Cardiomyocyte Expression of a Polyglutamine Preamyloid Oligomer Causes Heart Failure

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Background—To determine whether soluble preamyloid oligomers (PAOs) are toxic when expressed internally in the cardiomyocyte, we tested the hypothesis that cardiomyocyte-restricted expression and accumulation of a known PAO is cytotoxic and sufficient to cause heart failure.

Methods and Results—Intracellular PAOs, the entities believed to cause toxicity in many neurodegenerative diseases, have been observed in cardiomyocytes derived from mouse and human heart failure samples. Long (>50) polyglutamine (PQ) repeats form PAOs and cause neurotoxicity in Huntington disease and other neurodegenerative diseases, whereas shorter PQ peptides are benign. We created transgenic mice in which cardiomyocyte-autonomous expression of an 83 residue–long PQ repeat (PQ83) or a non–amyloid-forming peptide of 19 PQ repeats (PQ19) as a nonpathological control was expressed. A PQ83 line with relatively low levels of expression was generated, along with a PQ19 line that expressed ≈9-fold the levels observed in the PQ83 line. Hearts expressing PQ83 exhibited reduced cardiac function and dilation by 5 months, and all mice died by 8 months, whereas PQ19 mice had normal cardiac function, morphology, and life span. PQ83 protein accumulated within aggresomes with PAO-specific staining. The PQ83 hearts showed increased autophagosomal and lysosomal content but also showed markers of necrotic death, including inflammatory cell infiltration and increased sarcolemmal permeability.

Conclusions—The data confirm the hypothesis that expression of an exogenous PAO-forming peptide is toxic to cardiomyocytes and is sufficient to cause cardiomyocyte loss and heart failure in a murine model. (Circulation. 2008;117:2743-2751.)

Key Words: amyloid ■ heart failure ■ cardiomyopathy ■ heart diseases ■ cardiovascular diseases

Amyloidoses are well characterized in many tissues, including the heart, and are generally thought of as a heterogeneous syndrome characterized by the formation and accumulation of extracellular proteinaceous fibrils. A number of inherited forms of systemic amyloidoses, such as those associated with mutations in the serum protein transthyretin,1 can have diverse effects on heart function and result in dilated or restrictive cardiomyopathy or diastolic dysfunction.2,3 Some well-known neurodegenerative diseases, including Alzheimer’s, Parkinson’s, and Huntington’s disease, share the pathological characteristic of accumulation of extracellular, insoluble aggregates of misfolded amyloid proteins.4 Until recently, the extracellular, insoluble protein aggregates observed in these diseases were thought to be causal for disease presentation, but multiple studies have shown that aggregate content correlates poorly with disease severity, and pathological alterations often precede amyloid deposition.5–8 Recently, it has been proposed that cytoplasmic, soluble oligomeric forms of amyloidogenic proteins or preamyloid oligomers (PAOs) are the primary toxic entities responsible for disease. PAOs are a diverse set of proteins of divergent sequences that share a common conformational structure, which may impart a common mechanism of toxicity.9,10 PAOs represent an intermediate step in amyloid fibril formation that begins when a native soluble protein becomes misfolded. The misfolded but soluble protein then self-associates to form soluble oligomers (PAOs), which may subsequently go on to form protofibrils and other insoluble intermediates in the amyloid fibril pathway. PAOs from numerous amyloid-forming proteins can cause cellular toxicity in neural cells,10 consistent with the hypothesis that PAOs are the primary cytotoxic entities in several amyloidogenic diseases.11,12

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It is important to draw a distinction between the presence of PAO, an intracellular, soluble precursor amyloidogenic entity, and the final extracellular amyloid plaques/fibrils themselves. Classically characterized cardiac amyloidoses describe the accumulation of the extracellular, proteinaceous amyloid fibrils between myofibers, whereas PAOs accumulate as intracellular, soluble amyloid precursors. Although the
majority of studies on the PAO mechanism of toxicity have been restricted largely to nerve cells and neurodegenerative diseases, our recent data suggest that a mechanism of PAO-induced toxicity may also play a role in cardiomyocyte death, cardiac disease, and heart failure. In a model of desmin-related cardiomyopathy produced by cardiomyocyte-specific transgenic (TG) expression of a mutant αB-crystallin (CryAB[R120G]), PAO-positive aggregates were observed within cardiomyocytes.13,14 The presence of intracellular PAOs in the heart was closely associated with loss of cardiac function and heart failure, and the silencing of CryAB[R120G] expression resulted in rescue of cardiac function and a concomitant significant reduction in cardiomyocyte PAO levels. We have also observed high intracellular PAO levels in a number of human heart failure samples of various pathogenesis.14

On the basis of those data, we undertook a series of experiments to test whether direct expression and cardiomyocyte accumulation of an ectopic PAO-genic protein could cause dysfunction and cardiac disease. PAOs consisting of expanded polyglutamine (PQ) repeats are toxic when expressed in neural cells,10 and expanded PQ repeats are responsible for 9 neurodegenerative diseases, including Huntington’s disease.15 Proteins with expanded PQ tracts confer a gain of toxic function in a length-dependent manner, with longer tracts (>50) leading to neurotoxicity, whereas shorter tracts are benign.16 On reaching a toxic length, the PQ proteins undergo a conformational change, oligomerizing into toxic PAO intermediates.15,17 To assess the role of PQ repeat peptides in the mouse heart, we generated TG mice expressing either a PAO-forming peptide of 83 PQ repeats (PQ83) or a non–PAO-forming peptide of 19 PQ repeats (PQ19) under the control of the α-myosin heavy chain promoter (MyHC) to drive cardiomyocyte-specific expression. PQ83 hearts developed cardiac dysfunction and dilation with a concomitant increase in PAO-positive staining and formation of insoluble aggresomes within the cardiomyocytes, and the mice invariably died before reaching 8 months of age. PAO-induced heart failure was due to cardiomyocyte loss, and although apoptotic indices were unchanged in PQ83 hearts, ultrastructural analysis revealed increased autophagic and lysosomal content indicative of increased autophagy. PQ83 hearts also showed evidence of necrotic death, including inflammatory cell content and sarcosomal permeability. The data confirm the hypothesis that PAO-forming peptides are toxic when they accumulate in cardiomyocytes and that cardiomyocyte-based PAO accumulation causes heart failure.

Methods

TG Mice
A hemagglutinin epitope tag (HA) was added at the carboxyl terminus of each PQ construct. Those cDNAs were placed behind the α-MyHC promoter and used to generate TG FVB/N mice expressing PQ19-HA or PQ83-HA. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)–approved germ-free barrier facility, and all experiments were approved by the Children’s Hospital Animal Review Board.

Echocardiography
Cardiac ultrasound was performed on isoflurane-anesthetized mice from 2 to 7 months of age with a VisualSonics Vevo 770 Imaging System (Toronto, Canada) with a 30-MHz transducer. Heart rate and body temperature were monitored continuously. Images of the left ventricle (LV) were collected along the parasternal short axis and stored on the system’s hard drive. Two-dimensional directed M-mode echocardiographic images were recorded to determine LV systolic function and expressed as shortening fraction. M-mode measurements of LV end-diastolic and end-systolic chamber size were calculated as follows: shortening fraction = (LVIDd − LVIDs)/LVIDd, where LVIDd indicates LV internal dimension during diastole and LVIDs, LV dimension during systole.

Transcript and Protein Analyses
Total ventricular RNA was isolated with TRI Reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer’s protocol. RNA dot blots hybridized with transcript-specific oligonucleotides were used to quantify TG expression. Levels of hypertrophic markers were measured by 5′-nuclease TaqMan assays (Applied Biosystems, Foster City, Calif) after cDNA synthesis. Total ventricular protein was isolated by homogenization in radioimmunoprecipitation assay (RIPA) buffer. SDS-PAGE and Western blots were run on solubile fractions. Aggregate filter assays were performed on RIPA-insoluble fractions and immunoblotted with anti-HA antibodies (Y-11, Santa Cruz Biotechnology, Santa Cruz, Calif, and 3F10, Roche, Indianapolis, Ind), as described previously.2 RIPA-insoluble fractions were further solubilized by treatment with 95% formic acid at 37°C for 1 hour and then were lyophilized, neutralized, and resuspended in Laemmli buffer for SDS-PAGE and immunoblot analyses. Immunoblots were performed with antibodies against total caspase-3, cleaved caspase-3, and anti-IRE1 (Cell Signaling Technologies Boston, Mass); anti-caspase-8 (BioVision, Inc, Mountain View, Calif); anti-Grp78 (BD Biosciences, San Jose, Calif); anti Xbp-1 (Biolegend, San Diego, Calif); anti-Atg5 (Cosmo Bio Co, Tokyo, Japan); anti-HA, anti-PARP, anti-Lamp1, anti-ATF6, cathepsin D (C-20), and anti-PERK (H-300; Santa Cruz Biotechnology); and anti-LC3 (Nanotools, Munich, Germany).

Immunohistochemistry
Immunohistochemical analyses were performed as described previously.14,18 Primary antibodies were purchased as follows: anti-HA, anti-Lamp1, and anti-cathepsin D (Santa Cruz Biotechnology); anti-PAO and A11 antibody (courtesy of C. Glabe, University of California, Irvine); anti-Cd45 (R&D Systems, Inc, Minneapolis, Minn); anti-mac-3 (BD Pharmingen, San Jose, Calif); and anti-desmin (Biomeda, Foster City, Calif), TO-PRO-3, phalloidin, and Alexa 488- or 568-conjugated antibodies were from Invitrogen (Carlsbad, Calif). Stained sections were examined by confocal microscopy (PCM 2000, Nikon, Melville, NY) and images captured with Simple PCI version 4 (Compix Inc, Sewickley, Pa). Cd45- and Mac-3–positive cells were quantified with MetaMorph 7 software (Molecular Devices, Downington, Pa) from >10 images per genotype.

Apoptosis Assays
To quantify apoptotic nuclei via immunofluorescence, an in situ cell death detection kit (Roche) was used for direct TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling) staining. Paraffin whole-heart sections (n = 3 per genotype) were deparaffinized and boiled in 0.1 mol/L sodium citrate buffer (pH 6.0) for 20 minutes. After antigen retrieval, sections underwent the labeling reaction with fluorescein-dUTP. Sections were counterstained with desmin and TO-PRO to detect myocytes and nuclei and visualized by confocal microscopy. To quantify DNA laddering due to apoptotic endonucleases, genomic DNA was isolated from 3 hearts per genotype. One microgram of DNA was ligated with adaptor primers overnight followed by polymerase chain reaction for 30 or 40 cycles. Amplified products were separated on a 2% TAE gel, along with positive and negative controls. The polymerase chain
reaction–based DNA laddering kit was purchased from Maxim Biotech (San Francisco, Calif).

Mitochondrial Swelling
Whole hearts were collected from 4.5-month-old mice (n=4 per genotype) to analyze mitochondrial swelling. Mitochondrial swelling assays were performed as described previously. 19

Evans Blue Staining
Evans Blue dye was diluted in sterile saline to 10 mg/mL and sterile-filtered. Isoflurane-anesthetized mice were given intraperitoneal injections of 5 μL of dye per gram of body weight. Six hours after injection, mice were anesthetized again, and hearts were perfused with cardioplegic buffer in 4% paraformaldehyde. After perfusion, hearts were drop-fixed in the same buffer for 1 hour and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Fixed hearts were bisected and incubated in 15% sucrose for 2 hours and then in 30% sucrose overnight at 4°C for cytoprotection. Hearts were mounted in OCT, cut into 5-μm cryosections, and visualized by fluorescence microscopy.

Statistical Analyses
Data are expressed as mean±SEM and analyzed with 1-way ANOVA, in which a significant difference was P<0.05, after a Tukey post hoc adjustment. Longitudinal echocardiographic data were analyzed with 2-way ANOVA with repeated measures. A Kaplan–Meier log rank test was used to assess significant differences in survival between genotypes.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results
To test the hypothesis that PAO expression in cardiomyocytes leads to cell dysfunction and heart failure, we generated TG mice with cardiomyocyte-specific expression of either PQ19 (as a negative control) or PQ83 under control of the α-MyHC promoter (Figure 1A). Both constructs contained an HA tag at the carboxyl terminus for identification. Several TG PQ19 founders were obtained, and the highest-expressing PQ19 line (∼9-fold relative to the PQ83 line used) was analyzed in parallel with the PQ83 mice. Six TG PQ83 founders were produced, but only a single TG founder line survived to breeding age and yielded TG progeny before reaching breeding age. The 5 other mice positive for the presence of the transgene all died of heart failure before reaching breeding age. PQ83 expression by RNA dot blot in the surviving line was barely measurable under normal conditions but could be detected if 5-fold the normal amount of RNA was applied to the membrane for hybridization analysis (Figure 1B). Neither PQ19 or PQ83 protein could be detected in the soluble cytoplasmic fraction with Western blots probed with an anti-HA antibody (Figure 1C). However, insoluble PQ83 peptide could be quantified on a filter-trap assay (Figure 1D), and formic acid solubilization of the aggregates enabled us to detect HA-positive protein in the PQ83 hearts (Figure 1E).

Histological analysis of PQ83 mice showed progressive cardiac dilation from 5 to 7 months with no morphological changes in PQ19 hearts relative to nontransgenic (NTG) animals (Figure 2A). Echocardiography revealed a significant loss in cardiac contractile function and chamber dilation in the PQ83 hearts by 7 months (Figure 2B). Cardiomyocyte loss, hypertrophy, and infiltrating inflammatory cells were all apparent as early as 3 months in PQ83 hearts, none of which were observed in either the PQ19 or NTG hearts (Figure 2C). Trichrome staining showed modest increases in fibrosis and interstitial space in the dilated PQ83 hearts (Figure 2D). Those hearts showed activation of hypertrophic markers at the molecular level (Figure 2E) and overt hypertrophy as measured by a significant increase in the heart weight/body weight ratios (Figure 2F).

Cardiac function was measured longitudinally by echocardiography (Tables 1 and 2). PQ83 hearts underwent a progressive loss of cardiac function between 4.5 and 7 months, whereas PQ19 and NTG hearts maintained normal function (Figure 3A). All PQ83 mice died between 5.5 and 8 months, whereas the PQ19 mice appeared functionally and anatomically normal in all respects and had normal life spans of ∼2 years with no heart failure apparent at 22 months (data not shown; Figure 3B). Thus, PQ83-expressing mice died due to diminished cardiac function and dilated cardiomyopathy.

We used immunohistochemistry to detect the presence of PAO within the cardiomyocytes. Although neither the NTG nor the PQ19 cardiomyocytes contained significant amounts

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Figure 1. TG expression of PQ83 and PQ19 in the mouse heart. A, Construct design. The mouse α-MyHC promoter was used to drive PQ peptides of 19 or 83 repeats, fused with an HA tag and flanked by a human growth hormone polyadenylation signal (hGH polyA). B, RNA dot blots of PQ19 and PQ83 transgene expression. Ventricular RNA was probed for hGH. GAPDH levels were used for normalization (n=6 per genotype). Note that to detect the low levels of transcript expressed in the PQ83 line, 10 μg of RNA as opposed to 2 μg for the PQ19 sample was used. C, Immunoblotting with an anti-HA antibody shows that the bulk of PQ83 protein is insoluble. The PQ19 polypeptides were not retained on the gel under the electrophoresis and transfer conditions used. D, Aggregate filter-trapped protein probed with anti-HA antibody shows that PQ83 protein is retained in the insoluble aggregates. E, Formic acid treatment of insoluble protein fractions revealed HA-specific bands for PQ83 samples, which confirmed protein expression despite the very low levels of transcript that were detected. M indicates a lane containing a protein mass marker.
of PAO. PAO-positive material accumulated in the PQ83 cardiomyocytes (Figure 4A) in the characteristic aggregates observed in other cardiomyopathic models with high levels of PAO.14 Immunohistochemistry confirmed that PQ83-expressing hearts generated HA-positive intracellular aggregates as early as 1 month of age (Figure 4B).

In our previously characterized CryAB<sup>R120G</sup> cardiomyopathic model, which is also characterized by high PAO levels, we noted activation of apoptosis in the cardiomyocytes.19 To determine whether this was a common characteristic for PAO-induced cardiomyocyte toxicity, we assayed for indices of apoptosis in the PQ83 hearts. We could not detect any increases in caspase-3, caspase-8, or poly(ADP-ribose) (PARP) activation in the PQ83 samples relative to PQ19 and NTG controls (Figure 5A), and polymerase chain reaction–based DNA laddering assays confirmed the absence of apoptosis in the PQ83 samples (Figure 5B). Cardiomyocyte-specific in situ nick end-labeling (TUNEL) staining showed no apoptotic activation in the PQ83 cardiomyocytes (Figure 5C). We concluded that apoptotic-based cell death is not necessarily a commonly shared pathway for PAO-based cardiomyocyte death but rather may be dependent on the specific primary pathogenic entity responsible for triggering cardiomyocyte PAO accumulation.

Table 1. M-Mode Measurements for Parasternal Short-Axis Protocol

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>IVS/d, mm</th>
<th>LVID/d, mm</th>
<th>LV PW/d, mm</th>
<th>IVS/s, mm</th>
<th>LVID/s, mm</th>
<th>LV PW/s, mm</th>
<th>%EF</th>
<th>%FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG</td>
<td>3.5</td>
<td>0.764±0.017</td>
<td>4.20±0.08</td>
<td>0.635±0.019</td>
<td>1.165±0.040</td>
<td>2.72±0.10</td>
<td>1.000±0.035</td>
<td>64.5±1.5</td>
</tr>
<tr>
<td>PQ19</td>
<td>3.5</td>
<td>0.743±0.026</td>
<td>4.12±0.12</td>
<td>0.633±0.025</td>
<td>1.113±0.051</td>
<td>2.62±0.09</td>
<td>0.965±0.030</td>
<td>66.1±1.1</td>
</tr>
<tr>
<td>PQ83</td>
<td>3.5</td>
<td>0.714±0.018</td>
<td>4.24±0.09</td>
<td>0.603±0.019</td>
<td>1.081±0.040</td>
<td>2.76±0.09</td>
<td>0.910±0.045</td>
<td>64.4±1.4</td>
</tr>
<tr>
<td>NTG</td>
<td>5</td>
<td>0.776±0.035</td>
<td>4.10±0.08</td>
<td>0.684±0.029</td>
<td>1.234±0.043</td>
<td>2.58±0.07</td>
<td>1.016±0.019</td>
<td>67.3±1.1</td>
</tr>
<tr>
<td>PQ19</td>
<td>5</td>
<td>0.767±0.058</td>
<td>4.25±0.11</td>
<td>0.707±0.018</td>
<td>1.182±0.091</td>
<td>2.67±0.03</td>
<td>1.037±0.029</td>
<td>67.1±1.3</td>
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<tr>
<td>PQ83</td>
<td>5</td>
<td>0.836±0.039</td>
<td>4.30±0.08</td>
<td>0.701±0.035</td>
<td>1.238±0.047</td>
<td>2.97±0.12*</td>
<td>0.994±0.036</td>
<td>58.6±2.8*</td>
</tr>
<tr>
<td>NTG</td>
<td>6.5</td>
<td>0.801±0.037</td>
<td>4.11±0.08</td>
<td>0.650±0.016</td>
<td>1.189±0.054</td>
<td>2.58±0.07</td>
<td>1.004±0.016</td>
<td>67.6±1.3</td>
</tr>
<tr>
<td>PQ19</td>
<td>6.5</td>
<td>0.766±0.032</td>
<td>4.09±0.12</td>
<td>0.616±0.028</td>
<td>1.206±0.017</td>
<td>2.44±0.19</td>
<td>0.986±0.047</td>
<td>71.3±3.9</td>
</tr>
<tr>
<td>PQ83</td>
<td>6.5</td>
<td>0.706±0.033</td>
<td>4.89±0.15†‡§</td>
<td>0.530±0.019†‡§</td>
<td>1.045±0.046§</td>
<td>3.98±0.17†‡§</td>
<td>0.666±0.032†‡§</td>
<td>37.3±5.2†‡§</td>
</tr>
</tbody>
</table>

IVS/d indicates interventricular septal thickness during diastole; LVID/d, LV internal dimension during diastole; LV PW/d, posterior wall thickness during diastole; IVS/s, interventricular septal thickness during systole; LVID/s, LV dimension during systole; LV PW/s, posterior wall thickness during systole; %EF, ejection fraction; and %FS, fractional shortening.

M-mode measurements of the LV were taken from a parasternal long-axis view. Data are presented as mean±SEM.

*P<0.05 vs NTG, †P<0.05 vs PQ19, ‡P<0.05 vs 3.5-month-old PQ83, and §P<0.05 vs 5-month-old PQ83 by 2-way ANOVA with repeated measures.
The CryABR120G model of cardiomyopathy previously was shown to have impaired mitochondrial swelling.19 Neither the PQ19 or PQ83 heart mitochondria demonstrated any swelling defect (Figure 5D). Elevated markers of endoplasmic reticulum stress have also been noted in some aggregate-forming diseases,20,21 but multiple markers of the endoplasmic reticulum stress pathway were not activated as heart failure developed in the PQ83 hearts (Figure 5E and 5F).

With apoptosis ruled out as a primary cause of cardiomyocyte death, we examined other potential death pathways. Ultrastructural analyses revealed that PQ83 hearts had an increased number of small mitochondria. The cardiomyocytes showed anatomic hallmarks of autophagy, with autophagic vesicles, multilamellar bodies, and lysosomes (Figure 6A). The PQ83 aggresomes frequently could be observed being engulfed by autophagosomes, which further suggests an autophagic means of degradation. Lysosomal markers cathepsin D and LAMP1 were increased significantly in failing PQ83 hearts (Figure 6B and 6C). The active forms of cathepsin D, a lysosomal proteolytic enzyme, have molecular weights of ~44 and ~33 kDa,22 and the ~44-kDa species was increased 2.8-fold in the 7-month-old PQ83 hearts relative to NTG or PQ19 (Figure 6B and 6C). LC3-II, a protein that integrates into autophagosomal membranes, was also significantly elevated (2.4-fold) in the 7-month-old PQ83 hearts relative to NTG samples (P<0.05); however, other autophagic markers, such as Atg5, were unchanged. Immunohistochemical analyses showed high levels of cathepsin D staining in degenerating PQ83 cardiomyocytes, consistent with lysosomal permeabilization, and elevation of LAMP1 staining was apparent (Figure 6D). On the basis of the above data, and particularly the ultrastructural identification of autophagosomes, we conclude that PQ83-mediated PAO degradation.

A unique characteristic of necrotic death is the infiltration of inflammatory cells. The infiltrating cells apparent in the histological analyses of the PQ83 hearts were confirmed to be leukocytes and macrophages, by use of antibodies against CD45 and Mac-3, respectively. Increased staining for both types of inflammatory markers was evident in PQ83 hearts compared with PQ19 and NTG controls (Figure 7A and 7B). PQ83 hearts had significantly more CD45-positive cells per view (241 ± 18 versus 122 ± 18 and 121 ± 6, respectively) than PQ19 and NTG hearts (P<0.001). Mac-3–positive cells were also increased significantly (P<0.05) in PQ83 hearts relative to NTG (55 ± 5 versus 35 ± 4 cells per view) but did not differ from PQ19 (48 ± 5 cells per view). In addition to increased infiltration of inflammatory cells (Figure 7), another early feature of necrotic cell death is the loss of plasma membrane integrity. To assess cardiac sarcolemmal integrity, adult mice were injected with Evans blue dye 6 hours before death. The dye is taken up by cardiomyocytes with leaky sarcolemmal

### Table 2. B-Mode Measurements for Endocardial Protocol

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>Endocardial Area D, mm²</th>
<th>Endocardial Area S, mm²</th>
<th>Endocardial FAC, %</th>
<th>Endocardial Area Change, mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTG 3.5</td>
<td>12.4 ± 0.3</td>
<td>7.89 ± 0.42</td>
<td>49.2 ± 1.7</td>
<td>7.80 ± 0.34</td>
</tr>
<tr>
<td>PQ19 3.5</td>
<td>12.3 ± 0.4</td>
<td>7.71 ± 0.73</td>
<td>46.4 ± 1.4</td>
<td>6.87 ± 0.43</td>
</tr>
<tr>
<td>PQ83 3.5</td>
<td>12.4 ± 0.4</td>
<td>7.71 ± 0.49</td>
<td>45.8 ± 1.6</td>
<td>6.95 ± 0.33</td>
</tr>
<tr>
<td>NTG 5</td>
<td>14.8 ± 0.45</td>
<td>7.53 ± 0.45</td>
<td>49.3 ± 1.9</td>
<td>7.28 ± 0.28</td>
</tr>
<tr>
<td>PQ19 5</td>
<td>14.4 ± 0.7</td>
<td>7.13 ± 0.53</td>
<td>50.6 ± 2.5</td>
<td>7.28 ± 0.45</td>
</tr>
<tr>
<td>PQ83 5</td>
<td>16.0 ± 0.9</td>
<td>9.50 ± 0.79†</td>
<td>41.3 ± 1.8†</td>
<td>8.68 ± 2.22</td>
</tr>
<tr>
<td>NTG 6.5</td>
<td>13.8 ± 0.8</td>
<td>6.90 ± 0.64</td>
<td>50.6 ± 2.0</td>
<td>6.94 ± 0.26</td>
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<tr>
<td>PQ19 6.5</td>
<td>11.6 ± 1.0</td>
<td>5.43 ± 0.60</td>
<td>52.1 ± 4.1</td>
<td>6.39 ± 0.64</td>
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<tr>
<td>PQ83 6.5</td>
<td>19.8 ± 0.9‡§</td>
<td>14.60 ± 0.82‡§</td>
<td>26.2 ± 2.7‡§</td>
<td>5.21 ± 0.56‡§</td>
</tr>
</tbody>
</table>

D indicates during diastole; S, during systole; and FAC, fractional area change. B-mode measurements were made to increase resolution of cardiac structures.

*P<0.05 vs NTG, †P<0.05 vs PQ19, ‡P<0.05 vs 3.5-month-old PQ83, and §P<0.05 vs 5-month-old PQ83 by 2-way ANOVA with repeated measures.

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A unique characteristic of necrotic death is the infiltration of inflammatory cells. The infiltrating cells apparent in the histological analyses of the PQ83 hearts were confirmed to be leukocytes and macrophages, by use of antibodies against CD45 and Mac-3, respectively. Increased staining for both types of inflammatory markers was evident in PQ83 hearts compared with PQ19 and NTG controls (Figure 7A and 7B). PQ83 hearts had significantly more CD45-positive cells per view (241 ± 18 versus 122 ± 18 and 121 ± 6, respectively) than PQ19 and NTG hearts (P<0.001). Mac-3–positive cells were also increased significantly (P<0.05) in PQ83 hearts relative to NTG (55 ± 5 versus 35 ± 4 cells per view) but did not differ from PQ19 (48 ± 5 cells per view). In addition to increased infiltration of inflammatory cells (Figure 7), another early feature of necrotic cell death is the loss of plasma membrane integrity. To assess cardiac sarcolemmal integrity, adult mice were injected with Evans blue dye 6 hours before death. The dye is taken up by cardiomyocytes with leaky sarcolemmal
of the mutant CryAB.13,14,18 The PQ83 model represents a heart failure as a result of cardiomyocyte-specific expression of the related gene that precede visible plaque accretion rather than extracellular accumulation of amyloid plaques. Although the inherent toxicity of the amyloid-containing aggregates has been assumed in a wide range of human conditions such as light-chain amyloidosis, the spongiform encephalopathies, Alzheimer’s disease, Parkinson’s disease, and others,24 it is now generally thought that amyloid plaques are a consequence of a long pathogenic process, representing “tombstones” rather than being directly cytotoxic.9,25 Our recent studies show a strong correlation between PAO levels in cardiomyocytes and cardiac dysfunction in both mice and humans13,14 and confirm that PAO-induced toxicity and pathology are not restricted to neurodegenerative conditions but can also cause cardiomyocyte-based pathogenesis and lead to heart failure. Both previous and current TG mouse models confirm the hypothesis that PAO expression and accumulation leads to cardiomyocyte loss that results in heart failure.

Cardiomyocyte-specific expression of CryABR120G led to high intracellular PAO levels and subsequent activation of apoptosis.19 Similarly, the PQ83 data support the idea that PAO-forming proteins can induce toxicity in multiple cell types but illustrate that not all PAOs activate apoptosis as a mode of cell death. Many proteins are potentially amyloidogenic9,26 and therefore are potentially pathogenic if the protein accumulates as oligomeric amyloidogenic intermediates and is not cleared by the normal degradation pathways. Proteasome-based degradation and recycling via autophagy and lysosomal digestion represent 2 distinct pathways for clearance of misfolded proteins. The narrow barrel structure of the proteasome precludes the entry of large protein oligomers or aggregates for degradation, and thus, these substrates must necessarily be processed via autophagic/lysosomal degradation.27 Autophagy is the catabolic process whereby cellular contents are sequestered by double-membrane vacuoles, termed autophagosomes, and delivered to the lysosomes for degradation. Autophagy can function as a survival mechanism in starving cells, but under certain circumstances, it also serves as a form of programmed cell death, and dysregulated or upregulated autophagy can contribute materially to pathogenic processes in a variety of cell types under stress, including the heart.28 We observed increased autophagic and lysosomal content in PQ83 hearts; however, whether it is beneficial or maladaptive in this system is presently unclear, and further studies will be needed to determine whether autophagy contributes to PAO-induced cardiomyocyte toxicity and death.

Although upregulation or dysregulation of autophagy in the cardiomyocytes may be potentially pathogenic, particularly when the heart is stressed, autophagy appears to be essential for basal cardiac function. When the autophagy-related gene atg5 was ablated in mouse cardiomyocytes, the cells exhibited decreased autophagy but hypertrophied rap-
idly, and the animals developed severe heart failure, which illustrates the essential nature of this process in conserving cardiomyocyte function and homeostasis. Although the exact mechanism of PAO toxicity remains unclear, several PAOs have been shown to create pores in cellular membranes, thereby causing a loss in membrane permeability. The present data are consistent with this primary pathogenic mechanism, and PQ83-expressing hearts show increased sarcolemmal permeability.

Polyglutamine PAO expression in cardiomyocytes is clearly toxic and leads to increased sarcolemmal permeability, inflammation, and cell death via autophagy and necrosis. Although autophagy and necrosis have largely been studied as independent pathways of death, recent data have implicated autophagy in necrotic cell death. Consistent with the present observations in the PQ83 model, a study in Caenorhabditis elegans demonstrated a marked increase in autophagosomal formation early during necrotic processes and showed that both genetic and pharmacological inhibition of autophagy effectively suppressed necrotic cell death. Further work will be needed to dissect the relationships between autophagy and lysosomal proteolytic mechanisms as

Figure 5. Cell death in PQ83 cardiomyocytes is not due to apoptosis, mitochondrial swelling, or endoplasmic reticulum stress. A, Immunoblots against total and cleaved caspase-3, caspase-8, and total and cleaved PARP. GAPDH was used as a loading control. The positive control (+) consisted of HeLa cells treated with staurosporine (n=4 per genotype). B, Polymerase chain reaction–based DNA laddering shows no evidence of apoptotic laddering in PQ83 hearts after 30 amplification cycles. Extended amplification (40 cycles) was also negative (data not shown). M indicates a lane containing a protein mass marker. C, TUNEL staining shows no evidence of apoptotic activation in PQ83 cardiomyocytes. The rare TUNEL-positive cells are denoted (arrows). D, Mitochondrial swelling assays show no impairment in PQ83 samples; hearts were assayed at 4.5 months of age (n=4 per genotype). E, Immunoblots detecting markers of endoplasmic reticulum stress failed to show increases in protein levels in the PQ83 samples (n=4 per genotype). I/R indicates ischemia/reperfusion. F, Bar graphs of endoplasmic reticulum stress protein densitometry normalized to GAPDH levels. *Significant difference vs NTG (P<0.05), †significant difference vs PQ19 (P<0.05) within an age group by 2-way ANOVAs.
contributors to necrotic death within the general context of cardiomyocyte loss and heart failure. The data in the present study underscore the importance of understanding the mechanism(s) by which intracellular PAOs cause cardiomyocyte death. Considering the high levels of PAO that we have detected in human hearts that failed for a variety of reasons, further study of these toxic entities in cardiomyocyte-based models and human heart failure is warranted.

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Disclosures
None.

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**CLINICAL PERSPECTIVE**

We have previously published evidence that preamyloid oligomers, the substances thought to be toxic in a variety of neurodegenerative disorders, are generated in certain mouse models of cardiac disease and can also be found in cardiomyocytes derived from the failing human myocardium. This study tested the hypothesis that preamyloid oligomers can cause cardiomyocyte death that leads to heart failure. A preamyloidogenic protein comprising only glutamine was ectopically expressed in the mouse heart with transgenesis. Very low levels of this protein led to cardiomyocyte death and heart failure in young adult mice. Future work will be directed at understanding the role of preamyloid oligomer accumulation in human heart disease and failure.
Cardiomyocyte Expression of a Polyglutamine Preamyloid Oligomer Causes Heart Failure
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