Manipulation of Death Pathways in Desmin-Related Cardiomyopathy

Alina Maloyan, Jennifer Sayegh, Hanna Osinska, Balvin H.L. Chua, Jeffrey Robbins

Rationale: Transgenic mice with cardiac specific overexpression of mutated αB-crystallin (CryABR120G) display Desmin-related myopathy (DRM) with dilated cardiomyopathy and heart failure. Our previous studies showed the presence of progressive mitochondrial abnormalities and activation of apoptotic cell death in CryABR120G transgenic hearts. However, the role of mitochondrial dysfunction and apoptosis in the overall course of the disease was unclear.

Objective: We tested the hypothesis that prevention of apoptosis would ameliorate CryABR120G pathology and decrease morbidity.

Methods and Results: We crossed CryABR120G mice to transgenic mice with cardiac specific overexpression of Bcl-2. Sustained Bcl-2 overexpression in CryABR120G hearts prolonged CryABR120G transgenic mice survival by 20%. This was associated with decreased mitochondrial abnormalities, restoration of cardiac function, prevention of cardiac hypertrophy, and attenuation of apoptosis. CryABR120G misfolded protein aggregation was significantly reduced in the double transgenic. However, inhibition of apoptotic signaling resulted in the upregulation of autophagy and alternative death pathways, the net result being increased necrosis.

Conclusion: Although Bcl-2 overexpression prolonged life in this DRM model, in the absence of apoptosis, another death pathway was activated. (Circ Res. 2010;106:1524-1532.)

Key Words: heart disease ▪ mitochondria ▪ apoptosis ▪ protein misfolding ▪ autophagy ▪ necrosis
blocking the intrinsic cell death pathway, but did not prevent their ultimate death as the death process shifted toward necrosis.

Methods

Transgenic Mice

CryABR120G mice and Bcl-2 overexpressing mice were used as described previously,2,9 except that the Bcl-2 TG mice were first bred into the FVB/N background by back-crossing the original Bcl-2 TGs to FVB/N animals for at least 7 generations. Three experimental groups were studied: single CryABR120G, Bcl-2 overexpressing double TG CryABR120G/Bcl-2, and age matched nontransgenic (NTG) littermates. TG and double TG mice were identified by PCR analysis of genomic DNA isolated from tail clips. Animals were housed in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care, and experiments were approved by the Institutional Animal Review Board.

Antibodies and Reagents

Anti-tubulin and monoclonal anti-receptor-interacting protein (RIP)1 were purchased from Santa Cruz Biotechnology, anti–caspase-3 was from Cell Signaling, and monoclonal anti-RIP1 was purchased from BD Biosciences. Anti-Beclin 1 was obtained from Stressgen, and anti–light chain (LC3) from Novus. Recombinant human tumor necrosis factor (TNF) was purchased from Sigma.

Evans Blue Staining

Fluorescent staining with Evans blue dye (EBD) was used to assess the impairment of sarcolemmal membrane integrity. Six-month-old mice were injected with EBD (10 mg/mL in PBS) intraperitoneally, and any heart tissue was excised 8 hours later. Heart tissue was embedded in OCT Tissue Tek (Sakura Finetecnical), and snap-frozen in liquid nitrogen. Seven-μm sections were cut, washed with PBS, and counterstained with phalloidin (Invitrogen). Stained sections were visualized using the fluorescence microscope, and areas of red auto-fluorescence (EBD positive areas) were selected and imaged.

Cell Culture

3T3 fibroblasts were cultured in DMEM supplemented with 10% FCS and antibiotics (25 U/mL penicillin, 25 μg/mL streptomycin) (Invitrogen) in 5% CO2 in air at 37°C. Replication-deficient adenoviruses for CryABR120G and Bcl-2 were generated using the AdEasy adenoviral system (Stratagene). Cells were typically infected with adenovirus at a multiplicity of infection of 10 for 2 hours at 37°C. The cells were then cultured for a further 24 hours in virus-free media before treatment. Cell death was induced with 10 ng/mL of TNF for 10 minutes. For cell death analysis, the Cell Proliferation Kit from Roche was used according to the protocol of the manufacturer.

For autophagy assays, rat neonatal cardiomyocytes were used. Cells were plated 24 hours before the infection. On the next day, cells were infected with GFP-LC3 adenovirus (provided by J. Sadoshima, University of Medicine and Dentistry of New Jersey), in combination either with AD-CryABR120G, AD-Bcl-2, or both. Forty-eight hours later, cells were washed, fixed, excited with a 488-nm laser light and imaged on a time delay integration CCD camera. Images of fixed cells collected on the ImageStream (Amnis), were analyzed using ImageStream Data Exploration and Analysis Software (IDEAS). Because fluorescent probes have broad emission spectra, spectral compensation was digitally performed on a pixel-by-pixel basis before data analysis. After compensation, similarity analysis and spot counting was done in in-focus single cell images. Three samples from each group were analyzed using 10,000 cells per sample.

Statistical Analysis

All data are expressed as means±SE. Comparisons between experimental were determined by 1-way or 2-way ANOVA where appropriate, followed by Student’s t test. A probability value of <0.05 was considered statistically significant.

Results

Overexpression of Bcl-2 Increases Lifespan and Ameliorates Cardiac Dysfunction in CryABR120G Mice

Mice that overexpress Bcl-2 specifically in the cardiomyocytes have been described.8 We crossed those mice repeatedly into the FVB/N background, the strain in which our other transgenic mice were made, to avoid strain specific phenotypes. Cardiomyocyte-restricted Bcl-2 overexpression in an FVB/N background was benign with no effects observed on heart structure or function (data not shown). The Bcl-2 transgene continued to be strongly expressed in the hearts of transgenic mice as well (Figure 1A), with quantitation of the signals indicating overexpression levels of ~5-fold in animals carrying 1 copy of the Bcl-2 transgene and 9-fold in animals with 2 copies (homozygous for the transgene). We used double transgenic animals that carried 1 copy of each transgene (Bcl-2 and CryABR120G) for all experiments to avoid any effects attributable to insertional mutagenic events. As shown previously, cardiac-restricted expression of CryABR120G leads to premature lethality by the age of 7 to 8 months (Figure 1B) resulting from HF.2 Lifespans of the CryABR120G/Bcl-2 mice were significantly increased when compared to the CryABR120G mice. However,
all of these mice also died prematurely with no survivors by 8.5 to 9 months of age (Figure 1B).

We previously showed that overexpression of CryABR120G contributes to the LV dysfunction characterized by the progressive transition from concentric hypertrophy to dilated cardiomyopathy at the age of 6 months.2 In association with their prolonged lifespan, CryABR120G/Bcl-2 mice displayed an attenuated cardiac hypertrophic response (Figure 1C). To determine whether overexpression of Bcl-2 was sufficient to attenuate adverse cardiac remodeling in CryABR120G mice, we examined cardiac structure using standard morphometric analyses and 2D-directed M-mode echocardiography (Figure 1C and the Table). Figure 1B shows that heart weight-to-body weight ratios of the single CryABR120G mice at 4 and 6 months were significantly greater than age-matched NTG and CryABR120G/Bcl-2 mice. In addition, marked disparities in cardiac function between the CryABR120G and CryABR120G/Bcl-2 mice were apparent as well (Table). At 6 months of age, when the CryABR120G animals were already in failure, the heart rate and shortening fraction of the CryABR120G/Bcl-2 mice were conserved with significantly better LV function (Table).

**Bcl-2 Expression Prevents Mitochondrial Swelling and Blunts the Apoptotic Response in CryABR120G Mice**

Constitutive overexpression of mutant CryABR120G leads to mitochondrial dysfunction, a precocious and lasting 50% reduction in Complex I activity, opening of the MPTP and activation apoptotic cell death.3 MPTP opening results in mitochondrial swelling,3 but the mitochondria derived from Bak,15 we first analyzed this potential beneficial effect in the CryABR120G/Bcl-2 hearts. Mitochondria isolated from the CryABR120G/Bcl-2 hearts showed the same deficits apparent in the CryABR120G-derived material, showing that

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### Table. Echocardiography and Tissue Doppler Measurements

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<td>0.014 0.006</td>
<td>0.014 0.006</td>
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<tr>
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<td>15.66 4.5</td>
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A. Western blots showing expression levels in the single and double transgenic mice. When placed in the FVB/N background, the Bcl-2 transgene drove expression levels of between 5-fold (heterozygote) and 10-fold (homozygote, that is, 2 copies of the transgene) greater than present in NTG mice, with those levels maintained throughout the adult states (4- and 6-month levels are shown). Placement of the Bcl-2 transgene in the CryABR120G background did not affect expression of either CryAB or Bcl-2. B. Kaplan–Meier curves for NTG, CryABR120G, and CryABR120G/Bcl-2 mice. C. Cardiac hypertrophy from 2 to 6 months in CryABR120G/Bcl-2 mice compared to CryABR120G. *P<0.05 vs NTG; †P<0.05 vs CryABR120G.
Bcl-2 overexpression is not able to rescue the respiratory deficits in the CryABR120G mitochondria (Figure 2B).

To begin to explore the mechanisms responsible for Bcl-2-mediated attenuation of cardiomyocyte apoptosis we evaluated the extent of DNA fragmentation longitudinally in the NTG, CryABR120G and CryABR120G/Bcl-2 hearts by quantitating TUNEL-positive nuclei located in identified cardiomyocytes. We observed significant decreases in the number of TUNEL-positive nuclei in 2- and 6-month-old CryABR120G/Bcl-2 hearts as compared to CryAB R120G (Figure 2C). We confirmed that we were observing decreases in apoptosis by measuring reduced levels of activated caspase-3 in the CryABR120G/Bcl-2 but not in CryABR120G hearts (Figure 2D).

We then measured the release of cytochrome c from mitochondria into the cytosol in the hearts of 5-month-old CryABR120G and CryABR120G/Bcl-2 mice. Representative Western blots derived from the mitochondrial and cytosolic proteins showed that in CryABR120G/Bcl-2 mouse hearts, cytosolic cytochrome c levels were significantly lower than in the CryABR120G hearts although they remained higher than the level observed in the NTG littermates (Figure 2E).

**Protein Aggregation in CryABR120G/Bcl-2 Mice Is Reduced**

CryABR120G-mediated DRM is a protein misfolding disease characterized by accumulation of insoluble protein deposits, whose accumulations can have both toxic and protective functions. Although expression of Bcl-2 in the CryABR120G hearts did not completely prevent aggregate formation, electron microscopy revealed major disparities in protein deposition architecture. The aggresomes in the CryABR120G hearts appeared to be largely homogenous, consisting of uniformly solid, granulofilamentous bodies, whereas in the CryABR120G/Bcl-2 hearts, they appeared as heterogeneous masses of small electron-dense material interspersed with mitochondria (Figure 3A). Immunofluorescent staining for CryAB was used to quantitate the cytoplasmic area occupied by the aggresomes (Figure 3B) and confirmed significant decreases in their accumulation at all ages measured (Figure 3C).

Protein aggregates may form by abnormal folding or by disturbance of intracellular protein degradation pathways. Although autophagy is known to play a critical role in the major degradation pathways for cellular protein and organelles, it is becoming increasingly apparent that autophagy is an important cellular process that can be activated in response to the accumulation of misfolded proteins. Autophagy is upregulated in rat neonatal cardiomyocytes infected with CryABR120G adenovirus and blunting autophagy in CryABR120G hearts accelerated and increased development of the cardiac pathology.

Given those data and the observation that CRYABR120G/Bcl-2 hearts displayed significant decreases in protein aggregate levels, we wished to evaluate and compare the autophagic response in CryABR120G and CryABR120G/Bcl-2 hearts. Beclin 1 is considered to be a key player in autophagy. Beclin 1 promotes autophagic activity and is involved in the recruitment of membranes to form the autophagosome. However, in contrast to our expectation that autophagy would be
increased in both the CryABR120G and CryABR120G/Bcl-2 hearts, we observed modest but significant reductions in Beclin 1 levels in both the CryABR120G and CryABR120G/Bcl-2 hearts relative to NTG (Figure 4A).

Given the complex nature of autophagy, we analyzed additional markers of the process. Formation and maturation of autophagosomes is accompanied by intracellular translocation and processing of microtubule-associated protein 1 LC3,21 an important component of the autophagosome membrane and one of the most reliable markers of autophagy. Western Blot analysis showed accumulation of cleaved LC3-II exclusively in the hearts of CryABR120G/Bcl-2 but not CryABR120G mice, suggesting a significant augmentation of an autophagic response in the double transgenic mice (Figure 4B).

To gain a qualitative assessment of autophagy, we carried out transmission electron microscopy on hearts derived from the single and double transgenic hearts at 5 months. As expected, the CryABR120G hearts showed the usual pathology at this stage with significant accumulations of large, granulofilamentous aggregates, disrupted mitochondria with massive cristae lysis and sarcomere disruption. However, strikingly, there was a paucity of any structures that could be identified as either intermediates or late stages in the autophagic pathway (Figure 5A). In contrast, the fields from the double transgenic hearts contain numerous intermediates/participants in the autophagic pathway, including amphisomes, the autophagic vacuoles formed on fusion between autophagosomes and endosomes, lysosomes and autolysosomes (Figure 5B through 5D).
To quantitate the increase in autophagic activity in response to Bcl-2 overexpression, neonatal rat cardiomyocytes were infected with GFP-LC3 autophagy-reporter adenovirus, followed by infection with AD-CryABR120G, AD-Bcl-2, or a combination of both. Forty-eight hours later cells were fixed, visualized by fluorescent microscopy and analyzed using the Image Stream system (Figure 6). LC3 undergoes a significant redistribution during autophagy, changing from a diffused cytosolic signal to punctate dots that mark the mature autophagosome.22 After 48 hours, the abundance of punctuated LC3 was almost 5-fold higher in the cardiomyocytes infected with both AD-Bcl-2 and AD-CryABR120G in comparison to AD-CryABR120G only (Figure 6B). Blockade of autophagosome formation using 3-MA significantly reduced the accumulation of punctuated LC3 in R120G/Bcl-2 infected cardiomyocytes.

Blunting the Apoptotic Response Leads to Necrosis in CryABR120G/Bcl-2 Mice

Our data showed that overproduction of the antiapoptotic protein Bcl-2 increased the lifespan of the CryABR120G animals, indicating that the intrinsic apoptotic pathway does play a pathogenic role in DRM. However, the animals still die prematurely, so abrogation of apoptosis is clearly not sufficient to rescue the phenotype. Historically, apoptosis has been defined as a type of cell death that is morphologically distinct from other types of cell death such as necrosis.23 Despite the long held view that necrosis is an accidental and uncontrolled form of cell death, recent studies have revealed that necrosis is probably tightly controlled, and intriguing interactions between the apoptotic and necrotic machinery have been uncovered.24 Eliminating genes encoding proteins controlling apoptosis resulted in the conversion of an apoptotic phenotype to a necrotic one, both in vitro and in vivo.25 We hypothesized that by overexpressing Bcl-2 and decreasing apoptotic flux in the absence of not affecting the primary genetic lesion (expression of CryABR120G); we might have shifted the cell death balance to necrosis.

EBD analyses were performed to detect necrotic cell death-related membrane abnormalities in the myocardium of NTG, CryABR120G and CryABR120G/Bcl-2 hearts (Figure 7A). NTG cardiomyocytes were impervious to EBD (not shown). In the hearts from 6 month CryABR120G mice, which are undergoing extensive apoptotic cell death,3 sporadic EBD-positive myocytes were observed (Figure 7A). However, the number of EBD-positive cells in CryABR120G/Bcl-2 hearts were significantly increased (Figure 7A, bottom, and 7B). Pharmacological and genetic evidence suggests that necrosis can occur in a tightly regulated manner.26 Cell death inducers such as TNF promote either apoptosis or necrosis depending on the specific experimental setting.27 When apoptotic cell death is inhibited by antiapoptotic factors, a process of programmed necrotic death can occur.28 Although the proteins regulating necrosis are largely undefined, recent data indicate that some regulatory mechanisms may be shared or intersect with apoptosis,29 and RIP1 kinase may play an important role in deciding between necrosis and survival when the apoptotic signal is blocked.29 Consistent with this hypothesis, we found significantly higher RIP1 levels in 6-month-old CryABR120G/Bcl-2 hearts compared to NTG and CryABR120G/Bcl-2 mice. A, Evans blue permeability of cardiomyocytes derived from CryABR120G and CryABR120G/Bcl-2 hearts. Sections were counterstained with phallloidin (green) and visualized by fluorescent microscopy at magnifications of x10 and x40. B, Quantification of EBD-positive cells. *P<0.005 vs CryABR120G. C, Protein derived from CryABR120G and CryABR120G/Bcl-2 hearts were electrophoresed in SDS-PAGE, Western blotted, and probed with anti-RIP1 and anti-tubulin antibodies. Shown below the blot is quantification of RIP1 levels using ImageJ software. *P<0.005 vs NTG; ^P<0.005 vs CryABR120G.
CryAB<sub>R120G</sub> samples (Figure 7C and 7D). Interestingly, CryAB<sub>R120G</sub> hearts showed markedly reduced RIP1 levels.

We tested the hypothesis that coexpression of Bcl-2 and CryAB<sub>R120G</sub> is sufficient to cause increased levels of RIP1. 3T3 cells were infected with adeno-CryAB<sub>R120G</sub> (R group), adeno-Bcl-2 (B group), or with both (RB group) and RIP1 accumulation determined 24 hours later by immunohistochemistry (Online Figure I, A, in the Online Data Supplement, available at http://circres.ahajournals.org). Confirming the hypothesis, RB cells showed more than a 7-fold accumulation of RIP1 compared to R, B and control cells (Online Figure I, B).

We next tested whether accumulation of RIP1 was sufficient to increase the sensitivity of infected 3T3 cells to TNF-mediated necrosis (Online Figure I, C). Cells were treated with low levels of TNF (Methods) and the level of cell death was evaluated. Whereas nontransfected R120G and Bcl-2-infected cells showed only minor responses to TNF, cell death in RB cells increased by 100%, indicating a significantly elevated sensitivity of RB cells to TNF-mediated necrosis. However, when the effect of TNF was analyzed in the presence of necrostatin, a specific inhibitor of necrosis, is mediated through an integrated cascade, which under conditions of inhibited apoptotic cell death has been previously documented in Bak/Bax knockout mice.40 The precise role of autophagy in the progression of HF remains unclear. In some HF models, amplification of the autophagic response led to pathological hypertrophy, decreased cardiac performance, and promotion of autophagic cell death.41 In other studies, autophagy protected the heart by scavenging and eliminating misfolded proteins.42 It appears that autophagy can lead to different outcomes depending on disease context and severity. Future studies using genetic approaches are underway to define the exact role of autophagy in our model at the different disease stages.

Necrosis has been often viewed as an accidental and unregulated event but data are now rapidly accumulating that suggest necrosis can also be thought of as programmed cell death.43 The cell death program, whether by apoptosis or by necrosis, is mediated through an integrated cascade, which can be assessed at multiple junction sites and propagated through numerous branch points.29 Recent studies point to RIP1 as a possible “molecular switch” that can determine whether the cell succumbs through apoptosis or necrosis.44 In our model, overproduction of Bcl-2 led to increased RIP1 with a concomitant increase in necrosis and these admittedly preliminary but intriguing data point the way for further exploration of whether these markers are general or more specific to different but related cell death processes.

That overexpression of Bcl-2 leads to a reversal of calcium sensitivity of the mitochondria and may lead to a switch from apoptosis to autophagy and necrosis argues strongly for the existence of complex regulatory interactions between these scavenger/cell death mechanisms and could explain the limited success of antiapoptotic therapies. An additional complication is the different roles these processes could play at different times in disease progression and presentation. Although considerable work will be required to explain the observations outlined in this article, an appreciation of the
complexities and understanding the underlying molecular pathways will help define effective combination therapies.

**Sources of Funding**

This work was supported by NIH grants P01HL69799, P50HL074728, P50HL077101, P01HL059408, and R01HL087862 (to J.R.).

**Disclosures**

None.

**References**


Novelty and Significance

What Is Known?

- Mutations in αB-crystallin can cause a dilated cardiomyopathy and heart failure.
- Although apoptotic cell death contributes to the development of cardiac pathology, its necessity and sufficiency in the cardiac pathogenesis is not known.

What New Information Does This Article Contribute?

- In vivo evidence that death pathways other than apoptosis are capable of causing cardiomyopathy and heart failure in this animal model.
- This is the first demonstration in this animal model showing that abrogation of the intrinsic pathway of apoptosis in the heart is possible and that, although life is prolonged, the animal still dies prematurely.
- Indirectly supports a model for the interlinking of different death pathways in the pathogenic process that leads to cardiac death.

The missense R120G mutation in the small heat shock protein α-B-crystallin (CryABR120G) causes a desmin-related myopathy (DRM). This skeletal and cardiac disease is characterized by the formation of desmin- and CryAB-containing aggregates within muscle fibers, including those within the heart. Mice with cardiac-specific overexpression of CryABR120G develop severe cardiomyopathy at 3 months of age and die at 6 to 7 months from heart failure. Previous studies showed that overexpression of CryABR120G results in formation of perinuclear aggresomes, and accumulation of preamyloid oligomer. Preamyloid oligomer is considered to be the cytotoxic entity in many of protein misfolding-based diseases, and we noted extensive apoptosis in these hearts. Because of this, we asked whether we could impact materially on disease progression by interfering with apoptosis and so, we crossed the CryABR120G mice with a mouse that specifically expressed the antiapoptotic molecule, Bcl-2, in cardiomyocytes. To our surprise, although apoptosis was largely ameliorated, the mice still died although they lived 20% longer than mice that did not express increased levels of Bcl-2. To understand the cause of death, we explored whether other death pathways were activated and found that, indeed, although apoptosis was shut down, necrosis was activated. These findings have implications for using the death pathways as therapeutic targets and indicate that multiple pathway interactions will need to be considered and tested for any potential therapeutics aimed at a single pathway.
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Circ Res. 2010;106:1524-1532; originally published online April 1, 2010;
doi: 10.1161/CIRCRESAHA.109.212639

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/106/9/1524

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/04/01/CIRCRESAHA.109.212639.DC1
Supplemental Materials

Manipulation of Death Pathways in Desmin Related Cardiomyopathy

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A

Co-infection of CryAB\textsuperscript{R120G} and Bcl-2-containing adenovirus leads to increased accumulation of RIP1. A, 3T3 cells were infected with adenovirus carrying Bcl-2 (B), R120G (R), or a combination of both (RB). Forty-eight hours later the cells were fixed and stained with anti-RIP1 antibody (green). The cells were counterstained with phalloidin (red). We confirmed that the RIP1 staining was perinuclear using Western blots of proteins derived from the nuclear, cytosolic and mitochondrial compartments. RIP1 was present only in the cytosol (data not shown). Incubation with necrostatin did not affect RIP1 accumulation (RBN).

B, RIP1 quantitation

C

Cell Death Rate

Online Figure I. Co-infection of CryAB\textsuperscript{R120G} and Bcl-2-containing adenovirus leads to increased accumulation of RIP1. A, 3T3 cells were infected with adenovirus carrying Bcl-2 (B), R120G (R), or a combination of both (RB). Forty-eight hours later the cells were fixed and stained with anti-RIP1 antibody (green). The cells were counterstained with phalloidin (red). We confirmed that the RIP1 staining was perinuclear using Western blots of proteins derived from the nuclear, cytosolic and mitochondrial compartments. RIP1 was present only in the cytosol (data not shown). Incubation with necrostatin did not affect RIP1 accumulation (RBN). B, RIP1 quantitation C, Bcl-2-induced inhibition of apoptosis increases the sensitivity of 3T3 cells to necrosis mediated by TNF. Cell death rate is normalized to that level observed in uninfected controls, which was arbitrarily set at 1. *\textit{P}<0.005 versus non-infected cells.