for TUNEL and activated caspase-3. This demonstrates a caspase-independent cell death. These autophagic cardiomyocytes were also negative for C9 (data not shown) which is a marker for necrotic cell death.

3.2. TUNEL labeling of synthetic versus non-synthetic cardiomyocytes

TUNEL-labeled synthetic cardiomyocytes showed signs of RNA synthesis/splicing as demonstrated by their intensive nuclear staining for SC-35. TUNEL-labeled myocytes colocalized with SC-35 (Fig. 3) and were negative for ssDNA. Using fluorescence TUNEL labeling which is less sensitive, the number of labeled cardiomyocytes was much lower. Pretreatment with proteinase K strongly increased the number of TUNEL-labeled cardiomyocytes detected by both the peroxidase and fluorescence technique. These cardiomyocytes showed no association with markers for activated caspase-3 but stained intensively for SC-35. This indicates that irrespective of the labeling (peroxidase or fluorescence) used in the TUNEL reaction, non-apoptotic nuclei with signs of active gene transcription (SC-35-positive) can become TUNEL-positive. The percentage of SC-35-positive cardiomyocytes was approximately 90% in all sections. Cardiomyocytes that were SC-35 negative (about 10%) remained strictly TUNEL negative even after proteinase K treatment. These SC-35 negative cardiomyocytes were negative for activated caspase-3 and C9, and did not show signs of autophagic cell death.

3.3. Interstitial located TUNEL-positive nuclear fragments

TUNEL-labeled fragments could be recognized in the interstitial regions of ICM and DCM hearts. These labeled interstitial fragments stained for ssDNA and were negative for SC-35 (Fig. 3). To test the hypothesis that these fragments were derived from apoptotic interstitial cells, we performed colocalization studies with markers for endothelial cells (CD31), macrophages (CD68), vascular smooth muscle cells (α-SMC), leukocytes (LCA), and desmin. Colocalization could not be detected indicating that these TUNEL-labeled interstitial fragments are probably not derived from interstitial cells. Moreover, the TUNEL-labeled interstitial fragments were desmin and activated caspase-3 negative.

3.4. Intracellular located TUNEL-positive small nuclear fragments (SNUFS)

Interestingly, small nuclear fragments (SNUFS) labeled intensively by the TUNEL technique could also be ob-
Fig. 1. TUNEL and cleaved caspase-3 signal in the distal outlet segment of the embryonic mice heart at gestation of 13 days. (A) An overview of the distal outlet segment (DO). Atf is the embryonic atrium, PO is the embryonic proximal outlet segment. (B) Fluorescence TUNEL labeling (arrow) in the distal outlet region of the embryonic heart. Counterstained with Hoechst. (C) The peroxidase TUNEL labeling (arrow). (D) Activated caspase-3 (arrow heads) in the DO region of the embryonic mice heart. Note that the number of activated caspase-3 labeled cells is higher than the number of TUNEL labeled cells. Bar is 20 μm.

Fig. 2. (A, B) Cardiomyocytes of ICM and DCM hearts containing granular cytoplasmic ubiquitin inclusions (arrows) demonstrating autophagic cell death. Counterstained with hematoxylin. The cardiomyocytes containing these granular cytoplasmic ubiquitin inclusions (C) could not be colocalized with in situ activated caspase-3 (arrow heads) (D). Moreover, we could not detect colocalization of cardiomyocytes showing autophagic cell death (E) with TUNEL labeling (open arrow heads) (F).

Table 1
Apoptotic versus autophagic cell death in cardiomyopathies

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The table summarizes the staining differences between caspase-dependent cell death present in embryonic cardiomyocytes and caspase-independent cell death in cardiomyocytes of ICM and DCM hearts. Cells undergoing caspase-dependent cell death stain both for activated caspase-3 and TUNEL but are negative for SC-35. These cells show classic apoptosis. Cells undergoing caspase-independent cell death contain granular cytoplasmic ubiquitin inclusions indicating autophagic cell death. These cells could be stained by ubiquitin and do not stain for TUNEL or activated caspase-3. TUNEL-positive cardiomyocytes detected in ICM and DCM hearts as well as in the control hearts could be colocalized with SC35 indicating cardiomyocytes with active RNA synthesis/splicing. These cardiomyocytes are not in apoptosis but show an aspecific TUNEL labeling.
Fig. 3. TUNEL-labeled cardiomyocyte of a ICM heart (arrow heads) (A) colocalized with SC35 (B) indicating that these cardiomyocytes are showing active RNA transcription/splicing. (C, D) TUNEL labeling (small arrows) followed by RNA synthesis/splicing in the interstitial region of the myocardium. (E, F) Cardiomyocyte that extrudes a TUNEL-positive small nuclear fragment (large arrow) (SNUFS) that is negative for RNA synthesis/splicing compared to the nuclei. (G) TUNEL-positive small nuclear fragment (large arrow) that could not be stained for in situ activated caspase-3 (3H). Also the TUNEL-positive nuclei (double arrow head) is negative for activated caspase-3. Bar is 20 μm.

Taining these SNUFS were strictly negative for activated caspase-3. Moreover, colocalization studies with a marker for ssDNA revealed that these SNUFS stained for ssDNA (Fig. 4). The diameter of these SNUFS was approximately 1.5 μm and they were often found adjacent to perinuclear lipofuscin granules. When fluorescence TUNEL labeling was used these SNUFS could not be distinguished from the auto-fluorescent lipofuscin in the intact cardiomyocytes. However, using the peroxidase TUNEL labeling these SNUFS could clearly be distinguished from the lipofuscin granules. The fragments were observed in six of the 20 patients studied (two patients suffering from ICM and four patients suffering from DCM).

3.5. Quantification of TUNEL labeling and autophagic cardiomyocytes

Cardiomyocyte apoptosis in heart failure detected by the combination of TUNEL and in situ caspase-3 activation was 0.0014±0.002% in IHD and 0.0018±0.002% in DCM hearts (Fig. 5A). The percentage of autophagic cardiomyocytes was 0.352±0.058% in ICM and 0.343±0.105% in DCM hearts and differed significantly from normal cardiac tissue (P<0.01) (Fig. 5B). The percentage of the TUNEL-positive labeled interstitial

Fig. 4. Representation of a cardiomyocyte showing ‘kernfractur’ as drawn by Linzbach in 1947 (A). Note that a nuclear body is drawn in a non-apoptotic cardiomyocyte (small arrow) of a DCM heart. Using modern staining procedures such a nuclear body could be recognized by a stringent TUNEL technique (small arrow) (B) and by ssDNA (C) indicating that intact cardiomyocytes contain TUNEL-positive small nuclear fragments (SNUFS). Bar is 20 μm.
3.6. Western blot analysis

To test the hypothesis whether cardiomyocytes of the ICM and DCM hearts undergo caspase-dependent cell death Western blot analysis was performed combined with a human U-937 cell line (Fig. 6). No cleavage of both caspase-3 and caspase-7 could be found in the cardiomyocytes of ICM and DCM hearts. However, the U-937 cell line showed a cleavage of both caspases if the cells were treated with TNFα and CHX.

4. Discussion

The present study gives arguments that the terms TUNEL labeling, caspase activation and DNA fragmentation describe related but not necessarily congruent events in cell death of cardiomyocytes. Moreover, it was demonstrated that cardiomyocytes could exhibit one or more of these characteristics independently and that adult cardiomyocytes undergo preferentially autophagic cell death during heart failure. Table 1 summarizes the observations of TUNEL labeling combined with other techniques in the detection of cell death pathways both in embryonic and adult cardiomyopathies.

4.1. Caspase-dependent cell death versus caspase-independent cell death

The distal outlet segment of the heart is the region of the heart that forms the aorta and pulmonary trunk during cardiac development [24]. Apoptosis in the distal outlet segment of the embryonic heart can be deduced from quantitative analysis [25] and has already been described.
by light microscopy [26]. Both the fluorescence TUNEL labeling and the peroxidase-based TUNEL reaction can detect apoptotic cardiomyocytes in the distal outlet segment of the heart. Moreover, most of the TUNEL-labeled cells and caspase-3-positive cells are present in the same restricted region of the embryonic heart. This supports the involvement of a caspase-dependent cell death in the remodeling of the distal outlet segment during cardiac development as suggested by Watanabe and coworkers [27].

In the failing heart, apoptotic cell death is below 0.005% confirming the data of Schaper et al. [8]. Moreover, it has been demonstrated that classic apoptosis by chromatin condensation could not be found in dedifferentiated cardiomyocytes of the hibernating myocardium [28,29]. In this study, in situ labeling of active caspase-3 could not be detected in cardiomyocytes of both ICM and DCM hearts. Nevertheless, it cannot be excluded that very low levels of apoptosis are present which are difficult to detect since caspase activation is transient. Western blot analysis of both caspase-3 and caspase-7 showed no cleavage in cardiomyocytes of ICM and DCM hearts compared to U-937 stimulated cells. The discrepancy between our findings and the observation of Narula et al. [30] could be a consequence of technical aspects of the Western blot. In our experiment we used the same human U-937 cell line as a positive control for caspase activation. However, we used the cell lysates of the control cell line and tissue homogenates of both DCM and ICM hearts on the same blot (Fig. 6). Therefore, we had a good control in the same blot of the exact size of the cleaved caspase fragments. Using this method none of the cardiomyopathy tissues showed cleavage of caspase-3 or caspase-7. It has been reported that cell death in denervated skeletal muscle cells is distinct from classical apoptosis [21]. Interestingly, recent data have demonstrated that skeletal muscle loses Apaf-1 during differentiation and there are arguments that cardiomyocytes also lose Apaf-1 in the adult heart [31]. Apaf-1 is important for caspase activation via the mitochondrial pathway, although other pathways of caspase activation exist. However it is also possible that caspase-independent cell death pathways are activated in heart failure.

Caspase-independent cell death has been reported under different physiological or pathological situations and is an important topic in modern cell death research [20]. Recently, self-controlled cytoplasmic proteolysis not associated with nuclear degradation or cellular cleavage has been observed in cardiomyocytes of human hearts with chronic hemodynamic overload [32]. Moreover, genes responsible for degradation and disassembly of myocardial proteins, like ubiquitin, are upregulated in the failing human heart as detected by high-density oligonucleotide arrays [33]. Interestingly, in DCM and ICM hearts we observed cardiomyocytes with signs of autophagic degeneration that were TUNEL and activated caspase-3 negative. Autophagic cell death was demonstrated by granular cytoplasmic ubiquitin inclusions, an established marker of autophagic degeneration in neurons [34] and degenerating insect flight muscle [35]. The time process of autophagic cell death is not known yet although prominent autophagic responses occur between 2 and 7 days after strenuous exercise in skeletal muscle [36] suggesting that it is a slow process. The autophagic myocytes were also negative for C9, which is a marker for necrotic cell death [37]. This fits with findings that autophagic cell death could occur independently from apoptosis or necrosis [38,39]. Moreover, there is growing evidence that autophagic cell death is indeed caspase-independent [40,41]. However, the exact molecular mechanism of autophagic cell death as well as other specific indicators for autophagic cell death in heart failure should be further investigated.

4.2. Aspecific TUNEL labeling

All TUNEL-positive cardiomyocytes observed in myocardial tissue of ICM and DCM hearts colocalized with SC-35 indicating synthetic cardiomyocytes. When sections were pretreated with proteinase K the number of TUNEL-positive nuclei increased in both the peroxidase and fluorescence-based TUNEL technique. It has been suggested that the use of proteinase K could give false positive TUNEL labeling as a result of the release of endogenous endonucleases [17]. Recently, Gal et al. [18] found that proteinase K can induce 50-kb fragments of DNA that can also result in TUNEL labeling of non-apoptotic cells. Interestingly, these 50-kb cleaved fragments of DNA coincide with a class of DNase I hypersensitivity regions that are a hallmark of gene transcription [18,42]. Kanoh and coworkers [11] demonstrated that TUNEL-positive cardiomyocytes were living cells showing signs of DNA repair. Furthermore, chromatim is conformationally changed during transcription, replication and DNA-repair [43,44]. The more open structure of the chromatim during transcription or repair could give access to TdT incorporation during the TUNEL procedure resulting in aspecific TUNEL labeling [9,15].

4.3. Interstitial located TUNEL-positive nuclear fragments

TUNEL-positive labeled fragments could be observed in the interstitial region of both ICM and DCM hearts but not in the normal heart. These interstitial fragments could not be colocalized with activated caspase-3 or SC-35. It has been reported that in the center of myocardial infarctions 60% of TUNEL-positive labeled interstitial cells were apoptotic leukocytes, macrophages, endothelial cells, vascular smooth muscle cells or apoptotic fibroblasts and that approximately 40% remained unclassified [45]. In the present study no colocalization exists between the TUNEL-positive interstitial fragments and markers for leukocytes (LCA), macrophages (CD68), endothelial cells (CD31) and vascular smooth muscle cells (α-SMC). The
observed fragments could neither be colocalized with desmin indicating that all TUNEL-positive interstitial fragments remain unclassified.

4.4. Intracellular located TUNEL-positive small nuclear fragments (SNUFS)

Another intriguing finding was the occurrence of intracellular located TUNEL-positive small nuclear fragments (SNUFS) in cardiomyocytes in the failing heart. These SNUFS could not be distinguished from the perinuclear lipofuscin when the fluorescence TUNEL technique was used. This could be the reason why other observers that are totally committed to fluorescence TUNEL labeling did not detect these fragments. However, if the peroxidase TUNEL reaction was used these SNUFS could be distinguished from the autofluorescent lipofuscin granules. These SNUFS colocalized with markers for ssDNA pointing to damaged DNA that is extruded from the cardiomyocyte nucleus. Interestingly, these SNUFS were reported during autophagic degenerating neurons and were associated with secondary lysosomes [46]. In our study the autophagic cardiomyocytes did not show an increased number of SNUFS. However, small micronuclei particles in hypertrophied cardiomyocytes were reported 40 years ago by Linzbach [47]. Linzbach stated that these fragments were not the consequence of amitosis but could be a consequence of ‘kernfractur’. The micronuclei particles observed by Linzbach and the TUNEL-labeled SNUFS observed in the present article show surprisingly high similarities (Fig. 4). Although speculative, we think that these intracellularly located TUNEL-positive small nuclear fragments (SNUFS) are oxidative damaged nuclei and we hypothesize that these SNUFS are transported to the interstitial region of the myocardium explaining the relatively high percentage of the interstitial TUNEL-labeled fragments found in both ICM and DCM hearts.

In conclusion, cardiomyocytes undergoing classic apoptosis via a caspase-dependent pathway, are abundant during embryonic heart development but are below 0.005% in heart failure. TUNEL-labeled fragments could be observed in the interstitial region and in the cytoplasm (SNUFS) of non-apoptotic hypertrophic cardiomyocytes in the failing human heart. Interestingly, cardiomyocytes in heart failure show caspase-independent autophagic cell death rather than apoptotic cell death. Therefore, we conclude that the terms apoptosis, DNA fragmentation, TUNEL labeling, caspase activation, and autophagic cell death are not congruent events in cardiomyocytes of pathological human hearts.

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