On-line Supplementary Information

Delayed activation of caspase-independent apoptosis during heart failure in transgenic mice overexpressing caspase inhibitor, CrmA

Soochan Bae, Parco M. Siu, Sangita Choudhury, Qingen Ke, Jun H. Choi, Young Y. Koh, Peter M. Kang

Cardiovascular Division, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA.

Supplemental Methods

Western immunoblot analyses

Western immunoblot analyses were preformed as described previously (3-5). Total protein and cytosolic AIF were probed with an anti-AIF rabbit polyclonal antibody (BD Pharmingen, San Diego, CA). The purity of the subcellular fractionations was assessed by immunoblot analysis with anti-GAPDH (a cytosolic protein; RDI, Flanders, NJ) and anti-COXIV (a mitochondrial protein; Molecular Probes, Eugene, OR) antibodies. The inhibitor of caspase-activated DNase (ICAD) antibodies were obtained from BD Pharmingen (San Diego, CA).

Immunohistochemistry

Frozen 5 μm-thick ventricle muscle cross sections were cut in a freezing cryostat at -20°C and placed on the same glass slide to control for processing differences (e.g., incubation

time, temperature, etc.). The sections were air dried at room temperature, fixed in 4% paraformaldehyde in PBS, pH 7.4 at room temperature for 20 min. The sections were then blocked in 10% donkey serum in PBS at room temperature for 30 min following permeabilization with 0.5% Triton in 0.02% saponin/PBS for 10 min. After washes in PBS, sections were incubated with an anti-AIF rabbit polyclonal antibody (Cell Signaling Technology, Danvers), overnight at 4°C followed by AlexaFluor488 secondary antibody incubation (Invitrogen, Carlsbad, CA) for 2 h at room temperature. To distinguish cardiomyocyte from non cardiomyocyte nuclei, we triple stained for nuclei (4',6-diamidino-2-phenylindole (DAPI) staining), AIF-translocated nuclei (AIF staining), and cardiomyocytes (α-actinin staining), and analyzed under a confocal fluorescence microscope (BioRad 1024 with Nikon E800)..

Caspase and PARP activity assay

The activities of caspase-3, -8, and -9 were determined with colorimetric assay kits (R&D Systems, Minneapolis, MN) as described previously (2-4). Briefly, protein samples were added to substrates of Acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (for caspase-3), Acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide (for caspase-8) and Acetyl-Leu-Glu-His-Asp-*p*-nitroanilide (for caspase-9). The enzyme-catalyzed release of *p*-nitroanilide was measured at 405 nm. PARP activity was measured at 450 nm by incorporation of biotinylated poly (ADP-ribose) using colorimetric assay kits (R&D Systems) as described previously (1).

Apoptosis assays

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using *in situ* fluorescein-based Cell Death Detection Kits, (Roche Applied Science,

Indianapolis, IN) as described previously (5). To distinguish cardiomyocyte from non-cardiomyocyte nuclei, we triple stained for nuclei (4',6-diamidino-2-phenylindole (DAPI) staining), apoptotic nuclei (TUNEL staining), and cardiomyocytes (α-actinin staining), and analyzed the stained sections using confocal microscopy. A minimum of ~10 high power fields with ~200 nuclei/field was counted for each sample.

Supplemental Table 1. Baseline morphological findings for WT and CrmA Tg mice

	WT	CrmA
Body Weight (g)	29.7 ±0.4	29.6 ±1.1
Heart Weight (mg)	134 ± 0.3	123 ±0.3
Lung Weight (mg)	157 ± 0.4	151 ±0.8
TL (mm)	16.9 ±0.1	16.6 ±0.1
HW/TL (mg/mm)	7.5±0.3	7.4 ±0.1
LW/TL (mg/mm)	8.9 ±0.4	9.1 ±0.4

TL = tibial length, HW/BW = heart weight to body weight ratio, HW/TL = heart weight to tibial length ratio. $^{*}P < 0.05$ compared to WT. n=6

Supplemental Table 2. Baseline echocardiographic findings for WT and CrmA Tg mice

	WT	CrmA
HR (beats/min)	506 ±21	509 ±32
IVSd (mm)	0.85 ±0.03	0.86 ±0.05
LVIDd (mm)	3.6 ±0.01	3.5 ±0.02
LVPWd (mm)	0.97 ±0.05	0.94±0.08
FS (%)	53.4 ±1.3	52.0 ± 1.7

HR = heart rate, IVSd = intraventricular septum in diastole, LVIDd = left ventricular internal diameter in diastole, LVPWd = left ventricular posterior wall in diastole, FS = Fractional Shortening. $^{*}p < 0.05$ compared to WT. n=6