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Myocyte apoptosis occurs early during the development of pressure-overload hypertrophy in infant myocardium

Yeong-Hoon Choi, MD,[a,b,d] Douglas B. Cowan, PhD,[a,d] Adrian M. Moran, MBBS,[c] Steven D. Colan, MD,[c] Christof Stamm, MD,[b] Koh Takeuchi, MD,[b] Ingeborg Friehs, MD,[b] Pedro J. del Nido, MD,[b] and Francis X. McGowan, Jr, MD[a,d]

Objective: Abnormal hemodynamic loading often accompanies congenital heart disease both before and after surgical repair. Adaptive and maladaptive myocardial responses to increased load are numerous. This study examined the hypothesis that myocyte loss occurs during compensatory hypertrophic growth in the developing infant myocardium subjected to progressive pressure overload.

Methods: Pressure-overload left ventricular hypertrophy was induced in 7- to 10-day-old rabbits by banding the thoracic aorta. Left ventricular function and mechanics were quantified by serial echocardiography and noninvasive left ventricular wall stress analysis. Left ventricular tissue sections were examined for fibrosis by using Masson's trichrome stain and for myocyte apoptosis by using a myocyte-specific DNA fragmentation assay and caspase-3 activation (specific fluorescent substrate).

Results: Significant myocyte apoptosis ($198 \pm 37/10^6$ myocytes, $P < .01$ vs control) and caspase-3 activation were present in early hypertrophy when left ventricular contractility was preserved and compensatory hypertrophy had normalized wall stress. By 6 weeks, multiple indices of left ventricular contractility were reduced, and left ventricular wall stress was increased. Myocyte apoptosis was accelerated ($361 \pm 56/10^6$ myocytes), caspase-3 activity further increased, and the estimated total number of left ventricular myocytes was significantly reduced by $18\% \pm 4\%$.

Conclusion: In experimental infant left ventricular hypertrophy, myocyte apoptosis is initiated in the face of normalized wall stress and preserved contractility. The ongoing rate of apoptosis causes a measurable decrease in myocyte number that is coincident with the onset of ventricular dysfunction. It thus appears that pressure overload, even at its earliest stages, is not well tolerated by the developing ventricle.

Supplemental material is available online.

Congenital heart disease is commonly associated with abnormal hemodynamic loading (pressure or volume). Because complete structural and hemodynamic correction is frequently impossible, abnormal loading is a persistent problem in many patients. In the setting of sustained pressure overload, ventricular adaptation typically progresses through stages that include compensatory hypertrophy caused by the parallel addition of sarcomeres, which results in increased myocyte width and ventricular wall thickness. According to LaPlace's Law, myocardial wall stress can be calculated from the formula (pressure $\times$ radius)/(2 $\times$ wall thickness), and thus an increase in wall thickness can ameliorate increased wall stress caused by an increase in pressure. Because ventricular ejection is directly affected by afterload, it is believed that normalization of systolic stress (afterload) via increasing wall thickness is an important mechanism to maintain systolic performance in the face of increased systolic pressure. Failure to do so results in afterload mismatch where the degree of hypertrophy can no longer compensate for increased afterload.[1,2]

Numerous changes in gene expression and protein synthesis accompany this response. They include alterations in sarcomeric protein isoforms and activity; calcium handling and regulatory proteins (eg, the sarcoplasmic reticular Ca-ATPase, SERCA-2); substrate and energy metabolism; adrenergic, muscarinic, and angiotensin receptor species and activity; growth factor and cytokine production; and stimulation of other pleiotropic signaling pathways, such as calcineurin.[2-8] Abnormalities in parameters such as extracellular matrix signaling, matrix turnover, coronary blood supply, and angiogenesis have also been described and implicated in both the compensatory and pathologic hypertrophic responses.

The specific molecular mechanisms underlying the failure of continued hypertrophic compensation and the progression of contractile dysfunction are not well understood,

From the Departments of Anesthesiology and Perioperative and Pain Medicine,[a] Cardiac Surgery,[b] Cardiology,[c] and the Anesthesia/Critical Care Medicine Research Laboratory,[d] Children's Hospital Boston, and Harvard Medical School, Boston, Mass.

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Address for reprints: Yeong-Hoon Choi, MD, Children's Hospital Boston and Harvard Medical School, Department of Cardiac Surgery, 300 Longwood Avenue, Boston, MA 02115. Current address: Heartcenter of the University of Cologne, Department of Cardiothoracic Surgery and Center of Molecular Medicine Cologne, Kerpener Str 62, 50924 Cologne, Germany. (E-mail: yh.choi@uk-koeln.de).

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Abbreviations and Acronyms

ESS = end-systolic stress
FS = fiberstress
LV = left ventricular
mw = midwall
SF = shortening fraction
SSI = stress shortening index
SVI = stress velocity index
TUNEL = terminal deoxynucleotide transferase-mediated dUTP nick-end labeling
VCFc = rate of circumferential fiber shortening

especially in the immature, developing ventricle. Myocyte apoptosis has been implicated in numerous states associated with reduced myocardial performance, including acute and chronic ischemia, diabetes, myocarditis, Adriamycin toxicity, hypertrophy, and heart failure.[8-19] Although the data showing that apoptosis occurs in these settings are significant, their functional importance remains unclear, particularly with regard to the adaptation to chronic pressure load. Several studies have quantified cardiomyocyte apoptosis in late-stage chronic hypertrophy of adult myocardium,[20,21] but there is no evidence of this process in the developing heart. Cell growth, survival, and death signaling pathways are different than in the adult in both the normal developing myocardium and that exposed to pressure overload. We therefore examined the hypothesis that hypertrophic growth would stimulate myocyte apoptosis in the developing ventricle independently from the onset of heart failure.

MATERIALS AND METHODS

Animal Model

The institutional animal care and use committee approved these experiments. New Zealand white rabbits (aged 7–10 days) were used to create a model of progressive left ventricular (LV) hypertrophy. After anesthesia was induced with intramuscular ketamine (20 mg/kg) and xylazine (0.5 mg/kg), a left thoracotomy was performed under sterile conditions. A 2-0 silk suture was placed around the descending aorta just distal to the ligamentum arteriosum, with care taken to make the suture as snug as possible without acutely causing stenosis of the descending aorta. After chest closure, air was evacuated from the left thoracic cavity by catheter aspiration. After recovery from anesthesia, animals were returned to their mothers and allowed to feed in the normal manner. With growth, an aortic coarctation gradually develops. In preparation for later evaluations, weekly shaving of the right upper limb and thorax, as well as sham echocardiograms, were performed to accustom the animals to the procedure and reduce the stress response to actual echocardiography, thus avoiding the need for anesthesia or sedation during serial echocardiographic examinations.

For controls, age- and litter-matched New Zealand white rabbits were sham-operated and otherwise handled identically. Echocardiography and noninvasive blood pressure determination were performed at weekly intervals in the banded and age-matched control animals (see Appendix). A separate group of normal animals was used to determine the normal relationship in the rabbit of shortening fraction (SF) to afterload (end-systolic stress [ESS]) (stress shortening index [SSI]), rate of circumferential fiber shortening to ESS (afterload) (VCFc to ESS; stress velocity index [SVI]), and their midwall equivalents (SF_{mw} and $VCFc_{mw}$) compared with fiberstress (FS) to determine SSI_{mw} and SVI_{mw}. Blood pressure was determined serially and noninvasively using optical plethysmography (Finapress, Ohmeda, Englewood, Colo). The Finapress was applied to the shaved right forearm after application of topical alcohol over the region of the palpable brachial artery. The accuracy of the Finapress versus direct central arterial cannulation (carotid artery) to measure arterial blood pressure and calculate load-adjusted echocardiographic indices was validated in a separate set of experiments (see Appendix for details).

Histochemical Methods

Animals were sacrificed at weekly intervals, and hearts were immediately perfusion fixed in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, and paraffin embedded and sectioned. Mid-LV myocardial sections were deparaffinized, hydrated in a descending alcohol series, and boiled 3 times for 5 minutes in 1 mmol/L EDTA pH 8.0 (antigen retrieval) before staining. Slides were imaged on a Zeiss Axiovert microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) equipped with visible/ultraviolet/fluorescent objectives (4–100×), xenon light source/Sutter filter wheel (Sutter Instrument Company, Novato, Calif), and appropriate excitation/emission filter sets. Images were recorded using a Princeton Instruments cooled CCD camera (Princeton Instruments, Trenton, NJ) or Leica digital color camera (Leica Microsystems Inc., Bannockburn, Ill), depending on the probe type; images were stored and analyzed using MetaMorph and MetaFluor software (Molecular Devices, Sunnyvale, Calif).

Quantification of Apoptosis

Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining of DNA stand breaks was done with enhanced fluorescent detection by labeling cleaved double-stranded DNA in tissue sections following the Fluorescein FragEL detection kit (Oncogene, Boston, Mass). Positive and negative controls of this assay were previously performed in in vitro cell culture models using rabbit thoracic lymph nodes (data not shown). For DNA staining of nuclei, 10 mg/mL Hoechst 33258 in phosphate-buffered saline/50% glycerol was used. Myocytes were identified by co-staining with mouse anti-desmin monoclonal antibody (Sigma, St Louis, Mo) at a dilution of 1:100 in phosphate-buffered saline containing 5% horse serum and detected with Alexa 568 goat anti-mouse secondary antibody (Molecular Probes, Eugene, Ore). By using MetaMorph software, the following parameters were quantified: 1) total nuclei (Hoechst-positive), 2) total myocyte nuclei (Hoechst-positive in desmin-positive cells), and 3) number of TUNEL-positive myocyte nuclei (TUNEL+/Hoechst+/desmin+). For maximum specificity, a ''positive'' result (ie, apoptotic cardiomyocyte) was counted *only* when a TUNEL-positive signal (green) was co-localized to nuclear DNA (blue) that was located within a cardiomyocyte (red).

Activation of caspase-3 was used as another index of proapoptotic signaling. Total LV protein was extracted by homogenizing 20 to 30 mg of LV myocardium in 4°C lysis buffer A (consisting of 25 mmol/L HEPES [pH 7.5], 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L ethylene glycol bis aminoethyl ether tetra-acetic acid, 1 mmol/L benzamidine, 1 mmol/L dithiothreitol, and 1 mmol/L phenylmethylsulfonyl fluoride) containing 1% [v/v] Triton X-100). After centrifugation at 100,000*g* for 60 minutes at 4°C, supernatant protein concentrations were determined using the Bradford method according to the manufacturer's instructions (BioRad, Hercules, Calif). Protein extract (25 mg) and 25 mL reaction buffer (0.1 mol/L HEPES pH 7.0, 10% polyethylene glycol, 0.1% cholamidopropyl dimethylammonio propanesulfonate, 10 mmol/L dithiothreitol) were mixed with 1 mL of 5 mmol/L fluorogenic caspase-3–specific substrate (DEVD-AFC; BioMol, Plymouth Meeting, Pa). After incubation at 37°C for 2 hours in 96-well fluorescence-specific plates, fluorescence was quantified in a microplate reader (Perkin Elmer,

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