Reversal of amyloid-induced heart disease in desmin-related cardiomyopathy


*Division of Molecular Cardiovascular Biology and ¹Howard Hughes Medical Institute, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH 45229-3039; and ²Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697-3900

Amyloid oligomers, similar to the toxic entities found in Alzheimer’s disease patients and in other amyloid-based diseases, are present in cardiomyocytes derived from human heart-failure patients and in animal models of desmin-related cardiomyopathy (DRM). The R120G mutation in α-B-crystallin (CryAB) causes DRM and is characterized by aggresomes containing CryAB¹₂₀G and amyloid oligomer. In this study, we show that aggresome levels do not correlate with disease. Blocking aggresome formation results in increased levels of toxic amyloid oligomer and decreased cardiomyocyte viability. We confirmed the primary toxicity of intrasarcoplasmic amyloid accumulation in the cardiomyocytes by ectopic expression of the amyloidoogenic peptide PQ81, which consists of multiple repeats of a polyglutamine tract. We then addressed the issue of disease reversibility by placing CryAB¹₂₀G under inducible cardiomyocyte-specific expression in transgenic mice. The mice developed aggresomes and contained high concentrations of amyloid oligomer in the heart, resulting in cardiac disease. Cessation of CryAB¹₂₀G expression in symptomatic mice improved cardiac function and rescued all of the animals from premature death. Rescue was accompanied by significant decreases in amyloid oligomer without a significant reduction in aggresomes. Blocking cardiac amyloid oligomer formation, even after cardiac dysfunction presents, may be a therapeutic strategy in DRM as well as in other types of cardiac disease in which significant amyloid accumulation occurs.

Many systemic and neurodegenerative disorders whose etiologies are linked to misfolded or unfolded proteins are characterized by the accumulation of intracellular or extracellular protein deposits or aggregates known as aggresomes (1–3). Although the pathologies of these diseases are complex, the accumulation of the misfolded and unfolded proteins in the cells is causal for at least some of these neurodegenerative and systemic diseases (4). Some specific mechanisms have been defined, linking protein-misfolding to decreases in proteasome system activity and induction of reactive oxygen species, thus compromising mitochondrial activity (3). Alterations in the metabolism of the amyloid-β (Aβ) precursor protein are associated with altered mitochondrial function (5), and Aβ interacts directly with the Aβ-binding alcohol dehydrogenase mitochondrial protein (6). However, the direct relationship between protein deposition and disease pathology remains controversial. Although protein deposition is associated with neurodegenerative diseases such as Huntington’s disease and Alzheimer’s disease (7–10), there are numerous reports that indicate no correlation or even a negative correlation between deposition and pathology (11–13).

Although a relationship between the age-related neurodegenerative diseases and the accumulation of misfolded proteins as amyloid fibrils is recognized, the actual toxic entity remains obscure. In Huntington’s disease, expansion of the polyglutamine (PQ) tract results in large accumulations of aggregates containing these peptides. In Alzheimer’s disease, Aβ-peptide plaques and misfolded τ-neurofibrillary tangles are found, whereas Parkinson’s disease is characterized by α-synuclein accumulation. Although these and other neurodegenerative diseases involve the accumulation of disparate proteins, they are linked by the accumulation of abnormal aggregates containing a β-sheet structure. These β-sheet aggregates presumably form because of the protein(s) intrinsic mutation or because of alterations in correct processing, and the assembly pathways themselves have recently become an object of investigation as the prefibrillar intermediates have come under suspicion of being intrinsically toxic (14). Recent reports suggest that Aβ protein and other amyloidogenic proteins exert their cellular toxicity as soluble amyloidoligomeric intermediates (amyloid oligomer) but not as insoluble aggregates or fibrils (15–18). For example, when the small soluble prefibrillar form of Aβ was added to organotypic mouse-brain slice cultures, they acted as potent neurotoxins (19). Soluble Aβ-amyloid oligomers also are found in the cerebrospinal fluid of Alzheimer’s disease patients (20), with the soluble Aβ content of the human brain being a better indicator of disease severity than the content of plaques (21, 22). The cellular toxicity of soluble amyloid induced by the mutated huntingtin protein, which contains an expanded PQ repeat, also has been linked to the pathogenic cascade of Huntington’s disease. Microscopic analysis showed that inclusion-body formation induced by mutant huntingtin was a predictor for improved survival and led to decreased soluble mutant huntingtin levels in neurons, suggesting that inclusion-body formation is actually protective against the toxic mutant protein (23). These results indicate that the soluble amyloid oligomer may be more important in pathogenesis than the insoluble fibrillar amyloid deposits. An antibody that specifically recognizes a structure of the amyloid oligomer reacts with oligomers generated from all types of amyloidogenic proteins and peptides, such as Aβ, α-synuclein, PQ, and prions (18). This result implies that the amyloid oligomer has a shared structure between diverse proteins and also may share a common mechanism of pathogenic action.

The R120G missense mutation in the small heat-shock protein α-B-crystallin (CryAB) causes desmin-related cardiomyopathy (DRM) (24). DRM, which is characterized by the formation of aggregates containing CryAB and desmin, can be recapitulated in transgenic (TG) mice by expression of the mutant protein specifically in the heart (25). We showed that CryAB¹₂₀G led to perinuclear aggresome formation and that these aggresomes...
contained amyloid oligomer, suggesting that CryAB-DRM is a subclass of the aggresomal and amyloid-related diseases (26). These mice invariably developed heart failure and exhibited 100% mortality by 5–7 months. Somewhat surprisingly, the amyloid oligomer also was present in cardiomyocytes derived from multiple human dilated and hypertrophic cardiomyopathies, indicating that intrasarcoplasmic amyloidosis might be a prevalent contributor to cardiac disease (26).

In this study, we characterize the molecular basis of the cardiac pathology by analyzing the relationships between aggresomes, oligomeric amyloid formation, cardiac function, and survival in CryABR120G/DRM in vitro as well as in an inducible TG mouse model in which CryABR120G can be reversibly expressed or suppressed specifically in cardiomyocytes. Inducible CryABR120G expression led to the accumulation of CryAB-positive aggregates that contained the amyloid oligomer, with the mice showing cardiac dysfunction and premature death by heart failure. Deinduction of CryABR120G expression in symptomatic mice =2–3 weeks before death had no significant effect on aggresome levels. However, cessation of CryABR120G expression resulted in improved cardiac function, prevention of death, and decreases in cardiomyocyte amyloid.

Materials and Methods

TG Mice. Male FVB/N mice with cardiac-specific overexpression of CryABWT or CryABR120G driven by the α-myosin heavy-chain promoter were described in ref. 25. To generate mice with cardiac-specific inducible overexpression of CryABR120G, the CryABR120G cDNA was inserted into a modified myosin-promoter cassette as described in ref. 27. The responder CryABR120G mice were crossed with tetracycline-controlled transcriptional activator (tTA) mice to generate the tTA/CryABR120G double-TG mice.

Cardiomyocyte Cultures and Adenovirus Infection. After isolation of rat neonatal cardiomyocytes (RNCs), cells were grown in two-well chambered glass slides coated with gelatin. Replication-deficient recombinant adenoviruses were made by using the AdEasy system (Stratagene) (28). cDNAs were isolated by using RT-PCR and were used to generate adenoviral constructs as described in ref. 26. Constructs containing the expanded PQ fragment were provided by James R. Burke (Duke University, Durham, NC). A hemagglutinin epitope (HA) at the carboxyl terminus of the PQ was added, and the DNA was used to generate adenovirus constructs (29). The cells were normally transfected at a multiplicity of infection of 10 for each virus, except where indicated. To distinguish the TG products from the endogenous CryAB protein, a FLAG-epitope was introduced at the N termini of CryABWT and CryABR120G. Cotransfection of CryABWT led to equal levels of expression but pathogenic in others. We explored the potential cytotoxicity of the aggresomes by using RNCs. Amyloid oligomer was undetectable in untreated cardiomyocytes and barely detectable in CryABWT-transfected myocytes (Fig. 1A). In contrast with these data, when CryABR120G was expressed at equivalent levels (Fig. 1B Upper) in the transfected cardiomyocytes, perinuclear aggresomes were apparent, and these were stained intensely for both CryAB and amyloid oligomer (Fig. 1A). To determine whether increasing the level of chaperone-competent CryAB decreased aggresome formation, we performed a series of cotransfections with CryABWT and CryABR120G. Cotransfection of CryABR120G with CryABWT led to equal levels of expression (Fig. 1B) and resulted in a diffuse pattern of staining in which large, perinuclear CryAB-positive aggregates were reduced. However, the amyloid oligomer remained distributed throughout the cytoplasm with overall levels increased (Fig. 1A, D, and E). Cotransfection and expression of CryABWT led to a significant decrease in aggresomal accumulation (Fig. 1B and C). Quantitation of amyloid accumulation showed that the fraction of cytoplasm occupied by amyloid oligomer-positive material more than doubled, with a striking increase in the signal intensity (Fig. 1 D and E). Strikingly, decreased aggresomal formation and enhanced amyloid oligomer formation led to a significant increase in cytotoxicity (Fig. 1F). The apparent decrease in aggresomal accumulation, accompanied by decreased cell viability, implies that aggresome accumulation may be cytotoxic by lowering levels of the toxic amyloid oligomer in cardiomyocytes.

Supporting the commonality of the toxic oligomer hypothesis for amyloid-based disease is the observation that several distinct amyloidogenic proteins, including CryABR120G, α-synuclein, and PoQ129 (30) expansion proteins, share an epitope that is recognized by an oligomer-specific antibody (18, 32). To understand whether the cardiomyocyte CryABR120G data could be extended to other amyloidogenic proteins, we analyzed the consequences of expressing a peptide containing 81 repeats of glutamine (PoQ18), which is known to cause Huntington’s disease (33). A nonamyloidogenic peptide containing PoQ19 was used as a negative control. Cardiomyocytes transfected with PoQ19 showed PoQ-positive aggresomes in the cytoplasm, whereas no aggre-
CryAB were achieved by means of cotransfection with CryA-
typical nontransfected and transfected RNCs. Expression of CryABWT resulted
vs. LacZ-transfected myocytes (LacZ).

A FLAG tag to distinguish the TG from the endogenous crystallins. (B) Cardiomyocyte cultures. (C) Chaperone expression and amyloid accumulation. (A) Expression of CryABR120G and CryABWT, each of which is FLAG-tagged and migrates more slowly in PAGE, are expressed at equivalent levels in the respective transfected cardiomyocyte cultures. (B Lower) The quantitation of the aggresomes by using a filter-based assay (Materials and Methods) and subsequent detection of CryAB-positive material by using Western blot analysis. The histogram is shown in C. *P < 0.001 vs. WT; **P < 0.001 vs. CryABR120G-transfected myocytes. (D and E) Quantitative analysis of the amyloid oligomer. Average amyloid oligomer intensity (D) and intensity distribution (E) were determined by using METAMORPH software (version 6.3). (F) Cellularity of CryABR120G. Cellularity toxicity was determined by the release of adenylate kinase into the medium. Values shown are the -fold increase relative to uninfected cardiomyocyte cultures, whose value was set to 1. **P < 0.001 vs. control; #P < 0.05 vs. PQ81.

Reversibility of Amyloidosis in Vivo. We have modeled CryABR120G
DRM in vivo by using cardiac-specific transgenesis (25, 26). CryABR120G expression leads to the formation of protoaggres-
somes and amyloid accumulation within about 5 days. As the mice age, aggresomes and amyloid oligomer levels increase, and the mice die of heart failure between 5 and 7 months. We wished to determine whether amyloid formation was reversible and created a cardiac-specific inducible model of CryABR120G DRM by using the tTA system ("tet-off") and inducible α-myosin heavy-chain promoter construct (27). The biganic mice showed tight regulation of CryABR120G, with no detectable expression in the presence of doxycycline (DOX) and expression at or above the levels observed for a standard TG line (line 134) when the drug was removed (Fig. 4A). After 6 weeks, atrial natriuretic factor, a sensitive stress marker in the adult heart, is significantly elevated in both the standard and induced TG lines. DOX treatment reversibly shut down transgene expression and largely attenuated atrial natriuretic factor up-regulation (Fig. 4A).

Mice were allowed to express CryABR120G, and cardiac func-

somess were observed in myocytes transfected with PQ19 (Fig.
24). Again, when increased levels of chaperone-competent
CryAB were achieved by means of cotransfection with CryA-

Fig. 1. Chaperone expression and amyloid accumulation. (A) Shown are typical nontransfected and transfected RNCs. Expression of CryABWT resulted in increased amyloid oligomer accumulation relative to the cells transfected with CryA-R120G alone. Nuclei (blue) were stained with TO-PRO-3. All images shown were captured at equivalent gains and within the linear response range. Control transfections, in which cardiomyocytes were infected with
CryABWT at double the normal multiplicity of infection, showed that increased amyloid levels were not due merely to a double dose of CryAB virus and subsequent overexpression of normal protein, because those cultures (2WT) showed no amyloid accumulation. (B and C) Expression of CryABR120G and CryABWT and quantitation of aggresome levels. Constructs were labeled with a FLAG tag to distinguish the TG from the endogenous crystallins. (B upper) CryABWT and CryABWT, each of which is FLAG-tagged and migrates more slowly in PAGE, are expressed at equivalent levels in the respective transfected cardiomyocyte cultures. (B lower) The quantitation of the aggresomes by using a filter-based assay (Materials and Methods) and subsequent detection of CryAB-positive material by using Western blot analysis. The histogram is shown in C. *P < 0.001 vs. WT; **P < 0.001 vs. CryABR120G-transfected myocytes. (D and E) Quantitative analysis of the amyloid oligomer. Average amyloid oligomer intensity (D) and intensity distribution (E) were determined by using METAMORPH software (version 6.3). (F) Cellularity of CryABR120G. Cellular toxicity was determined by the release of adenylate kinase into the medium. Values shown are the -fold increase relative to uninfected cardiomyocyte cultures, whose value was arbitrarily set to 1. *P < 0.05; **P < 0.01, vs. LacZ-transfected myocytes (LacZ2).

results were observed in cardiomyocytes transfected with PQ19 (Fig.
2). Again, when increased levels of chaperone-competent
CryAB were achieved by means of cotransfection with CryA-

Fig. 2. PQ repeats lead to aggresome formation in cardiomyocytes. (A) Ectopic expression of an amyloidogenic protein leads to inclusion-body formation in RNCs. RNCs were transfected with an adenovirus containing sequence encoding PQ81 tagged with HA (PQ81-HA). No aggresome accumula-
tion was observed in cardiomyocytes transfected with PQ19-HA. Cotransfection with CryABWT (CryAB) prevented PQ81-positive aggregate formation. PQ-HA (green) and cTnI (red) identified the cardiomyocytes. (B) Quantitative analysis of the PQ-HA-positive aggregates. Aggregate levels were measured by using the filtration assay and were detected with HA antibody. Cotransfection with CryAB (CryAB) reduced the PQ-positive aggregates. (C) Histogram of data in B. Values shown are the -fold increase relative to uninfected cardiomyocyte cultures, whose value was set to 1. ***P < 0.001 vs. control; #P < 0.05 vs. PQ81.
characteristic of the standard CryABR120G line, including failure between 3.5 and 4 months, with visible signs of heart failure which the transgene was not inactivated went into acute failure the left ventricular weight DOX was 3.2 weight and biochemical analyses were performed. The left ventricular distress, ascites fluid, or pulmonary congestion.

survived until killed 1–3 months later, with no visible signs of cannot be observed in the cultures that were cotransfected with CryABWT gates that formed as a result of PQ81 expression. Although visible aggregates were determined by TnI staining (green). Amyloid oligomer (red) was observed in aggregates in PQ81-transfected myocytes (R120G). CTL, untreated cardiomyocytes. Cardiomyocytes were identified by TnI staining (green). Amyloid oligomer (red) was observed in aggregates in PQ81-transfected myocytes (R120G). CTL, untreated cardiomyocytes.

The mice were killed at 15–16 weeks, and immunohistology and biochemical analyses were performed. The left ventricular weight/body weight of non-TG (NTG) mice with or without DOX was 3.2 ± 0.02 mg/g and 3.1 ± 0.01 mg/g, respectively, and the left ventricular weight/body weight of the double-TG mice with or without DOX was 4.5 ± 0.04 mg/g and 4.4 ± 0.06 mg/g, respectively. The double-TG mice showed cardiac hypertrophy that was not completely attenuated by DOX treatment, even though normal shortening fractions were recovered. The double-TG mouse hearts contained massive accumulations of CryAB-positive aggresomes in the cardiomyocytes (Fig. 5 A and B). DOX treatment started at 12 weeks did not have an effect on aggresome levels (Fig. 5 C and D). In contrast with the aggresomes, levels of the amyloid oligomer were dramatically reduced in the animals that were treated at 12 weeks after significant amyloid accumulations had developed (Fig. 6). The in vivo data are consistent with the lack of cytotoxicity for the aggresomes and the correspondence of disease with elevated amyloid oligomer levels.

Discussion
A primary goal of this study was to understand whether cardiomyocyte aggresome accumulation was intrinsically pathogenic.

Fig. 3. Amyloid accumulation in PQ-induced cardiomyocyte amyloidosis. (A) Amyloid oligomer in PQ81-transfected myocytes. Cardiomyocytes were identified by TnI staining (green). Amyloid oligomer (red) was observed in aggregates that formed as a result of PQ81 expression. Although visible aggregates were determined by TnI staining (green). Amyloid oligomer (red) was observed in aggregates in PQ81-transfected myocytes (R120G). CTL, untreated cardiomyocytes. Cardiomyocytes were identified by TnI staining (green). Amyloid oligomer (red) was observed in aggregates in PQ81-transfected myocytes (R120G). CTL, untreated cardiomyocytes.

Fig. 4. Inducible CryABR120G TG mice. (A) Expression pattern of CryAB in the single- and double-TG hearts with (+) or without (−) DOX. For comparison, a TG mouse, in which CryABR120G is expressed in the heart by using the mouse &-myosin promoter (line 134, ref. 25), showed the expected increases in expression of CryAB and atrial natriuretic factor (ANF) that accompanied a hypertrophic response. DOX treatment reduced CryABR120G expression. (B) Shortening fractions of non-TG (NTG) and double-TG mice. NTG and tTA/R120G double-TG animals were aged for 2 months without DOX and initial functional measurements using echocardiography were made. At 2.5 months, the NTG and double-TG cohorts were each split, and half were treated with DOX. n = 5–20. (C) Survival curves. DOX treatment began at 2.5 months for a period of 1 month in the tTA/CryABR120G double-TG mice, effectively blocking transgene expression. +, P < 0.05 vs. NTG; #, P < 0.05 vs. tTA/CryABR120G double-TG mice.
CryAB WT would delay the onset of the disease (35). Our data are consistent with the hypothesis that a common structure of soluble nonfibrillar intermediates exists for different amyloidogenic molecules, resulting in common pathogenic mechanisms for widely varying primary etiologies (18, 26).

In our system, CryAB is not cytoprotective when the cardiomyocyte is challenged with either CryAB-R120G or PQ81, although it did prevent inclusion-body formation. The data suggest that, in the isolated cardiomyocyte, at least some chaperones can enhance amyloid oligomer levels by inhibiting aggregate formation. In contrast with these data, a number of in vitro studies showed that overexpression of heat-shock protein family members significantly attenuated Aβ-, PQ-, and α-synuclein-mediated toxicity (3), indicating that chaperone expression was cytoprotective against neurodegeneration. In fact, a number of genetic studies in animal models of human disease indicate that molecular chaperones are potent suppressors of neurodegeneration (36–38). The mechanism or mechanisms have not been defined, but a reasonable hypothesis is that the chaperone might decrease initial formation of the soluble but misfolded protein supramolecular structures that go on to develop amyloid-like characteristics. In fact, treatment of animal models of Parkinson’s disease, amyotrophic lateral sclerosis, or surgically induced...
neuronal degeneration, either with small molecules that induce heat-shock protein synthesis or with heat-shock proteins directly, can be neuroprotective and has been proposed as a treatment in some patients suffering from these diseases (3, 36). We think it likely that experimental outcome is dependent on the class of heat-shock protein expressed as well as the cell type. For example, in cell culture, rather than protecting neurons from Aβ1–40 toxicity, CryAB expression actually increased toxicity (39). With respect to cardiac disease, it appears that CryAB expression, while ablating fibril or aggresome formation, promotes the formation of the toxic oligomer, at least in the cardiomyocyte cultures.

It is well established for the neurodegenerative diseases that aberrant protein-folding can result in intracellular or extracellular aggregate accumulations and subsequent amyloid formation (3). The R120G mutation significantly impacts on CryAB’s ability to act as a chaperone (35), thus partially explaining the formation of large aggregates. Our data show that preventing aggresome formation in cardiomyocyte cultures by increased chaperone expression leads to increased levels of amyloid oligomer and increased cell death. These data, although not proving that cardiomyocyte aggresomes are cytoprotective, are consistent with the role of inclusion bodies in Huntington’s disease, in which their formation rendered the neuron more likely to survive (23).

Reversibility of CryABR120G Cardiac Disease. Aggregate formation by unfolded or misfolded proteins has been thought of as one of the typical pathologies in many degenerative diseases (3, 4). As previously shown by using the α-myosin heavy-chain promoter to drive cardiac-specific expression of CryABR120G, CryABR120G accumulation recapitulates DRM pathology, resulting in eventual heart failure and death. In the studies outlined above, it became apparent that amyloid oligomer levels were correlated with increased cytotoxicity. We, therefore, examined whether, in vivo after heart disease presented, amyloid levels could be decreased, and whether those decreases correlated with functional recovery. Abrogation of CryABR120G expression only a short time before death had a striking effect on preventing mortality: mouse viability was invariably linked to decreased amyloid oligomer levels, with little or no decrease in the aggresomes. Because we have determined that amyloid formation in cardiomyocytes appears to be common in heart failure patients (26), our experiments underscore the potential feasibility of a partial reversal of amyloid-induced pathology in many cardiovascular diseases if the endogenous clearance pathways that are present in the cardiomyocyte can be defined and regulated, or if effective pharmacologic means for amyloid clearance can be found.

This work was supported by National Institutes of Health Grants HL69779, HL65670, HL017428, HH61668, and HL52318 (to J.R.); a Howard Hughes Medical Institute Medical Research Training Fellowship (to C.V.); and a Grant-in-Aid from the American Heart Association (to A.S.).