Apoptosis in heart failure: Release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy

JAGAT NARULA*†‡, PRAMOD PANDEY§, ELOISA ARBUSTINI†, NEZAM HAIDER†, NAVNEET NARULA*, FRANK D. KOLODGIE, BARBARA DAL BELLO†, MARC J. SEMIGRAN*, ANNA BIELSA-MASDEU†, G. WILLIAM DEC*, SARA ISRAELS**, MANEL BALLESTER††, RENU VIRMANI¶, SATYA SAXENA**, AND SURENDER KHARBANDA§

†Hospital Sant Pau, 08025 Barcelona, Spain; ‡To whom reprint requests should be addressed at: Heart Failure/Transplant Center, Hahnemann University Hospital, Broad and Vine Streets, Mail Stop 115, Philadelphia, PA 19102. e-mail: Narula@auhs.edu.

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ABSTRACT Apoptosis has been shown to contribute to loss of cardiomyocytes in cardiomyopathy, progressive decline in left ventricular function, and congestive heart failure. Because the molecular mechanisms involved in apoptosis of cardiomyocytes are not completely understood, we studied the biochemical and ultrastructural characteristics of upstream regulators of apoptosis in hearts explanted from patients undergoing transplantation. Sixteen explanted hearts from patients undergoing heart transplantation were studied by electron microscopy or immunoblotting to detect release of mitochondrial cytochrome c and activation of caspase-3. The hearts explanted from five victims of motor vehicle accidents or myocardial ventricular tissues from three donor hearts were used as controls. Evidence of apoptosis was observed only in endstage cardiomyopathy. There was significant accumulation of cytochrome c in the cytosol, over myofibrils, and near intercalated discs of cardiomyocytes in failing hearts. The release of mitochondrial cytochrome c was associated with activation of caspase-3 and cleavage of its substrate protein kinase C δ but not poly(ADP-ribose) polymerase. By contrast, there was no apparent accumulation of cytosolic cytochrome c or caspase-3 activation in the hearts used as controls. The present study provides in vivo evidence of cytochrome c-dependent activation of cysteine proteases in human cardiomyopathy. Activation of proteases supports the phenomenon of apoptosis in myopathic process. Because loss of myocytes contributes to myocardial dysfunction and is a predictor of adverse outcomes in the patients with congestive heart failure, the present demonstration of an activated apoptotic cascade in cardiomyopathy could provide the basis for novel interventional strategies.

Loss of myocytes is a feature of the cardiomyopathic process that contributes to progressive decline in left ventricular function and congestive heart failure (1, 2). Recent studies have proposed that myocyte loss in cardiomyopathy can occur by apoptosis without an attendant inflammatory response (3). Apoptosis may be the consequence of prolonged growth stimulation of adult myocytes (4), which are terminally differentiated and are unable to divide (5). Growth stimulation of adult myocytes (4), which are terminally differentiated and are unable to divide (5). Growth stimulation initially occurs as a compensatory effort to meet chronically altered hemodynamic demands on the failing myocardium and is mediated by systemic and/or local up-regulation of mediators of adrenergic (6, 7) or renin-angiotensin (8, 9) axes and by various cytokines (10). Local up-regulation of angiotensin II induces immediate-early genes (9, 11), which may lead to increased protein synthesis (12) and myocardial hypertrophy (9, 13, 14) or, alternatively, may up-regulate expression of apoptotic proteins (such as p53) in myocytes (15). Similarly, certain cytokines (such as tumor necrosis factor α) can induce growth (16, 17) as well as apoptosis (18).

The induction of apoptosis as a form of cell death distinct from necrosis (19–23) is associated with activation of aspartate-specific cysteine proteases such as caspase-3 (CPP32/ Yama) (24, 25) and cleavage of poly(ADP-ribose) polymerase (PARP) (26), protein kinase C-δ (PKCδ) (27), and certain other proteins (28). Caspase-3 protein is detectable by immunostaining in various human tissues, including cardiomyocytes (29). Direct evidence for involvement of caspase-3 in apoptosis is derived from studies with the baculovirus protein p35, which directly inhibits this protease and blocks induction of apoptosis (30).

Recent studies have indicated that mitochondria may play a crucial role in apoptosis by releasing cytochrome c (31). Addition of purified cytochrome c and dATP to cytosolic extracts from proliferating cells activates caspase-3 responsible for cleavage of PARP (31). The finding that intact cells undergo apoptosis after release of cytochrome c into the cytosol has provided further support for an apoptotic function of this mitochondrial protein (31). Furthermore, recent studies have demonstrated that, in addition to cytochrome c, activation of caspase-3 also requires another protein, Apaf-1, a human homologue of the Caenorhabditis elegans ced-4 protein (32).

Although sequential activation of the apoptotic cascade has been well characterized in nematodes, in cell culture, and in cell free systems, little information is available in human diseases. To investigate the potential involvement of cytochrome c release from mitochondria and activation of caspases in the end-stage cardiomyopathy, a pathological state wherein apoptosis is known to play a role (3), we performed ultrastructural and biochemical analysis of explanted hearts from cardiac allograft recipients. The results support the constitutive activation of a cytochrome c-mediated apoptotic cascade in human cardiomyopathy.

MATERIALS AND METHODS

Myocardial Specimens from Cardiomyopathic and Normal Hearts. Sixteen explanted hearts were obtained from patients

Abbreviations: PARP, poly(ADP-ribose) polymerase; PKCδ, protein kinase C-δ; IDCM, idiopathic dilated cardiomyopathy; ISCM, ischemic cardiomyopathy.

A Commentary on this article begins on page 7614.

To whom reprint requests should be addressed at: Heart Failure/Transplant Center, Hahnemann University Hospital, Broad and Vine Streets, Mail Stop 115, Philadelphia, PA 19102. e-mail: Narula@auhs.edu.

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undergoing heart transplantation; 10 patients had idiopathic
dilated cardiomyopathy (IDCM), and the remaining 6 had
ischemic cardiomyopathy (ISCM). Normal myocardial tissues
also were obtained from five victims of road accidents and
from three donor hearts before transplantation. The control
tissues from accident victims were only accepted if obtained
within 6 hours of death. Similarly, donor-heart normal myo-
cardial tissues were accepted only when ischemic times were
<60 min.

Of these 16, explanted hearts from 7 patients either were
placed in 4% buffered formaldehyde fixative or were snap
frozen in liquid nitrogen. Four of the seven patients had
IDCM, and three suffered from ISCM. The formalin-fixed
tissue was processed by paraffin embedding and was used for
histopathologic characterization and for demonstration of
apoptosis by nick-end labeling. Histopathologic and end-
labeling studies from these seven patients have been reported
previously (3). The myocardial tissue from these hearts, which
was snap frozen in liquid nitrogen, was stored at −70°C for
biochemical characterization of cytochrome c release and
caspase activation. The results of the biochemical analysis were
compared with the myocardial tissues obtained from five
victims of motor vehicle accidents.

The myocardial tissue specimens from the remaining nine
hearts were obtained in 0.5% Karnovsky’s fixative for immu-
 noelectron microscopic examination and were compared with
the small ventricular tissue samples obtained from three donor
hearts. Ultrastructural examination was undertaken for pre-
cise localization of cytochrome c in mitochondrial and cyto-
plasmic compartments.

**Cell Culture Studies.** Human U-937 myeloid leukemia cells
and neonatal murine cardiomyocytes were used for positive
control studies. U-937 cells were grown in RPMI medium 1640
supplemented with 10% heat-inactivated fetal bovine serum,
100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM
L-glutamine. Irradiation was performed at room temperature
with a Gamma Cell 1000 (Atomic Energy, Ottawa) under
aerobic conditions with a 137Cs source emitting at a fixed-dose
rate of 13 Gy/min as determined by dosimetry and described
previously (33).

Neonatal rat myocytes were prepared from ventricular
muscle (34). Cardiocytes were plated in 25-ml Falcon flasks for
each experiment in high-glucose DMEM medium with 10%
fetal calf serum. The cells initially were incubated for 24 hours
at 37°C in 5% CO2. For hypoxic stress, the culture medium in
flasks was flushed with sterile nitrogen for 4 min, and flasks
were tightly capped and transferred to incubators as described
(35). The hypoxic conditions were maintained for 6 and 12
hours. After the indicated time periods, the cells were har-
vested from flasks by gentle scraping.

**Fig. 1.** Apoptosis in end-stage heart failure. Analysis of apoptosis was performed by end-labeling of DNA fragments in rat mammary tissue
(A; used as a positive control) and myocardial specimens from normal (B) and cardiomyopathic (C and D) hearts. The end-labeling of the DNA
fragments was obtained by addition of biotinylated dUTP in the presence of terminal deoxynucleotidyltransferase (TdT) (Trevigen, Gaithersburg,
MD). The nucleosomal DNA fragments were visualized with the help of avidin–biotin complex followed by chromogen substrate, which stained
the apoptotic nuclei blue (A, C, and D, arrowheads, which appear black here). The apoptotic nuclei (arrowheads) can be seen in acinar cells in
the mammary lobules (A). In contrast, apoptotic cells are not observed in the normal myocardial tissue (B) obtained from a victim of motor vehicle
accident. Eosin counterstaining demonstrates myocardial silhouette, and the unstained nuclear regions appear clear (open arrows). In a myocardial
specimen from a cardiomyopathic heart (C), two myocytes with apoptosis are shown by blue-stained nuclei (arrowheads, which appear black here).
The occurrence of apoptosis in myocytes (arrowheads) is further confirmed by double staining for α-muscle actin with antibody HHF-35 (brown
product, which appears gray here) (D).
Isolation of the Cytosolic Fraction. Myocardial tissue specimens from seven cardiomyopathic or five normal hearts, treated and untreated cultured neonatal cardiomyocytes, and treated and untreated cultured U-937 cells were homogenized in buffer A (20 mM Hepes, pH 7.5/1.5 mM MgCl2/10 mM KCl/1 mM EDTA/1 mM EGTA/1 mM DTT/0.1 mM PMSF/10 μg/ml leupeptin/antipain/pepsin A) (33). A uniform cell population was washed twice with PBS, and the pellet was suspended in 5 ml of ice-cold buffer A containing 250 mM sucrose. The cells were homogenized by douncing three times in a Dounce homogenizer with a sandpaper-polished pestle. After centrifugation for 5 min at 4°C, the supernatants then were centrifuged at 105,000 × g for 30 min at 4°C. The resulting supernatant was used as the soluble cytosolic fraction.

Immunoblot Analysis. Proteins from cytosolic fractions or total cell lysates were separated by SDS-PAGE, were transferred to nitrocellulose, and were analyzed by immunoblotting with anti-cytochrome c (provided by L. Procheska, Wright State Univ.) (36), anti-CPP32 (Santa Cruz Biotechnology), anti-PKCδ (Upstate Biotechnology, Lake Placid, NY), anti-PARP (Upstate Biotechnology), or anti-actin (Sigma) antibodies. The blots were developed by ECL chemiluminescence (Amersham Pharmacia). In mammalian cells, CPP32 normally exists as a 32-kDa inactive precursor that is converted proteolytically to active p17/12 subunits when cells are induced to undergo apoptosis (24). Anti-CPP32 antibody recognizes both the cleaved and uncleaved CPP32 proteins. PKCδ exists as a 70-kDa inactive protein and undergoes CPP32-mediated cleavage into a 40-kDa fragment in response to apoptotic inducer (27). On the other hand, PARP is a 116-kDa protein that also is cleaved by CPP32 during apoptosis. Signal intensities of various proteins and their fragments in Western blots were determined by densitometric analysis (UltraScan, LKB).

Immunoelectron Microscopy and Ultrastructural Examination. Immunoelectron microscopic studies were performed in the remaining 9 of the 16 hearts and in 3 myocardial tissue specimens obtained from donor hearts. The samples were fixed for 2 hours with 0.5% Karnovsky’s fixative, were postfixed with 1.5% OsO4 in 0.2 M cacodylate buffer (pH 7.3), and were dehydrated and embedded in Epon-Araldite. Ultrathin sections then were etched with 3% H2O2 for 10 min, and were treated for 30 min with 0.05 M normal goat serum in Tris buffer with 0.09% NaCl (pH 7.4), and were treated with anti-cytochrome c antibody overnight at 4°C. After rinsing in Tris buffer and Tris buffer containing 1% BSA, the sections were incubated for 1 hour at room temperature with the secondary antibody [gold-conjugated goat anti-mouse/anti rabbit IgG, particle size 10 nm (Dako)]. Negative controls were performed by substitution of the primary antibody with Tris buffer. The sections were counterstained with uranyl acetate and lead citrate.

RESULTS

Evidence of apoptosis in myocytes by nick end-labeling studies was only observed in the cardiomyopathic hearts and none of the normal myocardial tissue, as described (3) (Fig. 1). To assess the accumulation of cytochrome c in cardiomyopathic hearts, electron microscopic studies were performed by using immunogold labeling with anti-cytochrome c antibodies in nine hearts (six IDCM hearts; three ISCM hearts). The results demonstrate that cytochrome c was localized in mitochondria in donor heart biopsies (Fig. 2 Top). On the other hand, cytochrome c immunoactivity was observed both in mitochondrial and extramitochondrial compartments in cardiomyopathic hearts. The cytochrome c in these specimens was predominantly distributed in cytoplasm (Table 1), over contractile proteins and Z-bands (Fig. 2 Middle) as well as in the vicinity of intercalated discs (Fig. 2 Bottom). In addition, some anti-cytochrome c reactivity also was noted in the extracellular compartment in ISCM specimens. Of interest, ultrastructural changes of apoptosis had yet not occurred in the nuclei in a
These results support a mechanism that links mitochondrial cytochrome c release and cleavage of caspase-3 and PKC activity in myopathic hearts (Fig. 4A). Unlike U-937 cells, PARP cleavage was not seen in cardiomyopathic hearts (Fig. 4B). However, in cardiomyopathic hearts the activation of caspase-3 and cleavage of PARP was not observed in control myocardial specimens (Fig. 4C). These results support a mechanism that links mitochondrial release of cytochrome c to activation of caspase-3 in cardiomyopathic hearts.

**DISCUSSION**

Explanted hearts with end-stage cardiomyopathy exhibit release of cytochrome c from mitochondria into the cytoplasm. Cytochrome c is predominantly localized in cytoplasm around myofibrils and intercalated discs. The cytosolic accumulation of cytochrome c is associated with cleavage of caspase-3 and PKCδ. Cytochrome c release and cleavage of caspase-3 and PKCδ occur in both ischemic and idiopathic dilated cardiomyopathic hearts alike. Although evidence of cytochrome c depletion from mitochondria and caspase activation is almost universally seen, ultrastructural alterations of apoptosis in nuclei is not observed in these cells. The activation of nuclear substrate of caspase-3, PARP, also is not observed. The cytochrome c is confined to the mitochondria in normal myocardium, and there is no activation of caspase-3 in normal specimens.

**Table 1. Ultrastructural immunolocalization of cytochrome c in cardiomyopathic and control myocardial tissues**

<table>
<thead>
<tr>
<th>No.</th>
<th>Case</th>
<th>Mitochondria</th>
<th>Sarcomplasm</th>
<th>Z-bands</th>
<th>Intercalated discs</th>
<th>Extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Donor 1</td>
<td>+++ + + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>Donor 2</td>
<td>+ + + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Donor 3</td>
<td>+ + + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1.</td>
<td>ISCM1</td>
<td>+ + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>ISCM2</td>
<td>+ + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>ISCM3</td>
<td>+ + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>IDC1M</td>
<td>+ + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5.</td>
<td>IDC2M</td>
<td>+ + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>IDC3M</td>
<td>+ + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>IDC4M</td>
<td>+ + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td>IDC5M</td>
<td>+ + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9.</td>
<td>IDC6M</td>
<td>+ + + +</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

±, sparse, very few granules; +, few granules; ++, moderate amount; ++++, abundant granules.
The mammalian cysteine proteases are structurally similar to ced-3 and ced-4, which control programmed cell death in the nematode C. elegans. Similar to ced-3, caspase-3 exists in the cytosolic fraction as an inactive 32-kDa precursor that is proteolytically cleaved in apoptotic cells to active subunits (27). Caspase-3 inhibitors effectively block the ability of cytoplasm from the apoptotic cells to induce apoptotic changes in the normal nuclei in vitro (27). Once activated, the caspase-3 production is self-sustained by autocatalysis (27). However, the initial activation has recently been proposed to require release of mitochondrial respiratory chain protein, cytochrome c (31).

Cytochrome c is localized in the intermembrane space loosely attached to the surface of the inner membrane and, as expected, was observed only in the mitochondria (both biochemically and ultrastructurally) of the normal heart. On the other hand, an ~15- to 20-fold higher concentration was present in the cytoplasm of all myopathic tissues. Involvement of mitochondria in execution of apoptosis has been suggested by the existence of apoptotic process even in anucleate cells (37, 38) and the ability of mitochondria-rich fraction to induce apoptosis in nuclei in Xenopus egg extracts (39). Substantial quantitative release of cytochrome c in cytoplasm has been demonstrated in cultured U-937 cells undergoing apoptosis on exposure to DNA-damaging agents (33). During apoptosis, permeability transition pores are formed in the mitochondrial membrane (40) mediated by an inner mitochondrial membrane protein, adenine nucleotide translocator (41). Ligands of adenine nucleotide translocator, such as atractyloside and bongkrekic acid, alter the probability of permeability transition pore formation and modulate ability of mitochondria in induction of apoptosis in cell-free systems (40). It is interesting to note that adenine nucleotide translocator antibody has been frequently isolated from the sera in cardiomyopathy patients and may be involved in the pathogenesis of cardiomyopathy (42, 43).

Although protease cleavage and cytochrome c in the myocardial cytoplasmic extracts support the phenomenon of apoptosis in end-stage heart failure, presence of significant expression of upstream mediators of apoptosis is intriguing, especially when nuclear alterations of apoptosis is not seen in the same cells. This may suggest that the myocytes may be in the preapoptotic stage long before morphologic changes become manifest. One also can surmise that the presence of precursors of apoptosis indicates the commitment of these cells to programmed self-destruction and not the actual occurrence or execution of the process. It is also possible that externally induced apoptosis, especially in a differentiated cell, may follow an unconventional course, unlike classical, spontaneous, single cell-related physiological apoptosis. Furthermore, cleavage of cytoplasmic substrate of caspase-3 but absence of ultrastructural nuclear fragmentation also may suggest a possible dissociation of cytoplasmic and nuclear processes of apoptosis.

**CONCLUSIONS**

Continued loss of myocytes leads to myocardial dysfunction. Because decreased systolic function is a powerful predictor of adverse outcome in congestive heart failure, strategies that reduce the biological signals responsible for myocyte loss and chamber remodeling should improve clinical outcome. The intervention aimed at prevention of apoptosis may be a step in this direction, and documentation of the apoptotic cascade, therefore, becomes necessary for the development of novel therapeutic regimens.

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