Extensive Induction of Important Mediators of Fibrosis and Dystrophic Calcification in Desmin-Deficient Cardiomyopathy

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Mice lacking the intermediate filament protein desmin demonstrate abnormal mitochondria behavior, disruption of muscle architecture, and myocardial degeneration with extensive calcium deposits and fibrosis. These abnormalities are associated with cardiomyocyte hypertrophy, cardiac chamber dilation and eventually with heart failure. In an effort to elucidate the molecular mechanisms leading to the observed pathogenesis, we have analyzed gene expression changes in cardiac tissue using differential display polymerase chain reaction and cDNA atlas array methods. The most substantial changes were found in genes coding the small extracellular matrix proteins osteopontin and decorin that are dramatically induced in the desmin-null myocardium. We further analyzed their expression pattern both at the RNA and protein levels and we compared their spatial expression with the onset of calcification. Extensive osteopontin localization is observed by immunohistochemistry in the desmin-null myocardium in areas with massive myocyte death, as well as in hypercellular regions with variable degrees of calcification and fibrosis. Osteopontin is consistently co-localized with calcified deposits, which progressively are transformed to psammoma bodies surrounded by decorin, especially in the right ventricle. These data together with the observed up-regulation of transforming growth factor- β 1 and angiotensin-converting enzyme, could explain the extensive fibrosis and dystrophic calcification observed in the heart of desmin-null mice, potentially crucial events leading to heart failure. (*Am J Pathol* 2002, 160:943–952)

Ablation of desmin, the muscle-specific intermediate filament protein, by gene targeting in mice leads to transient cardiomyocyte hypertrophy and extensive cardiomyocyte death, followed by cardiac chamber dilation and heart failure.^{1–3} Similarly, missense mutations of desmin have been identified in humans suffering from idiopathic dilated cardiomyopathy⁴ as well as other more generalized myopathies with both skeletal and cardiac dysfunction.

^{5–8,9} The cellular and tissue pathology associated with cardiac dysfunction in desmin-null mice has been adequately addressed.^{1,3,10,11} Briefly, mice lacking desmin demonstrate disruption of muscle architecture with mitochondrial abnormalities, including loss of normal positioning, extensive proliferation and clumping, as well as compromised respiratory function. These abnormalities are followed by myocardial degeneration with extensive fibrosis and dystrophic calcification. The molecular mechanisms underlining the development of these abnormalities are mainly unknown.

The inappropriate biomineralization occurring in soft tissues is defined as ectopic calcification. In the absence of a systemic mineral imbalance ectopic calcification is typically called dystrophic calcification and is commonly observed in injury, disease, and aging.^{12,13} Although it can occur in all soft tissues, cardiovascular tissues seem particularly prone to dystrophic calcification. In arteries, calcification is correlated with atherosclerotic plaques with the known clinical consequences. Age-related dystrophic calcification in the human cardiovascular system can contribute significantly to cardiac dysfunction and is perhaps more prevalent than ischemic heart disease.¹² Despite the high prevalence and clinical significance, very little mechanistic data exist mainly because of lack of animal models.

Dystrophic calcification possesses several features of bone mineralization, including the presence of noncollagenous matrix proteins such as osteopontin, matrix Gla protein, osteocalcin, SPARC (osteonectin), and bone morphogenetic proteins, which all are thought to regulate also pathological calcification.^{12,13} Indeed mice lacking matrix Gla protein, by gene targeting inactivation, have extensive calcification of arteries and valves,¹⁴ thus supporting the idea that this protein is indeed a natural inhibitor of mineralization. Similar results have been obtained for the osteoprotegerin gene, a member of the transforming growth factor (TGF) receptor superfamily, known to regulate osteoclast differentiation.¹⁵ Although dystrophic calcification in all of the above cases is restricted to the vascular system, in desmin-null mice the cardiac muscle is the target tissue and can only be

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compared to dystrophic cardiac calcinosis. This is an age-related cardiomyopathy that occurs in certain inbred mouse strains that can also lead to congestive heart failure.^{16,17} There are also a few other cases in which mutations in sarcomeric proteins, among other cardiac abnormalities, demonstrated calcification but at minor levels.^{18,19} The molecular mechanisms underlying ectopic calcium deposition at sites of inflammation and/or necrosis is a fundamental but poorly understood element of not only dystrophic cardiac calcinosis and desmin-null cardiomyopathy but for any tissue response to injury. Desmin-null mice could serve as a good model to unravel the molecular mechanisms of cardiovascular degeneration, calcification, and the development of heart failure in these animals.

Because of the complexity of the observed pathology of desmin-null hearts, it is anticipated that alterations in multiple processes should be responsible for the development of the observed cardiomyopathy. To address this issue we analyzed general gene expression changes in cardiac tissue of the desmin-null mice, using differential display polymerase chain reaction (PCR) and cDNA atlas array methods. The most substantial changes were found for genes coding for extracellular matrix proteins and especially for the small matricellular proteins osteopontin and decorin.¹³ We connect their action to the extended inflammatory reaction first observed between the second and third week of the animal's life because of pronounced cardiomyocyte death. These data, together with the observed up-regulation of TGF- β 1 and angiotensin I-converting enzyme (ACE) could explain the extensive fibrosis and dystrophic calcification observed in the heart of desmin-null mice.

Materials and Methods

Animals

The procedures for the care and treatment of animals were according to institutional guidelines. Mice lacking desmin were generated by gene targeting via homologous recombination as previously described.¹ The mice used for this study were of the C57BL/6–129SV genetic background.

Isolation of RNA, Differential Display PCR, cDNA Arrays, and Northern Blots

Wild-type and desmin-null mice were anesthetized and blood-free hearts were pulverized into powder under liquid nitrogen. RNA was isolated usually from pools of 4 to 5 hearts using the Totally RNA isolation kit (Ambion, Austin, TX). PolyA RNA was isolated using oligo-dT cellulose (Ambion). The differential display PCR method was used essentially as described.²⁰ The mouse atlas 1.2 k (1185 genes) cDNA array (catalog no. 7853-1) analysis was performed by Clontech (Clontech, San Diego, CA), using pools of three hearts of 4-month-old desmin-null and wild-type animals. Northern blots were performed as previously described² using standard techniques. For

the ACE probe we have isolated a 0.95-kb fragment of the mouse ACE cDNA (accession no., J04947; area, 2093 to 3044) by RT-PCR amplification and similarly for the TGF- β 1 probe we have isolated a 1.54-kb fragment of the mouse cDNA (accession no., AJ009862; area, 414 to 1960). The mouse osteopontin cDNA was kindly provided by Dr. Larry Fisher, National Institute of Dental and Craniofacial Research, Bethesda MD.²¹

Protein Extracts and Western Blots

For the osteopontin Western blot, pulverized tissue (same as in RNA isolation) was extracted either with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2% SDS, 10% glycerol, 50 mmol/L Tris-HCl, pH 6.8) or with demineralizing buffer that contained all of the above plus 300 mmol/L ethylenediaminetetraacetic acid (EDTA). Bone extracts were prepared from the femur bone of 3-week-old animals. For the decorin Western blot, the pulverized tissue was extracted with guanidine buffer (6 mol/L guanidine isothiocyanate, 50 mmol/L Tris-HCl, pH 7.3, 5 mmol/L EDTA). For the chondroitinase digestion the guanidine extracts were dialyzed against (50 mmol/L Tris-HCl, 30 mmol/L sodium acetate, pH 7.3, 5 mmol/L EDTA, 3 mmol/L phenylmethyl sulfonyl fluoride) and centrifuged at $13,000 \times g$ for 20 minutes. The supernatant ($\sim 5 \mu\text{g}$ of total protein) was incubated with 0.04 U of chondroitinase ABC from Sigma (catalog no. C3667; Sigma, St. Louis, MO) in a final volume of 40 μL of 0.5 $\times$ phosphate-buffered saline (PBS) at 37°C for 3 hours.

The samples were analyzed by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed for decorin and osteopontin using the antibodies LF-113 and LF-123, respectively, in a 1:1000 dilution. The antibodies were kindly provided by Dr. Larry Fisher.²¹

Immunofluorescence

Blood-free mouse hearts were immersed in OCT compound (Miles Inc., Torrance, CA) and frozen in liquid nitrogen. Frozen tissue sections (7- μm -thick) fixed with 4% paraformaldehyde in PBS were used for immunolabeling as previously described.¹ The anti-decorin (LF-113), anti-osteopontin (LF-123), and anti-collagen- α 1 (LF-67) antibodies were kindly provided by Dr. Larry Fisher and the anti-laminin antibody was from Sigma (catalog no. L9393). All of the above polyclonal antibodies were used at a 1:300 dilution. The appropriate secondary antibodies (Alexafluor-594 and Alexafluor-488) were from Molecular Probes (Eugene, OR) and used in a 1:800 dilution.

Histology

Routine histological analysis and hematoxylin-eosin staining was performed as previously described.¹ For immunohistochemical analysis 5- μm -thick paraffin sections, from tissues fixed overnight in 2% paraformaldehyde solution in PBS, were used. The anti-decorin (LF-

Table 1. Induction of Fibrosis and Calcification Are Linked in the Heart of Desmin-Null Mice

Des+	Des-	Fold X	cDNAs
1	42	42.0	Osteopontin precursor (OP); bone sialoprotein 1
8	46	5.8	Decorin; bone proteoglycan II (PG-S2)
11	17	1.5	Bone morphogenetic protein 1 (BMP1); biglycan
1	4	4.0	Osteoblast-specific factor 2 (OSF-2)
5	17	3.4	Fibronectin
18	55	3.1	Angiotensin-converting enzyme (ACE)

General gene expression profile was determined in the heart of desmin-null and wild-type animals, using the mouse 1.2 k (1185 genes) Atlas Array from Clontech Lab. RNA was isolated from 4-month-old, wild-type (Des+) and desmin-null (Des-) animals. Data from extracellular matrix/cell structure proteins are presented. The numbers represent arbitrary units.

113) and anti-osteopontin (LF-123) antibodies were used in a 1:800 dilution. Reagents for the immunoperoxidase labeling were from DAKO (Carpinteria, CA). Substitution of primary antibodies by normal rabbit IgG was used as a negative control. Von Kossa staining for calcium detection was performed as described.²²

Results

Expression of Genes Coding for Small Extracellular Matrix Proteins, Decorin, and Osteopontin Is Induced in the Heart of Desmin-Null Mice

To identify genes that are differentially expressed in the heart of desmin-null mice, a mouse cDNA array was screened with RNA isolated from hearts of 4-month-old wild-type and null animals. Nine percent of the 1185 cDNAs examined displayed at least a twofold difference between wild-type and null animals with 60% of the differentially expressed genes being up-regulated in the null heart. These differentially expressed cDNAs belong to several functional groups (data not shown), but the

Figure 1. Osteopontin expression is dramatically induced in the heart of desmin-null animals. Expression profile of osteopontin mRNA in the heart of wild-type (+/+) and desmin-null (-/-) mice by Northern blot, reveals that osteopontin is induced early in the animal's life (21 days) and declines as the animal ages. The expression of osteopontin mRNA was undetectable in wild-type animals. D, days; Mo, months. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a loading control (exposure, 12 hours).

Figure 2. Decorin, ACE, and TGF- β 1 mRNAs are up-regulated in the heart of desmin-null mice. mRNA was isolated from the hearts of 2.5-month-old wild-type (+/+) and desmin-null (-/-) animals and analyzed by Northern blot using the corresponding cDNA probes. Shown are representative results for each mRNA. GAPDH was used as a loading control (exposure, 3 days).

most substantial changes were found in genes coding for extracellular matrix proteins (Table 1).

Osteopontin RNA, was markedly induced (42-fold) in the heart of desmin-null animals (Table 1). The cDNA array screen results were confirmed by Northern blot analysis (Figure 1), which indeed revealed a dramatic induction of osteopontin RNA in desmin-null hearts. The expression of osteopontin RNA in the heart of wild-type animal is undetectable, even for longer (5 days) exposures. Further analysis of the expression profile of osteopontin RNA at various ages shows maximum induction around 3 weeks of age and then a decline as animals age (compare 6- to 12-month-old animals in Figure 1). In contrast, no detectable expression of osteopontin RNA was observed in skeletal muscle (gastrocnemius) of both desmin-null and wild-type animals (data not shown).

The second highest increase (5.8-fold) in the heart of desmin-null animals revealed by the array screen (Table 1) was in the expression of decorin RNA that codes for a proteoglycan, another small extracellular matrix protein.²³ The up-regulation of decorin was initially observed by differential display PCR in which a fragment identical to the 1337- to 1763-bp region of the mouse decorin cDNA (accession no. X53928) was isolated. Differential expression of the decorin mRNA in desmin-null heart was also confirmed by Northern blot analysis (Figure 2) using the above fragment as a probe. The induction of the decorin mRNA in the heart of desmin-null animals is approximately threefold compared to the wild-type. This level of decorin induction could be observed as early as 9 days after birth and remains constant at least up to the age of 13 months.

Another gene linked to fibrosis, ACE, a key regulator of the renin-angiotensin system²⁴ was induced (3.1-fold, Table 1) in desmin-null hearts. ACE induction was also confirmed by Northern blot analysis (Figure 2). The two ACE messages observed, sizes 4.9 and 4.2 kb, are commonly found in Northern blots of somatic tissues.²⁵ Given the established connection between decorin, TGF- β 1, and fibrosis^{26,27} we wanted to see how the expression of TGF- β 1 is modulated in the desmin-null heart. Although the cDNA array screen did not reveal any difference,

Figure 3. Expression of osteopontin and decorin proteins is elevated in the heart of desmin-null ($-/-$) animals compared to wild-type ($+/+$). Heart extracts from 4-month-old animals were electrophoresed in a 9% SDS-PAGE and immunoblotted for osteopontin (**A**) and decorin (**B**). Osteopontin could be detected only in extracts from desmin-null heart as a high-molecular weight complex trapped in the stacking gel. Bone extracts were used as a positive control. For the decorin immunodetection, heart extracts were digested with (ABC+) chondroitinase ABC or not (ABC-) before analysis by SDS-PAGE. Note that in the undigested extracts decorin runs as a diffused band centering ~ 90 kD and is significantly up-regulated in the hearts of desmin-null animals. After chondroitinase ABC digestion the proteoglycan is converted to an ~ 48 -kD band clearly overexpressed in the desmin-null animal whereas in the wild-type only a faint band is observed.

Northern blot analysis did show increase in the expression of TGF- $\beta 1$ RNA in desmin-null hearts (Figure 2). The increase was ~ 2.5 -fold in 3-week-old and 4-month-old animals.

Analysis of Decorin and Osteopontin Expression by Western Blots

To further examine whether the observed increase in osteopontin and decorin RNA levels was accompanied by similar changes at the protein level, we performed Western blot analysis. Murine osteopontin has a predicted molecular weight of 35 kD. However, in SDS-PAGE it shows anomalous migration with an apparent molecular weight of 45 to 75 kD,²⁸ because of posttranslational modifications, depending on the tissue of origin. Western blot analysis of cardiac extracts from 4-month-old desmin-null animals using anti-osteopontin antibody, showed the presence of high amounts of the protein in a high-molecular weight complex form (Figure 3A). This complex is mainly retained in the stacking gel and has an apparent molecular weight ranging from ~ 130 to >200 kD. Even after treatment of the samples with demineralizing buffer for 12 hours, the complex remains the same indicating that osteopontin most possibly is in a stable polymeric form.²⁹ Extracts from wild-type hearts were negative for osteopontin protein. Two minor bands of ~ 40 - to 50 -kD molecular weight were also detected in both wild-type and desmin-null cardiac extracts at similar intensity. These could result from either cross-reactivity of the antibody that has been occasionally seen in preparations of

different tissues²⁸ or could be basic levels of nonmodified or degraded osteopontin (the antibody used recognizes the carboxy half of the molecule). Bone extracts were used as a positive control revealing a major band of ~ 65 -kD molecular weight and a diffused high-molecular weight band, remained on the top of the gel (Figure 3A) as in the case of desmin-null heart extracts.

Western blot analysis revealed that decorin was also significantly increased (approximately threefold) in the heart of desmin-null animal when compared to the wild type (Figure 3B). Heart extracts from 4-week-old, 4-month-old, and 8-month-old animals gave similar results (only data from 4-month-old animals are shown). In SDS-PAGE analysis of cardiac extracts, decorin runs as a diffused band with an apparent molecular weight of 85 to 105 kD. After digestion with chondroitinase ABC, decorin migrates at ~ 48 kD (Figure 3B), indicating the existence of a glycosaminoglycan chain as expected. The predicted molecular weight of the core protein is ~ 38 kD and usually is modified with one glycosaminoglycan chain and two or three N-linked oligosaccharides.²³ Analysis of protein extracts from skeletal muscle (tongue and gastrocnemius) did not show any obvious differences in decorin expression between wild-type and desmin-null animals (data not shown).

Localization of Osteopontin, Decorin, Collagen, and Laminin in Cardiac Tissue Sections

Immunohistochemical and immunofluorescence analysis was performed to determine the relationship in the spatial and temporal distribution of the different matrix proteins and the pattern of calcium deposits. Extensive osteopontin staining was first observed at the age of 3 weeks in the right ventricle of desmin-null animals (Figure 4, A and B) in areas with pronounced myocyte death, acute inflammatory infiltrate, and calcium precipitation with a gritty appearance (Figure 4, C and D). With the progression of the pathology the extensive myocyte death leads to further tissue remodeling with replacement of cardiomyocytes with fibrosis and accumulation of calcium precipitates in psammoma body structures (Figure 5). These structures of concentric calcium laminations were positive for osteopontin staining, and were observed very frequently. In the right ventricle degeneration and calcification can reach up to 80% of the myocardium thickness. In some cases, after work overload, the damage is so extensive that it leads to rupture of the cardiac wall.^{9,11} Various degrees of osteopontin staining and calcium precipitation could be also detected in areas with features of chronic inflammation (Figure 6), such as presence of lymphocytes, macrophages, and fibroblast-like cells. These areas could be found spontaneously in different regions of the cardiac tissue, such as the ventricles (Figure 6A), the interventricular septum (Figure 6C), and the papillary muscles. Immunostaining of cardiac tissue sections of wild-type animals for osteopontin were negative in all corresponding cases checked (not shown). In all cases studied, osteopontin seemed to co-localize with calcium deposits (Figures 4, 5, and 6), suggesting that

Figure 4. Extensive osteopontin localization is observed in the myocardium of desmin-null animals, in areas with acute inflammatory infiltrate, myocyte degeneration, beginning of calcium precipitation, and coagulative necrosis. **A** and **B** (higher magnification of **A**): Immunohistochemical localization of osteopontin in the right ventricle of a 3-week-old desmin-null animal. Wild-type animals were negative for osteopontin staining, (not shown). **D**: Von Kossa staining for calcium deposition indicates the beginning of calcium precipitation on the degenerating myocytes. **C**: H&E (Hem./Eos.) staining, indicating more clearly the infiltrating neutrophils-polymorphs, the degenerating myocytes, and the edema, typical characteristics of acute inflammatory reaction. **E**: Extended coagulative necrosis is more clearly observed a few days after the initial acute inflammatory infiltration. Degrading myocytes, necrotic cells, debris, and edema fluid are observed by H&E staining of a corresponding area in a desmin-null animal 5 days older than the one described in **A–D**. **F**: Negative control for the immunohistochemistry. Unrelated rabbit IgG was used as primary antibody. No staining is observed. **A–D** and **F** are from adjacent sections of a 3-week-old heart. For the immunoperoxidase staining (**A**, **B**, and **F**), diaminobenzidine was used as substrate and hematoxylin for nuclei counterstaining. Von Kossa staining for calcium deposition (**D**) is brown/black and counterstaining for nuclei by Kernechtrot is red.

this protein plays a crucial role during calcification. Comparison of osteopontin and decorin localization revealed that decorin does not co-localize with osteopontin inside calcified areas, but it is abundant in the immediate surrounding fibrotic region (Figure 5C).

Immunofluorescence localization of decorin in the heart of desmin-null animals gave more intense staining in the epicardium (not shown) and very strong and extensive staining in fibrotic areas (Figure 7B) in contrast to the wild-type animals, where decorin is localized mainly in the endomysium (Figure 7A). Extended areas of

decorin staining could be found in different compartments of cardiac tissue as early as in 4 weeks after birth and throughout the entire life of the animal.

Immunofluorescence localization of collagen- $\alpha 1(I)$ gave intense staining in the heart of desmin-null animals in fibrotic (Figure 7D) and perivascular areas, with a pattern very similar to decorin. On the other hand immunofluorescence localization of laminin ($\alpha 1, \beta 1, \gamma 1$), an abundant basement membrane protein that envelopes individual cardiomyocytes, did not reveal any obvious difference between wild-type (Figure 7E) and desmin-null

Figure 5. Immunohistochemical localization of osteopontin and decorin in advanced stage calcification. Osteopontin is detected in desmin-null animals in necrotic areas with extensive calcification and psammoma body morphology (**A and B**). These structures are large mineralized deposits with lamellated configurations surrounded by decorin-containing fibrotic tissue (**C**). **A**, **B**, and **C** are from serial sections in the outer surface of the right ventricle of a 6-month-old animal. Immunohistochemistry staining for osteopontin and decorin (**A and C**) and von Kossa staining (**B**) is same as in Figure 4.

animals (Figure 7F). The data revealed that laminin does not contribute to the extended fibrotic lesions in the heart of desmin-null animals (compare Figure 7; B, D, and F).

Discussion

In an effort to elucidate the molecular mechanisms by which the lack of desmin leads to the observed cardiac pathology, we have screened RNA from wild-type and desmin-null hearts for changes in gene expression. It was

Figure 6. Osteopontin co-localizes with calcium in areas with chronic inflammation. Immunohistochemical localization of osteopontin (**arrowheads**) in a hypercellular area of infiltrate in the right ventricle of desmin-null animal (**A**) together with mild granular calcium deposits as indicated by a serial section stained by von Kossa (**B**, **arrows**). Osteopontin (**arrowheads**) is also detected in calcified regions of other parts of the myocardium such as the septum (**C**). **A and B** are from a 6-month-old animal and **C** from a 2-month-old animal. Staining procedure is same as in Figure 4.

very encouraging to find that the most dramatic changes observed in gene expression were for osteopontin and decorin, molecules linked to fibrosis, calcification, and cardiomyocyte death, the hallmarks of the desmin-null heart pathology.

Osteopontin has been implicated in multiple diverse functions in both physiological and pathological processes as recently reviewed.^{30,31} Although normally it is expressed in bone and at epithelia surfaces,³² osteopontin is elevated during injury and inflammation in most tissues studied today, including calcification in athero-

Figure 7. Abnormal accumulation of decorin and collagen but not laminin in the myocardium of desmin-null animal. Immunofluorescence localization of decorin, collagen, and laminin was performed in cardiac tissue from wild-type (**A**, **C**, and **E**) and desmin-null (**B**, **D**, and **F**) animals. Extensive decorin staining could be seen in fibrotic areas (**arrows**) of desmin-null-only myocardium (**B**) but not in wild-type animal (**A**), where only staining of the endomysium (**arrowheads**) could be detected. **B**, **D**, and **F**: Staining of serial sections of desmin-null myocardium, for decorin, collagen, and laminin. Note that laminin shows normal pattern and is not a component of the fibrotic areas (compare with the wild-type in **E**). Decorin (**B**) has a similar staining pattern as collagen (**D**) and co-localizes with it in fibrotic areas of desmin-null myocardium. **Asterisks** in **B**, **D**, and **F** indicate corresponding spots in serial sections.

sclerotic plaques,³³ T-cell response to infection,³⁴ wound healing,^{35,36} and tumor growth.³⁷ These findings have suggested a role for osteopontin in modulating the inflammatory process, for example by stimulating macrophage infiltration. Also much like its role in bone, osteopontin's ability to interact with hydroxyapatite crystals^{38–40} probably serves both to interfere with bioapatite crystal growth through physical interactions, as well as to regulate host cell resorptive mechanisms of the ectopic calcification via receptor-mediated interactions.^{31,41}

Recent reports have shown expression of osteopontin by macrophages in cardiac tissue in response to myocardial necrosis caused by transdiaphragmatic freezing,³⁵ in cardiomyopathic Syrian hamster,⁴² and in spontaneously hypertensive rats.⁴³ On the other hand in a single report cardiomyocytes have also been found as the primary source of osteopontin in a hypertrophy model of rat heart by renovascular hypertension and aortic banding.⁴⁴ These differences could be just the consequence of different time periods studied. It is possible that at early stages of cardiomyopathy myocytes may be the source of osteopontin mRNA but later interstitial non-myocyte cells are taking over. In the present case the cell source of osteopontin was not revealed.

As described above, osteopontin is believed to act as an inhibitor of calcification.³⁹ However, in all cases tested in the desmin-null heart although osteopontin co-localizes with calcium deposits, the calcification does indeed progress overriding the inhibitory action of osteopontin or

osteopontin is in a form that cannot anymore act as inhibitor of calcification. This could be linked to our observation that in desmin-null myocardium, osteopontin forms high-molecular weight complexes (Figure 3A). These complexes, might represent polymeric forms of osteopontin, possibly covalently bound through the action of transglutaminase, as has previously been demonstrated.²⁹ This form of osteopontin has the increased ability to bind collagen,⁴⁵ thus it can serve in adhering together calcified deposits, the surrounding cells, and adhesive matrix. In such form it might be irreversibly bound to calcium deposits, thus promoting the isolation of these deposits by fibrotic tissue (Figure 5C) and eventually the formation of psammoma body structures.

The precise mechanism by which the absence of desmin leads to cardiomyocyte death and calcification, and the reasons why this does not happen that extensively in skeletal muscle, at the present, can be only speculated. Our recent studies have strongly suggested that desmin is very important for normal mitochondrial behavior and function.¹¹ There is plenty of evidence suggesting that impaired mitochondrial behavior and function could lead to cell death.⁴⁶ Because mitochondrial abnormalities are the earliest defects that have been observed in the desmin-null heart, we believe these defects are the main cause of death of these mice. The fact that heart muscle cells have the maximum content of desmin, 2% of total protein, compared to 0.35% of skeletal muscle cells,⁴⁷ and the highest volume density of

mitochondria of all mammalian cells (36% in mice),⁴⁸ could easily explain why these cells are mostly affected. When compared to fast glycolytic muscle, slow oxidative skeletal muscle, which has also more mitochondria, is more affected by the absence of desmin.¹¹ However, the ability of skeletal muscle to regenerate could explain the lack of necrotic tissue accumulation and pronounced calcification. Other explanations are not excluded.

One of the many ways by which mitochondrial abnormalities could lead to cell death in desmin-null heart could be the disturbance of intracellular calcium homeostasis. Ultrastructural studies in dystrophic cardiac calcinosis (DDC) mice, which show many common features with the desmin-null calcification,⁴⁹ have shown that initial events of calcification include granular calcium deposition in or around mitochondria.^{49,50} Mitochondria are able to take up large amounts of Ca^{+2} and buffer cytosolic Ca^{+2} levels. If this is compromised, excessive intracellular Ca^{+2} can contribute to cytotoxic events leading to formation of reactive oxygen species and cell death.⁵¹ Cytotoxic events such as increased phosphate concentration⁵² by overactivation of protein phosphatases and increased NO production by stimulated macrophages⁵³ can significantly induce the osteopontin expression. Another potential mechanism of osteopontin induction could involve angiotensin II, which can induce osteopontin expression in cardiac fibroblasts⁵⁴ and can directly increase both TGF- β 1 and osteopontin in the heart.⁵⁵ The present cDNA array studies revealed increased ACE expression in the desmin-null hearts, which might contribute to osteopontin induction through angiotensin II. In turn, osteopontin could modulate calcium levels by different ways including calcium mobilization,^{56,57} activation of Ca^{++} -ATPase pump,⁵⁸ Ca^{++} binding,⁵⁹ and by modulating hydroxyapatite crystal growth.³⁸ Thus, abnormalities in mitochondria apart from cytochrome c- and caspase-related prodeath events, can initiate and maintain the Ca^{++} /osteopontin cycle described above, which could lead to the extensive calcification in desmin-null hearts.

From our study it is evident that another noncollagenous matrix protein, decorin, is a prominent component of fibrotic areas in the myocardium of desmin-null animals. Up-regulation of decorin and its co-localization with collagen in the desmin-null heart could correlate with its ability to participate in the assembly of fibrillar collagen.⁶⁰ Thus decorin up-regulation could be a counteraction to tissue necrosis, interfering with tissue remodeling by fibrosis.^{26,27} Elevation in the expression of decorin was recently reported in two cases in myocardial infarction^{61,62} and one in cardiac hypertrophy,⁶³ all of which displayed fibrosis. The two more recent cases were also elaborated by global gene expression analysis.^{62,63} Decorin has the ability to bind and neutralize TGF- β 1 and potentially abrogate its effect on tissue fibrosis.^{26,27} On the other hand TGF- β 1 has the potential to initiate the production of decorin.^{64,65} Thus decorin in the heart of desmin-null mice could participate in a feedback loop that regulates TGF- β 1 action. A potential mechanism by which induction of both osteopontin and decorin could be

achieved in desmin-null hearts is by angiotensin II, directly or through TGF- β 1,⁵⁵ as a result of the increased ACE expression found in desmin-null hearts (see Table 1).

In addition to osteopontin and decorin, molecules such as bone/cartilage proteoglycan I precursor (also named biglycan) and osteoblast-specific factor-2, initially thought to only participate in normal osteogenesis are also up-regulated in the heart of desmin-null mice (see Table 1) and in other cardiomyopathy models.^{62,66} Except for osteoblast-specific factor-2, all of these molecules are also overexpressed in dystrophic vascular calcification,¹² suggesting that a common mechanism resembling osteogenesis may exist in vascular and cardiac calcification.

In conclusion, despite the complexity of the observed pathology in the desmin-null heart the data so far favor the possibility that the early observed mitochondria abnormalities, maybe through disturbance of Ca^{+2} homeostasis, can initiate a cycling cascade of events leading to extensive cell death and calcification. Among other interplayers of this cascade, the matricellular proteins osteopontin and decorin together with, or because of, increased TGF- β 1 and ACE expression can modulate fibrosis and calcification and thus the development of dilated cardiomyopathy and heart failure.

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