Remodeling of gap junctions and slow conduction in a mouse model of desmin-related cardiomyopathy

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Abstract

Objective: We studied a transgenic mouse model of human desmin-related cardiomyopathy with cardiac-specific expression of a 7-amino acid deletion mutation in desmin (D7-des) to test the hypothesis that impaired linkage between desmin and desmosomes alters expression and function of the electrical coupling protein, connexin43 (Cx43).

Methods: Expression of Cx43 and selected mechanical junctions proteins was characterized in left ventricles of D7-des and control mice by quantitative confocal microscopy and immunoblotting. Remodeling of gap junctions was also analyzed by electron microscopic morphometry. The electrophysiological phenotype of D7-des mice was characterized by electrocardiography and optical mapping of transmembrane voltage.

Results: Cx43 signal at intercalated disks was decreased by ~3-fold in D7-des ventricular tissue due to reductions in both gap junction number and size. Immunoreactive signal at cell–cell junctions was also reduced significantly for adhesion molecules and linker proteins of desmosomes and fascia adherens junctions. Electron microscopy showed decreased gap junction remodeling. However, immunoblotting showed that the total tissue content of Cx43 and mechanical junction proteins was not reduced, suggesting that diminished signal at cell–cell junctions was not due to insufficient protein expression, but to failure of these proteins to assemble properly within electrical and mechanical junctions. Remodeling of gap junctions in D7-des mice led to slowing of ventricular conduction as demonstrated by optical electrophysiological mapping.

Conclusions: These results illustrate how a defect in a protein conventionally thought to fulfill a mechanical function in the heart can also lead to electrophysiological alterations that may contribute to arrhythmogenesis.

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1. Introduction

The cytoskeleton mechanically couples cardiac myocytes by interconnecting cell–cell junctions into a network that transmits contractile force across cell borders and, thereby, coordinates the contractile activities of individual cells. Recently, we characterized expression of cell–cell
junction proteins in myocardium from patients with Carvajal syndrome and Naxos disease, familial diseases related to defective linkage between the cytoskeleton and cell−cell adhesion junctions [1,2]. These rare cardiocutaneous syndromes are characterized by abnormalities of hair and skin, and severe cardiomyopathies associated with a high risk of sudden death [1,2]. Carvajal syndrome is caused by a recessive mutation in desmoplakin [3], an abundant intracellular protein that links desmosomal adhesion molecules to desmin, the intermediate filament protein of the cardiac myocyte cytoskeleton [4]. Naxos disease is caused by a recessive mutation in plakoglobin (γ-catenin) [5], an intracellular protein of adherens junctions and desmosomes that links N-cadherins to actin and desmosomal cadherins to desmin [6]. In both diseases, we observed remodeling of gap junctions and diminished localization of selected mechanical junction proteins at intercalated disks [1,2]. These findings are consistent with the hypothesis that normal electrical coupling of ventricular myocytes via gap junctions depends on normal mechanical coupling via cell−cell adhesion junctions [7]. They suggest further that a defect in cell−cell adhesion or a discontinuity in the linkage between intercellular junctions and the cytoskeleton prevents normal localization of connexins in gap junctions, which could contribute to tachyarrhythmias and sudden death in patients with Naxos disease and Carvajal syndrome.

In the present study, we characterized expression and distribution of electrical and mechanical junction proteins in a mouse model of human desmin-related cardiomyopathy. Desmin appears to play a critical role in maintaining the structural integrity of muscle cells and transmitting force generated by contraction by forming a continuous network of filaments that link desmosomes at cell−cell adhesion complexes to intracellular components of the contractile apparatus [8]. Human desmin-related skeletal and cardiomyopathies have been attributed to a 7-amino acid deletion (R173 through E179) [9,10] and several missense mutations (A337P, A360P/N393I, L345P, N342B and R406W) in desmin [11−13]. To determine whether the R173 through E179 deletion mutation was sufficient to cause desmin-related cardiomyopathy, Wang et al. [14] created mice with cardiac-specific transgenic expression of the 7-amino acid deletion mutation in desmin (D7-des) implicated in the human disease. D7-des mice exhibited a phenotype with features of human desmin-related cardiomyopathy including intracellular accumulation of desmin, disruption of the desmin filament network, misalignment of myofibrils and diminished responsiveness to β-agonist stimulation [14].

In the present study, we hypothesized that interactions between desmin and desmoplakin are disrupted in D7-des mice, potentially causing defective linkage between cell−cell adhesion junctions and the cytoskeleton. Based on this hypothesis, we would predict altered expression of proteins responsible for both mechanical and electrical coupling of cardiac myocytes. To test this hypothesis, we characterized expression and localization of intercellular junction proteins in D7-des mice and determined whether this mouse model exhibited an electrophysiological phenotype. We observed marked remodeling of gap junctions, altered expression of selected mechanical junction proteins at intercalated disks and slowing of ventricular conduction. These results illustrate how a defect in a protein conventionally thought to fulfill a mechanical function in the heart can also lead to electrophysiological alterations that may contribute to arrhythmogenesis.

2. Materials and methods

2.1. Animals

Transgenic mice expressing wildtype desmin (WT-des, line 520) and mutant desmin (D7-des, line 641) under the control of the α- myosin heavy chain promoter were originally produced by Wang et al. [14]. Both lines express ~3-times the amount of desmin present in non-transgenic littermates [14]. Mice were genotyped using polymerase chain reaction methods described by Wang et al. [14]. All studies were performed in young adult animals (12−30 weeks of age). Experimental protocols were approved by the Animal Studies Committee of Washington University School of Medicine. This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Immunohistochemistry and quantitative confocal microscopy

Hearts of WT-des, D7-des and non-transgenic control animals were rapidly excised, rinsed in phosphate-buffered saline, and fixed in 10% neutral-buffered formalin in preparation for immunohistochemistry of cell−cell junction proteins. Primary antibodies included a rabbit polyclonal antibody against desmoplakin (Sertotec), a mouse monoclonal antibody against plakoglobin (Sigma), a rabbit polyclonal antibody against a conserved sequence in the N-cadherins (Sigma), a rabbit polyclonal antibody against connexin43 (C643) (Zymed), a mouse monoclonal antibody against desmocollin 2/3 (Zymed) and a rabbit polyclonal antibody against desmin (ScyTek). Appropriate secondary antibodies were conjugated with CY-3. Slide-mounted sections were deparaffinized, boiled in citrate buffer for 10 min, and incubated with primary and secondary antibodies as described in previous studies [15,16]. Immunofluorescent signal was analyzed by quantitative confocal microscopy using methods developed in our laboratory and validated by electron microscopy [16]. This method is specifically designed to measure the amount of signal at cell−cell junctions by quantifying the number of pixels concentrated in clusters showing high-intensity fluorescence. Five test
areas were analyzed for each antibody in each of 3–5 hearts from each genotype. Each test area encompassed \( \sim 26,500 \mu m^2 \) and included profiles of 30–50 cardiac myocytes. The amount of immunoreactive signal at cell–cell junctions in each test area was expressed as a percentage of total cell area by quantifying the total numbers of pixels in digitized images exceeding prospectively defined signal intensity thresholds, divided by the total number of pixels occupied by tissue. The number and size (area) of individual clusters of high intensity signal within each test area were quantified as described [16].

2.3. Western blotting

Homogenates of left ventricular tissue from D7-des and non-transgenic littermates were analyzed by immunoblotting to measure the total tissue content of Cx43, desmoplakin, plakoglobin and \( \alpha \)-cadherin according to methods described in detail previously [15,16]. Cx43 and plakoglobin were separated on 10% polyacrylamide gels. For the Cx43 blots, 7 \( \mu g \) of total protein was loaded in each lane, and 14 \( \mu g \) of total protein was loaded in each lane for the plakoglobin gels. \( \alpha \)-cadherin and desmoplakin were separated on 5–15% polyacrylamide gradient gels. For these proteins, 20 \( \mu g \) of total protein was loaded in each lane. Each blot was also probed with an antibody against GAPDH to control for variations in loading. Quantitative densitometric analysis was performed as previously described [15,16] to measure the amount of each protein corrected for loading. The amount of each protein in D7-des hearts was normalized to the level in control hearts which was assigned a value of 1.0.

2.4. Light and electron microscopy

Hearts of 4 D7-des mice and 4 non-transgenic litter mates were fixed in formalin, sliced at 1 mm intervals in the short axis from apex to base and embedded in paraffin for conventional light microscopy. Sections were stained with hematoxylin and eosin for assessment of general tissue morphology, and with a trichrome stain to identify potential areas of cellular damage and fibrosis. To directly analyze gap junction remodeling and independently confirm immunohistochemical observations, left ventricular tissues from 5 D7-des and 5 non-transgenic litter mates were processed for electron microscopy and analyzed morphometrically using methods described previously [16,17]. Technically suitable areas of ultra-thin sections of left ventricle composed of compact groups of myocytes cut in longitudinal section were selected for analysis by viewing left ventricle composed of compact groups of myocytes cut in longitudinal section were selected for analysis by viewing
ventricular myocytes. Thus, expression of mutant desmin appears to act in a dominant negative fashion to disrupt the normal distribution of desmin filaments in ventricular myocytes.

3.2. Distribution of cell–cell junction proteins in D7-des myocytes

The distribution of the major gap junction channel protein, Cx43, and proteins associated with fascia adherens junctions and desmosomes (the two types of cell–cell adhesion junctions of the intercalated disk) was characterized using immunofluorescence and quantitative confocal microscopy. Left ventricular tissue from D7-des mice showed a ~3-fold reduction in the amount of immunoreactive signal for Cx43 in gap junctions (Figs. 2 and 3). No significant change in junctional Cx43 signal was seen in WT-des transgenic mice (Figs. 2 and 3). The decrease in Cx43 signal appeared to occur uniformly and was not associated with redistribution of signal to the lateral borders of the cells. Additional immunohistochemical studies were performed to determine whether expression of Cx45, the other gap junction protein in ventricular myocytes, was altered in D7-des hearts. Only low levels of Cx45 expression were observed in control hearts and no change was seen in D7-des hearts (data not shown).

Diminished Cx43 signal in D7-mice was due to significant reductions in both the number and size of individual clusters of immunoreactive signal at cell–cell junctions (Table 1). The number of discrete signal clusters per test field was reduced by ~50% and the mean size of an individual cluster was reduced by ~30%. This indicates that D7-des mice have fewer and smaller gap junctions containing Cx43 than non-transgenic controls.

We also observed a marked decrease (~50%) in immunoreactive signal at cell–cell junctions for the desmosome-specific desmin linker protein, desmoplakin (Fig. 3). In addition, signal for N-cadherin, the intercellular adhesion molecule at fascia adherens junctions, was diminished by ~65%, and signal for the linker protein, plakoglobin, was reduced by ~35% in D7-des mice (Fig. 3). The distribution of these proteins was not altered in WT-des mice (Fig. 3).

No consistent pattern of intracellular staining for Cx43 or any of the mechanical junction proteins was observed by confocal microscopy. There may have been a diffuse increase in intracellular Cx43 signal (see Fig. 2) but this was difficult to recognize and impossible to quantify.
3.3. Immunoblotting analysis

Immunoblots showed no apparent reduction in the total left ventricular content of Cx43, desmoplakin, plakoglobin and N-cadherin in D7-des compared with wildtype mice (Fig. 4). These results indicate that D7-des ventricular myocytes contain a larger proportion of intracellular, as opposed to junctional, Cx43 and mechanical junction proteins than wildtype mice (this intracellular signal would not be detected by the quantitative confocal microscopy method used in this study). No apparent differences in the relative densities of the Cx43 bands were seen (Fig. 4), suggesting that remodeling of gap junctions in D7-des mice is not associated with major changes in Cx43 phosphorylation. Taken together, the results of immunoblotting and confocal studies indicate that D7-des mice contain normal levels of Cx43 and mechanical junction proteins, but these proteins fail to localize and/or assemble properly in gap junctions and mechanical junctions at intercalated disks.

3.4. Ultrastructural analysis of cell–cell junctions

Electron microscopy was used to examine the ultrastructure of intercalated disks in D7-des mice and to confirm immunofluorescence observations regarding gap junction remodeling. Intercalated disks appeared to be more convoluted in D7-des mice, perhaps reflecting the loss of registry of Z-disks (Fig. 5). No obvious, consistent ultrastructural alterations were observed in individual desmosomes or fascia adherens junctions in D7-des mice. However, as predicted by confocal analysis, ultrastructural morphometry showed a significant reduction in gap junction density (number of gap junctions per unit intercalated disk length) in D7-des mice compared with wildtype mice (7.54±3.31 vs 20.74±11.54 gap junctions/100 µm intercalated disk length; p<0.02). Aggregate gap junction length per unit intercalated disk length was also less in D7-des mice (5.46±3.7 vs 14.86±10.1 µm gap junction length/100 µm intercalated disk length) but because of the large standard deviations, this difference did not achieve statistical significance (p<0.08). These observations provide independent evidence that although Cx43 is present in normal amounts

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Table 1
Gap junction number and size

<table>
<thead>
<tr>
<th></th>
<th>Number of GJ/test area</th>
<th>GJ size (µm²)</th>
</tr>
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<tbody>
<tr>
<td>Non-transgenic</td>
<td>290±32</td>
<td>1.24±0.25</td>
</tr>
<tr>
<td>WT-des</td>
<td>249±56</td>
<td>1.20±0.22</td>
</tr>
<tr>
<td>D7-des</td>
<td>124±54*</td>
<td>0.75±0.15*</td>
</tr>
</tbody>
</table>

* p<0.001 compared with non-transgenic and WT-des.

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Fig. 4. Representative immunoblots and quantitative densitometric analysis (n=4 for each) of the total tissue content of Cx43, desmoplakin, plakoglobin and N-cadherin in non-transgenic control and D7-des left ventricular myocardium. Each blot was probed for GAPDH to control for loading. Signal in D7-des hearts was normalized to the level in controls.
in D7-des mice, it does not form normal gap junctions in ventricular myocytes.

3.5. Electrophysiological characterization of D7-des mice

Analysis of ECGs obtained by telemetric monitoring revealed no evidence of spontaneous arrhythmias in D7-des mice. Simultaneous lead I, II and III ECGs recorded in anesthetized animals showed significant prolongation of the P-wave duration in the D7-des mice compared with non-transgenic littermate controls (21 ± 2 vs 15 ± 3 ms; \( p \leq 0.01 \)) (Fig. 6). This observation is consistent with previously reported enlargement of the atria in D7-des mice [14]. There was a trend towards prolongation of QRS duration in D7-des mice compared to non-transgenic littermates (12.5 ± 1.8 vs 11.5 ± 1.3 ms, \( p = 0.17 \)).

Optical mapping revealed modest but significant slowing of ventricular epicardial conduction velocity in both transverse and longitudinal directions in D7-des compared with wildtype mice (Fig. 7). There was no change in the anisotropic ratio (1.6 ± 0.2 vs 1.5 ± 0.2; \( p = 0.16 \)) suggesting that epicardial conduction was diminished uniformly. Light microscopic analysis of D7-des hearts revealed no evidence of tissue damage, inflammation or fibrosis. Thus, potential changes in extracellular resistance related to fibrosis do not appear to contribute to the slow conduction phenotype in D7-des mice.

Fig. 5. Representative electron micrographs of intercalated disks in non-transgenic control (Con) and D7-des left ventricular myocardium (bar=1 μm). In control tissues, Z-disks were aligned and a typical intercalated disk included adherens junctions, desmosomes (D) and gap junctions (GJ). In D7-des tissue, sarcomeres were misaligned. Intercalated disks were highly convoluted and contained elongated adherens junctions, and fewer desmosomes and gap junctions (none contained in this image).

Fig. 6. Representative lead I ECGs from control and D7-des animals.

Fig. 7. Upper panel: Contour plots (1.5 ms isochrone lines) showing the sequence and pattern of impulse propagation from a point of stimulation in representative control (Con) and D7-des hearts. In both hearts, the impulse spread anisotropically according to fiber orientation. Although the patterns of conduction were similar, D7-des heart exhibits slower conduction velocity. Lower panel: Summary data showing modest but significant reduction of conduction velocity in D7-des hearts in directions longitudinal and perpendicular to the epicardial fiber axis.
4. Discussion

In the present study, we tested the hypothesis that defective linkage between desmin and desmosomes in D7-des mice causes a phenotype characterized not only by contractile dysfunction, as previously demonstrated by Wang et al. [14] but also by electrical abnormalities likely related to remodeling of gap junctions at intercalated disks. We observed marked reductions in the amount of immunoreactive Cx43 signal localized at cell–cell junctions and, using electron microscopy, we showed that ventricular myocytes of D7-des mice are interconnected by fewer gap junctions. We also demonstrated that remodeling of gap junctions in D7-des mice results in slowing of ventricular conduction, thus showing that expression of a mutant cytoskeletal protein can lead to structural and functional alterations in intercellular coupling and conduction.

The degree of conduction slowing observed in D7-des mice is consistent with results of previous studies in mice in which relatively modest conduction phenotypes have been associated with substantial reductions in Cx43 expression at gap junctions [20,21]. This has been attributed to the high degree of coupling in cardiac myocytes in general, and the smaller myocyte size and larger role of myoplasmic resistance in mice compared with other mammals [22]. The uniform change in longitudinal and transverse conduction in D7-des mice is consistent with diminished gap junction density at the ends of cells without redistribution to the lateral cell borders. This is supported by the immunohistochemical observations and is also reminiscent of the spatial pattern of gap junction remodeling seen in Carvajal syndrome and Naxos disease [1,2]. It is not surprising that there was no significant widening of the QRS complex in D7-des mice, in view of previous studies highlighting the limitations of the ECG in electrophysiological phenotyping [19]. The absence of changes in ECG parameters pertaining to depolarization in D7-des mice is consistent with conduction slowing due to diminished coupling rather than decreased tissue excitability.

In their original study of D7-des mice, Wang et al. [14] reported moderate biventricular hypertrophy (ventricular weight:body weight ratios increased by ~50%). Because our confocal measurements of Cx43 expression at gap junctions are expressed as a percent of total cell area occupied by junctional signal, enlargement of myocytes due to hypertrophy could have contributed to the decreased Cx43 signal observed in D7-des mice. However, the degree of hypertrophy reported by Wang et al. [14] can only account for a 20–25% decrease in Cx43 signal, less than the observed ~67% decrease. Furthermore, Wang et al. [14] found no evidence of a heart failure phenotype, and we saw no evidence of fibrosis in D7-des hearts. Taken together, these observations strongly suggest that loss of Cx43 signal and remodeling of gap junctions in D7-des mice is not related to tissue remodeling or ventricular dysfunction, but rather reflects pathological processes different than those in end-stage heart disease [23,24].

It should be stressed that the confocal methods used here are specifically designed to measure immunoreactive signal in intercellular junctions (both gap junctions and mechanical junctions). Because of the high concentration of Cx43 and other cell–cell junction proteins in their respective organelles within the intercalated disk, it is possible to selectively measure immunoreactive signal in discrete junctional structures while “ignoring” lower intensity signal in other subcellular organelles (e.g., endoplasmic reticulum, Golgi, lysosomes, or non-junctional sarcolemma). This does not imply that protein in non-junctional compartments is of no biological importance, but merely that the quantitative strength of the confocal method rests on being able to specifically measure protein within cell–cell junctions which is identified not only by its high signal intensity but also by its location at points of cell–cell apposition.

It is clear that there must have been increased amounts of Cx43 within non-junctional pools in D7-des mice because confocal analysis showed a marked reduction in the amount of Cx43 present in gap junctions while immunoblotting demonstrated roughly normal levels of Cx43 protein in ventricular myocardium. This suggests that diminished Cx43 in gap junctions in D7-des mice was not due to limited Cx43 expression but rather to an inability of Cx43 to localize normally at cell–cell junctions. A similar finding was made in Naxos disease myocardium in which Cx43 was expressed abundantly but failed to localize to gap junctions [2]. These observations stand in contrast to findings in mice with genetic deficiency in Cx43 expression [20,25] or in end-stage heart failure in humans [23,24] in which reduced Cx43 at gap junctions is associated with down-regulation of connexin expression. In D7-des mice and Naxos disease, there seems to be a problem with formation and/or maintenance of a normal number of large gap junctions which, presumably, arises because of a defect in mechanical coupling of cardiac myocytes.

In previous studies of Carvajal syndrome [1], we noted normal distribution of desmin within cardiac myocytes but a distinct absence of desmin signal at cell–cell junctions. The mutation responsible for Carvajal syndrome truncates the C-terminus of desmoplakin, a region of the molecule which includes the desmin binding domain [3,8]. In the present study, expression of mutant desmin in D7-des mice resulted in abnormal accumulation of desmin signal within cardiac myocytes and derangement of the normal distribution of desmin filaments within cells, which included a marked reduction in the amount of desmin signal present at cell–cell junctions. Taken together with results of studies of Carvajal syndrome, this observation suggests that diminished localization of Cx43 in gap junctions and remodeling of gap junctions in D7-des mice is related to mechanical instability caused by defective linkage between desmin and intracellular mechanical junctions.
Despite the marked remodeling of gap junctions, we did not observe arrhythmias in D7-des mice. However, the absence of arrhythmias in a mouse model does not preclude an arrhythmogenic effect in the human disease, especially when one considers the differences in heart size and heart rate. The fact that conduction is slowed in D7-des mice suggests that this mutation could contribute to arrhythmias known to occur in patients with desmin-related cardiomyopathies [26–28]. How defects in cell–cell adhesion or discontinuities in cytoskeletal linkage actually lead to gap junction remodeling is a complex and, as yet, largely unexplored question. The clinical phenotypes in Naxos disease and Carvajal syndrome (abnormalities confined to high mechanical stress tissues) are most consistent with a biomechanical basis for gap junction remodeling but even if this is true, the underlying molecular mechanisms remain undefined. Because Cx43 levels are roughly normal in D7-des mice, it appears that cells contain sufficient Cx43 but still cannot form or maintain normal gap junctions. Although no marked changes in Cx43 phosphorylation were apparent in immunoblots of D7-des myocardium, more subtle changes mediated by stress-activated signaling pathways were apparent in immunoblots of D7-des myocardium, more subtle changes mediated by stress-activated signaling pathways could diminish the stability of individual Cx43 channels or channel arrays in gap junctions. Recent studies have demonstrated molecular interactions between Cx43 and the cytoskeleton, most notably microtubules [29,30]. These observations raise the possibility that gap junctions may function to anchor the cytoskeleton [29,30]. Conversely, defects in the cytoskeleton could alter the distribution or function of connexins in gap junctions.

In conclusion, we have demonstrated marked remodeling of gap junctions and altered distribution of Cx43 in a mouse model of desmin-related cardiomyopathy. Although the clinical phenotype in the desminopathies is primarily one of contractile dysfunction, our results emphasize the functional links between electrical coupling and mechanical coupling and provide a new paradigm for linking arrhythmias and sudden death to defects primarily associated with contractile dysfunction.

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References


