Mouse Model of Desmin-Related Cardiomyopathy

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Background—The consequence of upregulation of desmin in the heart is unknown. Mutations in desmin have been linked to desmin-related myopathy (DRM), which is characterized by abnormal intrasarcoplasmic accumulation of desmin, but direct causative evidence that a desmin mutation leads to aberrant intrasarcoplasmic desmin accumulation, aggregation, and cardiomyopathy is lacking.

Methods and Results—Multiple transgenic mouse lines that expressed either murine wild-type desmin or a 7–amino acid deletion (R173 through E179) desmin (D7-des) mutation linked to DRM were made. The distribution of desmin protein was unchanged, and no overt phenotype was detected in the wild-type desmin transgenic mice. In contrast, the D7-des mouse heart showed aberrant intrasarcoplasmic and electron-dense granular filamentous aggregates that were desmin-positive and characteristic of human DRM. The desmin filament network was significantly disrupted, and myofibril alignment was visibly compromised. Although systolic function at the whole-organ level was substantially conserved in the young adult animals, the ability of the heart to respond to β-agonist stimulation, as measured in the intact animal, was significantly blunted.

Conclusions—Upregulation of desmin protein at moderate levels is not detrimental. However, the D7-des mutation is dominant negative, and expression of the mutant protein leads to the appearance of aggregates that are characteristic of and diagnostic for human desmin-related cardiomyopathy. (Circulation. 2001;103:2402-2407.)

Key Words: heart diseases □ cardiomyopathy □ molecular biology □ physiology □ pathology

Desmin, a muscle-specific intermediate filament protein, is encoded by a single gene and is evolutionarily conserved.1 Desmin filaments link myofibrils to one another, to the sarcolemma, and to the nuclear envelope.2 This filament network may play a role in mediating stress signaling.3 Upregulation of desmin in the heart occurs in a number of cardiac disorders such as cardiac hypertrophy and congestive heart failure,4–6 and desmin mutations are associated with desmin-related myopathy (DRM) and idiopathic dilated cardiomyopathy.7–10

The desminopathies are a heterogeneous group of human myopathies characterized by abnormal intrasarcoplasmic desmin accumulation.11 Clinically, DRM can present as a generalized myopathy, although often only cardiac muscle or cardiac and skeletal muscle is affected. To date, a putative 7–amino acid deletion (R173 through E179; D7-des) and missense mutations (A337P, A360P/N393I, L345P, N342D, R406W) of desmin deletion (R173 through E179; D7-des) mutation linked to DRM were made. The distribution of desmin protein was unchanged, and no overt phenotype was detected in the wild-type desmin transgenic mice. In contrast, the D7-des mouse heart showed aberrant intrasarcoplasmic and electron-dense granular filamentous aggregates that were desmin-positive and characteristic of human DRM. The desmin filament network was significantly disrupted, and myofibril alignment was visibly compromised. Although systolic function at the whole-organ level was substantially conserved in the young adult animals, the ability of the heart to respond to β-agonist stimulation, as measured in the intact animal, was significantly blunted.

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Both skeletal and cardiac myopathies have been described in desmin-null mice.13,14 However, the relationship of the desmin-null mutation to DRM is not clear because the total desmin complement, which consists of both normal and mutated protein, is significantly increased in a patient’s skeletal and cardiac muscles. Thus, the disease does not appear to be caused by the absence of protein. To unambiguously establish that the R173 through E179 deletion mutation is sufficient to cause DRM, we created an animal model of DRM using cardiac-specific transgenic (TG) expression of D7-des protein. TG expression of murine wild-type desmin (WT-des) mRNA and protein did not result in a discernible phenotype. In contrast, TG expression of D7-des protein led to aberrant intrasarcoplasmic desmin aggregation, perturbed myofibril alignment, and defective myocyte mechanical function, recapitulating aspects of human DRM.

Methods
The full-length desmin cDNA was cloned with reverse transcriptase–polymerase chain reaction. Multiple isolates were sequenced, and a
clone with the correct sequence (Genbank accession No. L22550) was placed in the α-myosin heavy chain promoter.15 The 7 amino acids deleted in the mutant linked to DRM, as well as the immediate flanking sequences, are essentially conserved (Figure 1a). The WT-des cDNA was used as a template to make the D7-des mutant, which was generated by polymerase chain reaction, and the cDNAs used to generate TG mice as described.13

Total ventricular RNA was isolated separately from 3 hearts of each line, and the integrity of the TG RNA was confirmed by Northern analysis and sequencing. RNA levels were quantified with dot blots hybridized to transcript-specific oligonucleotides. Total desmin protein levels were determined with SDS-PAGE followed by Western blots with a desmin mononclonal antibody (Sigma). Protein extracts were prepared from cytoskeletal (detergent insoluble) and soluble fractions of myocardial homogenates. Approximately 20–30 μg apical ventricular myocardium was homogenized on ice in PBS, pH 7.4, with 1% Triton X-100, EDTA (5 mmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), and 1 tablet/10 mL protease inhibitor cocktail (Roche). The homogenates were centrifuged at 14 000 g at 4°C, and the supernatant (soluble fraction) was separated from the pellet. The pellet was resuspended in SDS-PAGE loading buffer, boiled, and centrifuged, and the resultant supernatant (containing the desmin) was kept as the insoluble fraction. The ECF Western blotting protocol (Amersham Life Sciences) was used for quantification of desmin protein, and an enhanced chemiluminescent blotting protocol (Amersham Life Sciences) was used for quantification of desmin protein at ultrastructural level. The hearts were perfused with 1% glutaraldehyde/3% paraformaldehyde in cardioplegic buffer (5% dextrose, 30 mmol/L KCl in PBS), further fixed in the same fixative in cacodylate buffer, incubated in 0.1 mol/L glycine/PBS, dehydrated in series of N,N-dimethyl formamide, and embedded in LRWhite resin. Ultrathin sections were picked up on nickel grids, dried, and etched with a saturated solution of sodium m-periodate and 0.1N HCl. The immunolabeling procedure consisted of a PBS rinse; a 1.5-hour incubation in 1% BSA, 0.1% cold water fish skin gelatin, and 1% Tween 20 in PBS; overnight incubation in rabbit anti-desmin antibody (Biomeda) diluted 1:25 with 1% BSA/PBS; a short rinse in PBS; a 1.5-hour incubation in the blocking solution; and then a 2-hour incubation with goat anti-rabbit IgG tagged with colloidal gold (10-nm particle size, diluted 1:20 with 1% BSA/PBS; Aurion/Electron Microscopy Sciences) for 1 hour in PBS. After rinsing in PBS, postfixing in 2% glutaraldehyde, and rinsing in water, sections were counterstained with uranium/lead and viewed in a Zeiss Omega 912 at 100 kV. Indirect immunofluorescence staining was carried out on both fixed isolated myocites and myocardial cryosections.4 Hearts were excised, fixed by coronary perfusion with 4% paraformaldehyde, saturated with 30% sucrose solution, and embedded in Tissue Tek OCT (Sakura Finetek USA). Cryosections or mounted isolated myocytes were air dried, incubated with 0.1 mol/L glycine in PBS (pH 7.2) for 30 minutes, treated with 1% Triton X-100 for 60 minutes, and blocked with 0.5% BSA/10% goat serum in PBS. The specimens were then incubated with mouse monoclonal antibodies to α-actinin (Sigma) and/or rabbit polyclonal antibodies to desmin (Biomeda) overnight at 4°C and subsequently with TRITC-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG antibodies (Sigma) for 1 hour for confocal microscopy.

In addition to gravimetric analyses of different compartments of the heart, the size and area of isolated cardiomyocytes,17 transcripts characteristic of the hypertrophic response,18 and echocardiography16 of 8- to 10-week-old mixed-sex animals were used to determine the extent of hypertrophy.

Results

Morphology of D7-Des Hearts Recapitulates DRM

The secondary structure of desmin can be divided into 3 parts: a head, rod, and tail (Figure 1a). A D7-des in the initial part of rod segment 1B was selected for TG analysis. The genetic data concerning the dominance of this mutation are ambiguous,19 whereas the biochemical data indicate that the mutated protein should act as dominant negative.9 If the latter were correct, a phenotype should present with a TG approach. To rule out the possibility that alterations in the overall stoichiometry of the desmin transcript/protein pools could be responsible for any phenotype observed, TG lines expressing normal desmin (WT-des) were also made (Figure 1). Three WT-des lines (510, 520, 560) and 2 D7-des TG lines (83, 641) were generated. Normal mendelian ratios were observed in the subsequent breedings, indicating that no embryonic lethality occurred. Genomic Southern blotting showed that the transgene copy numbers of lines 510, 520, 560, 83, and 641 were 1, 14, 50, 3, and 15, respectively (data not shown).

Lines 520 (WT-des) and 641 (D7-des) were chosen for detailed characterization because analysis showed that both lines had ~3-fold the amount of desmin present relative to NTG littermates (Figure 1b and 1c). Diagnosis of DRM is based largely on morphological criteria; the hallmark of DRM is the presence of aberrant desmin aggregates in the myocytes of the affected muscles. These aggregates display a unique histology and ultrastructure that are diagnostic for DRM.11,19 To determine whether the D7-des mutation was causative for DRM, light microscopy, immunofluorescence confocal microscopy, transmission EM, and immuno-EM were used to characterize the TG hearts. At the light microscope level, no differences presented between the non-TG (NTG) littermates and the WT-des mice. Cell size, organization, and appearance were unremarkable. Confocal analysis confirmed that the WT-des staining pattern (Figure 2d through 2f) was essentially identical to the NTG controls (Figure 2a through 2c). α-Actinin and desmin staining colocalized and were restricted to the Z band (Figure 2c and 2f). In contrast, immunostaining of cryosections of the D7-des TG hearts showed abnormal desmin arrangements, confirming that the abnormal aggregates were desmin positive (Figure 2h and 2i). The aberrant desmin aggregates clearly disrupted the continuity and overall organization of the desmin network throughout the cell from the sarcolemma to the nuclear envelope (Figure 2i). Line 83, which had lower levels of mutant protein expression, also displayed some aggregates, but they were much less abundant (data not shown).

The aggregates appeared as electron-dense granular filamentous structures proximal to the nuclei and in the intermyofibrillary space (Figure 3). Some appeared to be associated with the nuclear envelope; others, with the Z band (Figure 3c and 3d). Numerous fragmented filaments with diameters characteristic of the intermediate filaments were found in the immediate surrounding area of the aggregates (Figure 3d). The aggregates and filaments were desmin positive as demonstrated by immuno-gold labeling (Figures 3e and 3f). Consistent with the hypothesis that desmin helps to maintain the structural integrity of the cardiomyocyte, both immunostaining of α-actinin and ultrastructural analyses showed that alignment of adjacent myofibrils was perturbed at the Z line in the D7-des TG hearts (Figures 2 and 3). The high degree of uniformity that is normally present at the boundary was absent (Figure 3c).
Desmin Mutation Causes Cardiac Hypertrophy

At the molecular level, activation of fetal genetic programs is common during the early stages of hypertrophy, with upregulation of atrial natriuretic factor and β-myosin and downregulation of α-myosin.18 The transcripts for phospholamban and the sarcoplasm reticulum Ca$^{2+}$ pump, which can often serve as markers for changes in calcium handling, were also determined. The relative amounts of these transcripts, endogenous desmin, and total desmin (Figure 4a) indicate that expression of D7-des protein but not overexpression of WT-des protein triggers a hypertrophic response. The response, as manifested by increases in the ratios of ventricle to body weight (Figure 4b), is most pronounced between 4 to 8 weeks of age but is progressively ameliorated in the adult. This apparent compensation is consistent with the lack of morbidity/mortality observed in the animals during the first 17 months of life.

To determine whether the cardiomyocytes themselves had hypertrophied, we analyzed the cell volume, profile area, length, sarcomere length, the transverse sectional area, and its minor and major diameters (Table I).17 In both ventricles, neither the sarcomere length nor the myocyte length was significantly increased (Table I); rather the increases observed in the D7-des cardiomyocyte size were due to increases in the transverse sectional area. In the left ventricle (LV), this was caused by growth in the minor diameter and in both the minor and major diameters in the right ventricle myocytes, indicating that a concentric hypertrophy at the cellular level occurs in both ventricles. Consistent with these data, echocardiography showed a significant increase in posterior wall thickness in the absence of significant changes in either diastolic and systolic LV chamber dimensions in the D7-des TG hearts (Table II). No abnormalities were observed in the cohort that overexpressed WT-des.

Myocyte and Cardiac Dysfunction

Cardiac function in patients with DRM is often significantly compromised.19 Considering the dramatic effects of D7-des protein expression on cellular structure and organization, we first determined the impact of D7-des or WT-des TG protein expression on cardiomyocyte function. Isolated cardiomyocytes are often more sensitive than whole-organ or whole-animal analyses in reflecting a primary pathology at the functional level because they are unloaded and isolated from potential compensatory mechanisms that the heart is able to bring to bear on the compromised cells through both internal and external mechanisms. Cardiomyocytes isolated from 8- to 10-week-old animals...
were field stimulated, and the cell mechanics were analyzed. Both the contractile and relaxation functions of the D7-des cardiomyocytes were significantly compromised in the D7-des TG group with respect to NTG cardiomyocytes (Figure 5a). The percent shortening of unloaded myocytes isolated from D7-des mice was decreased by 38% compared with the NTG controls \((P<0.05)\). The first derivatives of both shortening and relengthening were decreased by 42% and 35%, respectively \((P<0.05, P<0.01)\), in the D7-des group. Interestingly, the percentage shortening of ventricular myocytes isolated from age-matched WT-des mice was 27% greater than the NTG littermate controls \((P<0.05)\), whereas the first derivatives of the shortening and relengthening of these cells were unchanged compared with the control cohorts.

In intact mice instrumented with a Millar pressure transducer,16 LV diastolic function was markedly compromised in the D7-des TG group but not in the WT-des TG mice (Figure 5b and 5c). LV minimum \(dP/dt\) was significantly higher in the D7-des group. In addition, the response to the \(\beta\)-agonist dobutamine was substantially blunted in the D7-des TG animals. These data confirm that the deficits in ventricular performance described in the isolated myocytes extend to the intact animal and that systemic alterations in cardiovascular dynamics are unable to compensate for the myocardial deficit at this stage. It is interesting to note that baseline LV end-diastolic pressure was significantly elevated in the D7-des TG animals compared with the other 2 groups \((14.8 \pm 2.0 \text{ mm Hg in D7-des TG versus } 7.6 \pm 1.0 \text{ mm Hg in NTG and WT-des TG})\). The relatively large effect on \(dP/dt_{\text{min}}\) was confirmed by determining the time constant of relaxation, \(\tau\), a load-independent measure of diastolic function, which was markedly prolonged in the D7-des TG mice both at baseline and during dobutamine stimulation (Figure 5d).

**Discussion**

Significant increases in desmin expression in the heart can occur in human familial hypertrophic cardiomyopathy and end-stage heart failure, as well as in experimental pressure-overload cardiac hypertrophy and failure.4 However, the WT

**Figure 3.** Transmission EM. a, Eight-week-old NTG LV; b, 8-week-old WT-des TG LV; c, 8-week D7-des TG LV. Large accumulations of electron dense aggregates (asterisk) surrounding nucleus affect regular organization of sarcomeres. Z-band alignment is perturbed. d, Ultrastructural detail of electron dense aggregate and surrounding area in D7-des TG cardiomyocyte. Numerous 10-nm-diameter filaments are apparent (arrowheads). e, Immuno-gold labeling for desmin; aggregates and filaments are desmin positive. f, Lower magnification of immuno-gold–labeled section. Note concentration of grains over aggregates and in intermyofibril space at Z-band level. g, Immuno-gold labeling in NTG control heart. N indicates nucleus; Z, Z band.

**Figure 4.** Hypertrophic response. a, Three to four 8-week-old animals of mixed sex were used. Relative RNA levels of indicated transcripts were determined as described.18 Error bars indicate SD of mean. No statistically significant differences presented between NTG and WT-des TG mice, whereas changes in D7-des cohort were all statistically significant \((P<0.01)\). b, Both LV and right ventricle were used to determine degree of hypertrophy in 5 to 10 animals of mixed sex in each group at indicated times. Statistically significant differences \((P<0.01)\) were observed. \(\alpha\)-MyHC indicates \(\alpha\)-myosin heavy chain; \(\beta\)-MyHC, \(\beta\)-myosin heavy chain; PLB, phospholamban; Serca2A, sarcoplasm reticulum Ca\(^{2+}\) pump; and end, endogenous.
overexpressors show that a 12-fold overexpression of desmin mRNA by itself does not lead to a detectable pathology. Line 641 (D7-des), which was the focus of our studies, had a 4-fold increase in total desmin mRNA in mature animals. Despite the discrepancies in TG transcript levels between the 2 lines, the total desmin protein levels were comparable, indicating that the pathology observed in line 641 was not due to an absolute increase in desmin protein levels. A reasonable hypothesis is that the discrepancy in RNA versus protein levels between lines 641 and 520 may be due to a decreased rate of protein turnover in the animals carrying the mutant desmin. A large proportion of the protein in these cardiomyocytes is present in an aggregated form and may be less accessible to the normal enzymatic machinery involved in desmin turnover. We noted the appearance of aggregates in these mice as early as 1 month. By 8 to 10 weeks, they were fully developed and apparently reached a steady state because no further increases in abundance could be determined.

The α-helical rod domain of desmin is critical for the polymerization of protein and subsequent filament formation. In vitro studies showed that the ability of D7-des to assemble normally is compromised. In the D7-des cardiomyocytes, although the immunostaining pattern of desmin in the cortical region of the cell appeared to be overtly normal, the presence of aberrant desmin aggregates interrupted network architecture and presumably its function. Myofibril alignment is perturbed, even in areas that do not contain the aggregates. These data indicate that the mutant desmin protein disrupts desmin filament formation in vivo with a resultant myofibrillar misalignment at the Z-line level.

The structural defects caused by D7-des protein expression are accompanied by functional abnormalities. The percent shortening and the rates of shortening and relengthening of the unloaded isolated ventricular myocytes were significantly lower compared with both the NTG and WT-des TG controls. These findings demonstrate that cardiac function at the cellular level is compromised by structural abnormalities resulting from D7-des protein. Results from experiments in intact anesthetized mice at 8 months showed that the defective diastolic function persisted in vivo. The rates of ventricular contraction and relaxation were markedly decreased in D7-des TG animals under baseline conditions and during maximal β-adrenergic stimulation. We believe that it is likely that this impairment in contractility and relaxation leads to the observed increase in LV end-diastolic pressure (and presumably volume) that may further contribute to the development of cardiomyopathy.

Acknowledgments

This work was supported by NIH grants HL-56370, HL-41496, HL-56620, HL-52318, HL-60546, and HL-56620 and by the Marion Merrell-Dow foundation (Dr Robbins) and by the American Heart Association, Ohio Valley Affiliate (Dr Wang).

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Circulation. 2001;103:2402-2407
doi: 10.1161/01.CIR.103.19.2402
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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