Protein Aggregates and Novel Presenilin Gene Variants in Idiopathic Dilated Cardiomyopathy

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Background—Heart failure is a debilitating condition resulting in severe disability and death. In a subset of cases, clustered as idiopathic dilated cardiomyopathy (iDCM), the origin of heart failure is unknown. In the brain of patients with dementia, proteinaceous aggregates and abnormal oligomeric assemblies of β-amyloid impair cell function and lead to cell death.

Methods and Results—We have similarly characterized fibrillar and oligomeric assemblies in the hearts of iDCM patients, pointing to abnormal protein aggregation as a determinant of iDCM. We also showed that oligomers alter myocyte Ca2+ homeostasis. Additionally, we have identified 2 new sequence variants in the presenilin-1 (PSEN1) gene promoter leading to reduced gene and protein expression. We also show that presenilin-1 communoprecipitates with SERCA2a.

Conclusions—On the basis of these findings, we propose that 2 mechanisms may link protein aggregation and cardiac function: oligomer-induced changes on Ca2+ handling and a direct effect of PSEN1 sequence variants on excitation-contraction coupling protein function. (Circulation. 2010;121:1216-1226.)

Key Words: calcium ■ cardiomyopathy ■ genetics ■ heart failure ■ myocytes

In 1906, Alois Alzheimer discovered, in the brain of a patient suffering from early-age onset of dementia, aggregates of a proteinaceous material, later identified to be composed of amyloid fibers.1 Alzheimer disease (AD) is associated with several genetic defects and the abnormal accumulation of the amyloid-β protein (Aβ) and τ in the form of extracellular (senile plaques) and intracellular (neurofibrillary tangles) aggregates, respectively.2 In the heart, amyloid degeneration leading to dilated cardiomyopathy (DCM) has been limited to 3 conditions: (1) light-chain amyloidosis second- ary to multiple myeloma; (2) transthyretin cardiomyopathy3; and (3) desmin cardiomyopathy.4,5 In a smaller number of cases of nonischemic origin, the pathogenesis of heart failure (HF) is infective, toxic, or genetically determined. When 1 of these causative events cannot be recognized, the myocardial disease is classified as idiopathic DCM (iDCM).

Clinical Perspective on p 1226

Genetically, DCM has been found in only 35% of HF cases, indicating that the cause of this disease remains largely unknown.6 Mutations in genes encoding sarcomeric, cytoskeletal, and nuclear proteins as well as proteins involved in the regulation of Ca2+ homeostasis have been described.7,8 Allelic and locus heterogeneities occur in DCM,9 and these alterations, together with environmental factors, can disclose an otherwise silent genetic background as occurs in neurodegenerative diseases. Recently, 2 mutations in the familial early-onset AD-associated presenilin (PSEN1 D333G and PSEN2 S130L),2 have been found in 0.9% of tested DCM families (3/325).10 The presenilins (PS1 and PS2) are highly conserved polytopic membrane proteins that are required for γ-secretase activity, an enzyme responsible for proteolytic processing of the amyloid precursor protein to generate Aβ and a variety of membrane-associated proteins, including the Notch receptor.9 In the brain, presenilins also regulate Ca2+ in the endoplasmic reticulum and modulate ryanodine receptor function by cleavage of Sorcin.11 Both PSEN1 and PSEN2 are expressed in the heart and are essential for cardiac morphogenesis.12,13

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Because of the role that presenilins play in protein processing and Ca\textsuperscript{2+} homeostasis, the possibility was advanced that mutations in \textit{PSEN1} and \textit{PSEN2} may account for the cardiac phenotype found in a subset of patients affected by iDCM. These mutations may result in alterations of Ca\textsuperscript{2+} homeostasis and/or buildup of misfolded substrates, which alter myocyte mechanical behavior. In this study, we address whether the accumulation of protein aggregates and genetic alterations in the \textit{PSEN} and oligomeric fragments alter cardiomyocyte mechanics, Ca\textsuperscript{2+} homeostasis, and ultimately ventricular performance.

**Methods**

**Patients**

Myocardial tissue was collected from biopsy and explanted hearts from patients with iDCM or amyloid cardiomyopathies or from donor nonfailing hearts. All tissue from donor or transplant patients was collected in cold and oxygenated Wisconsin cardioplegic solution as soon as the heart was explanted from the patient. Nonfailing hearts were available from the National Disease Research Interchange supported by the National Institutes of Health. Tissues received as nonfailing hearts can be found to be unsuitable for transplantation for several reasons (eg, lack of identification of a suitable recipient, blood transfusion while in the emergency department, age of donor, need for resuscitation). All donations were made with family approval. Donor hearts ruled out from transplantation were used if no macroscopic, laboratory, or instrumental signs of cardiac diseases were present. The AD patients and non-AD affected family members were from the National Institute Genetics Initiative Alzheimer’s Disease Study Sample. Subjects were identified according to a standardized protocol applying the criteria of the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer’s Disease and Related Disorders Association for the diagnosis of AD.

**Sample Collection**

The hearts were dissected by regions, and the tissue was frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\). From the anterior wall, a branch of the left anterior descending artery was cannulated, and the tissue was perfused with 4\% paraformaldehyde-lysinum-sodium metaperiodate in 0.1 mol/L NaPO\textsubscript{4}. A sample of the left ventricular anterior wall of the hearts was also collected in 2.4\% glutaraldehyde for electron microscopy (EM).

**Structural Staining**

The frozen tissue was cut into 8-\textmu m slices. Samples were stained in a blinded fashion by the clinical pathology laboratory for protein aggregates with the use of modified Congo red\textsuperscript{14} or thioflavin-S. The Congo red sections were observed in random sequence under direct light as well as under polarized light. Polarized light microscopy could not, however, differentiate positive intracellular staining because of the natural anisotropy of the myosin filaments. Thioflavin-S slides were imaged with confocal microscopy (Leica TCS).

**Immunoblotting, Immunohistochemistry, EM, Immunogold EM, Polymerase Chain Reaction, Sequencing, and Luciferase Activity**

Standard techniques were employed for these methodologies and are detailed in the online-only Data Supplement. Specific aspects of the present study included the use of anti-oligomer (A11) antibody that recognizes a generic, sequence-independent epitope for prefibrillar amyloid oligomers at immunohistochemistry and EM. Anti-PS1 N-terminal and anti-\textalpha-B\textbeta antibody were used for immunohostaining. The entire coding and 5'-promoter regions of \textit{PSEN1} and \textit{PSEN2} genes were amplified from DNA for sequencing. Four pGL2 constructs were established containing \textit{PSEN1} \(-92C\) and \(-92delC\) and \textit{PSEN1} \(-21G\) and \(-21A\) for determining the transcriptional activity by luciferase reporter assay. The average transcriptional activities of \textit{PSEN1} \(-92delC\) variant versus \(-92C\) wild-type (WT) allele and \(-21G\) variant versus \(-21A\) WT allele from 4 repeated experiments were compared by Wilcoxon 2-sample test. A \textit{P} value <0.05 was taken to indicate statistical significance.

**Preparation of Homogeneous Populations of Amyloid Peptide Monomers, Oligomers, and Fibrils**

Soluble oligomers were prepared following the protocol kindly provided by Dr Charles Glabe. Ab\textbeta stock solutions (2 mmol/L) were obtained by dissolving the lyophilized peptide (from 1:1 H\textsubscript{2}O:acetonitrile) in hexafluorosopropanol and were sonicated. The resulting solution was added to double-distilled water in a siliconized Eppendorf tube. The solution was gently mixed and stirred, and the hexafluorosopropanol evaporated. Aliquots were taken at 6- to 12-hour intervals for the first 12 hours and then every 12 hours. Oligomers were obtained after 4 days. Oligomer formation was monitored by A11 dot blots. Annular protofibrils were obtained from the spherical oligomer preparation followed by the addition of 5\% hexane. Once fibril formation was complete, the solution was centrifuged, and the fibril pellet was washed 3 times with double-distilled water and then resuspended in phosphate-buffered saline. The morphology was verified by negative stain electron microscopy.

**Myocyte Oligomer Toxicity Measurements**

Adult myocytes were isolated from C57/B16 mice, and measurements were performed as described previously.\textsuperscript{15} Isolated cardiomyocytes were placed in a flow chamber on the stage of an inverted microscope, superfused with Tyrode solution, and electrically stimulated with a biphasic pulse (5 Hz, 50\% above threshold). After a stable contraction and Ca\textsuperscript{2+} transient were obtained, 0.5 or 5 \mu g of oligomer solution was applied on a cell by rapid release (ALA Scientific). In control cells, the buffer without oligomers was applied. Contraction amplitude and rates of contraction and relaxation were recorded online with the use of a video-edge detection system and data acquisition software (IonOptix Corp). The fluorescent Ca\textsuperscript{2+} indicator Fura-2 (Molecular Probes) was used to measure intracellular Ca\textsuperscript{2+}, as described previously.\textsuperscript{16}

**Results**

**Detection of Protein Aggregates**

To establish whether protein aggregates are present in iDCM, ventricular samples obtained from explanted failing (\(n = 23\)) and donor nonfailing (\(n = 12\)) hearts were stained for amyloid and amyloid-like structures. Samples from patients with cardiac amyloidosis (\(n = 6\)) were included as controls for the staining of misfolded proteins. Amyloid specific positive staining was detected in 17 of the 23 iDCM hearts (Figure 1A and 1B) and in 2 donor hearts. In the latter case, patients were aged 75 years, the oldest in the group.

To strengthen these observations and provide evidence that protein aggregation occurs in iDCM, fibrillar deposits were identified by EM within the myocyte cytoplasm and in the extracellular compartment (Figure 1C). The distribution of the protein aggregates resembled tangles and plaques commonly found in AD, respectively.\textsuperscript{17} To exclude the possibility that myocardial aging and end-stage HF were implicated in the accumulation of degradation products, biopsy samples collected from patients with early-stage iDCM (\(n = 5\)) were studied by EM. The mean age of our patient sample was 52 years (age range, 31 to 65) in the transplant population and 45 years (age range, 24 to 65) in the biopsy group (Table 1). Tangle- and plaque-like structures were found (Figure 1C and
1D), strongly suggesting that fibrillar deposits may constitute an integral component of the onset and progression of the pathology of iDCM.

Because in AD patients Aβ accumulates in the brain, tissue sections from all iDCM patients and controls were also tested for the levels of Aβ in the myocardium. We did not detect differences in the level of Aβ in iDCM patients compared with controls on immunohistochemistry (Figure 2).

**Oligomeric Assemblies and Their Function**

In the brain of AD patients, fibrillar deposits of β-amyloid represent the end product of misfolded peptides. Smaller oligomeric assemblies have been identified as soluble toxic aggregates that impair neural function.17,18 This observation raised the possibility that a similar mechanism may be operative in the failing heart, providing an unanticipated cause for at least a subset of patients affected by iDCM. Therefore, a structural antibody, specific for the recognition of oligomeric structure, first described in the brain of subjects with AD,17 was employed to detect oligomer fragments in iDCM hearts. We examined 6 cases of iDCM and 8 controls. Positive immunostaining was observed in 5 cases (Figure 3A) and in 37.5% of controls. A larger number of age- and harvest time–matched samples are required to assess the relative abundance of oligomers in the diseased hearts. Additionally, anti-oligo immunogold EM was utilized to confirm the

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**Figure 1.** A. Congo red staining of a case of senile amyloid (a and b), 2 nonfailing controls (c and d), and 3 cases of iDCM (e through h). B. Confocal microscopy of the thioflavin-S staining of iDCM (a and b), control (c and d), and light-chain (AL) amyloid cardiomyopathy (e and f). Panels a, c, and e show the sections under direct light; panels b, d, and f show the sections under fluorescence light. The light chain amyloid cardiomyopathy shows the distribution of the staining in the interstitial space, whereas in iDCM, the staining also occupies the cytosol of the myocytes. C. Electron micrographs of superthin sections from light chain amyloid cardiomyopathy (a and b) and iDCM left ventricle (c and d). Higher magnification of the fibrillar aggregates from light chain amyloidosis (b) and iDCM (d) is shown. The insert in panel d shows collagen fibers from the same heart for comparison. D. EM of myocardial diagnostic biopsies from iDCM patients showing extracellular aggregates (plaques) (panels a through c; panels b and c show higher magnification of panel a) and intracellular fibrils (tangles) (panels d through g). Panels e and g are higher magnifications of panels d and f, respectively.
| Congo Red/Genetically Tested | Explanted | Donors | Biopsies | |  | Heart Weight, g | Ejection Fraction, % | Associated Diseases | Medications |
|-----------------------------|-----------|--------|----------|---|---|---|---|---|---|---|---|
| 1/16                        | 53        | White female | 340    | 19 | Breast cancer | Diuretics, digitalis, BB, ACEI, antiarrhythmics, anticoagulants |
| 2                           | 60        | White male | 710    | 18 | Hypertension, gout, hyperlipidemia, hypotension | Diuretics, BB, digitalis, LL, nitrates |
| 3                           | 43        | White female | 320    | 15 | Anoxic brain injury | Diuretics, BB |
| 4                           | 63        | White female | 330    | 10 | Anemia, AF, MVR, IDDM, hypertension | Diuretics, aspirin, AB |
| 5/7                         | 56        | White male | 480    | 15 | NIDDM, PHT, AF | Diuretics |
| 6/8                         | 57        | White male | 620    | 20 | AF, DM, hypertension, hyperlipidemia | Diuretics, PEI, nitrates, LL, AII/RB, vitamin D |
| 7/9                         | 49        | White female | 420    | 20 | NIDDM, COPD, CRI | Diuretics, ACEI, nitrates, digitalis, inotropes |
| 8/10                        | 31        | Black female | 376    | 10 | Asthma | Diuretics, BB, ACEI |
| 9/11                        | 65        | Hispanic male | NR    | 15 | COPD | Diuretics, digitalis, ACEI, antiarrhythmics |
| 10/12                       | 48        | White male | 420    | 23 | Asthma, NIDDM, alcoholism | NA |
| 11/13                       | 54        | White male | 830    | 12 | Hypertension | Diuretics, digitalis, AII/RB, antiarrhythmics |
| 12/14                       | 45        | Hispanic male | 340    | 19 | Alcoholism, AF | Diuretics, CA, AB |
| 13/15                       | 44        | White male | 490    | 27 | POST TX | Diuretics, ACEI, nitrates |
| 14/17                       | 65        | White female | 570    | 10 | COPD, AF, alcoholism, hypotension | Diuretics, ACEI, digitalis, antiarrhythmics, LL |
| 15/18                       | 47        | White male | 470    | 15 | Hypertension, pulmonary hypertension, hyperlipidemia | Diuretics, ACEI, nitrates, digitalis |
| 16/19                       | 33        | White female | 590    | 23 | Hypertension, pulmonary hypertension, alcoholism | Diuretics, ACEI, digitalis |
| 17/20                       | 58        | White male | 535    | 22 | HTCM, gout, AF, CRI | Diuretics, digitalis, BB, aspirin, antiarrhythmics, anticoagulants |
| 18/21                       | 55        | White female | 250    | 22 | DM, Asthma, AF, pulmonary hypertension, hypotension | Diuretics, digitalis, ACEI, AII/RB |
| 19/22                       | 65        | White male | 520    | 41 | AFI, ASD, hyperlipidemia, CRI | Diuretics, BB, aspirin, LL, antiarrhythmics |
| 20/23                       | 28        | White male | 880    | 9  | Hypertension, NIDDM, AF, CRI | Diuretics, BB, ACEI |
| 21                          | 65        | White male | 600    | 9  | AF, hypertension | Diuretics, digitalis, BB, ACEI |
| 22                          | 43        | White female | 320    | 15 | None | Diuretics, BB, CA, AB |
| 23                          | 59        | White male | 450    | 18 | Hypertension | Diuretics, ACEI |

(Continued)
Table 1. Continued

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AF indicates atrial fibrillation; MVR, mitral valve regurgitation; IDDM, insulin-dependent diabetes mellitus; NIDDM, non–insulin-dependent diabetes mellitus; COPD, chronic obstructive pulmonary disease; CRI, chronic renal insufficiency; AFl, atrial flutter; ASD, atrial septal defect; MS, multiple sclerosis; NA, not applicable; BB, β-blocker; ACEI, angiotensin-converting enzyme inhibitor; LL, lipid-lowering drug; AB, α-blocker; PEI, phosphodiesterase inhibitor; AIRB, angiotensin II receptor blocker; PHT, pulmonary hypertension; POST TX, previous heart transplant; CA, calcium antagonists also known as calcium channel blockers; HTCM, myocardial hypertrophy; and MAOI, monoamine oxidase inhibitor.

presence of oligomeric peptide forms in biopsy samples of individuals in the initial phases of iDCM (Figure 3B). Similar pathology was found in the core of myocardium removed for left ventricular assist device implantation in a patient with end-stage iDCM.

However, these results leave unanswered the question of whether formation of abnormal oligomers in the heart depresses myocyte mechanics and thereby ventricular function. Because the toxicity of oligomers is linked to the structure rather than the polypeptidic sequence of the fragments,19 oligomers were generated from commercial lyophilized Aβ peptide (Figure 4A) to evaluate their effects on cardiomyocyte performance. Thus, freshly isolated adult mouse cardiomyocytes were exposed to oligomers (0.5 to 5 μg/mL) while Ca2+ transients and cell shortening were measured. Oligomers induced a sudden increase in peak systolic Ca2+ transient together with an enhanced velocity of Ca2+ release (Figure 4B).

Genetic Analysis

The coding region and promoters of PSEN1 and PSEN2 were sequenced in 20 patients with iDCM. In this report, we describe 4 variants in PSEN1 and PSEN2 genes (Table 2). Two novel PSEN1 5′ promoter region variants, −92delC (University of California–Santa Cruz Genome Chr 14 Database 72672839) upstream of alternative transcription initial exon 1A20 and −21G>A (University of California–Santa Cruz Genome Chr 14 Database 72673257) upstream of alternative transcription initial exon 1B, were recognized in patients 21 and 4, respectively (allele frequency 0.025) (Figure 5A, top panel). These variants were absent in our 14 controls. Patient 4 also possessed a previously reported PSEN1 missense mutation E318G21 (allele frequency 0.025). Additionally, a known PSEN2 missense mutation R62H22 (University of California–Santa Cruz Genome Chr 1 Database 225138072) was identified in patients 1 and 17 (allele frequency 0.05). The 4 patients who possessed PSEN1 promoter region variants or PSEN2 missense variants were white women.

The functional relevance of the new genetic variations was tested at the transcriptional level by luciferase assay and at the protein level by SDS-PAGE. We cloned the PSEN1 5′ promoter regions spanning −92delC and −21G>A variants into a reporter vector, and their transcriptional activity was measured. The constructs contained all known positive promoter elements that are required for PSEN1 expression, including 2 putative TATA boxes and 2 putative transcription initiation CAP sites upstream of exon 1A.23 In comparison to the basal level of PSEN1 promoter WT −92C allele, PSEN1 −92delC variant reduced transcriptional activity by 75% (P=0.002; Figure 5A, bottom panel). Constructs for PSEN1 −21G or −21A included sequences from the end of exon 1A to the end of alternative transcription initial exon 1B. With respect to −21A WT allele, PSEN1 −21G variant reduced transcriptional activity by 32% (P=0.002; Figure 5A, bottom panel). The construct containing PSEN1 WT −92C to alternative transcription initial exon 1A exhibited 5-fold higher transcriptional activity than the construct containing PSEN1 WT −21G to alternative transcription initial exon 1B. Consistently, these variants decreased the expression of PS1 protein in the myocardium (Figure 5B). Importantly, PS1 communoprecipitated with the cardiac isoform of the sarcoplasmic reticulum Ca2+-ATPase pump SERCA2a (Figure 5C), suggesting that changes in PS1 may be accompanied by defects in SERCA2a function, as were shown to occur in vitro for SERCA2b.24

Population Frequency

By single-nucleotide polymorphism analysis, PSEN1 −21G>A was not detected in 265 AD patients (0/530 chromosomes). PSEN1 −92delC was found in AD patients with an allele frequency of 0.005 (8/1632 chromosomes). PSEN1 E318G was previously detected in AD patients with an allele frequency of 0.005 (2/125832 chromosomes). PSEN1 R62H was segregated with AD affection status in AD patients from National Institute of Mental Health samples. PSEN2 R62H missense mutation was detected in AD patients with an allele frequency of 0.016 (5/320 chromosomes). The family members of identified AD patients who possessed PSEN1 −92delC, E318G, and PSEN2 R62H were also analyzed for segregation by single-nucleotide polymorphism genotyping. None of PSEN1 −92delC, E318G, or PSEN2 R62H was segregated with AD affection status in identified AD families.
Discussion

The mechanisms responsible for the onset of iDCM and its evolution to overt HF and death are currently unknown. The documentation in the present study that fibrillar deposits with the formation of tangle- and plaque-like structures within the myocardium occur in the early and late stages of the disease point to protein misfolding as an important determinant of the pathogenesis of iDCM. Protein misfolding impairs cell function in neurodegenerative disorders and other chronic diseases, suggesting that a similar effect may be operative in the human heart. Although the negative impact of protein aggregates on the mechanical and Ca²⁺-handling properties of human myocytes was not tested, these deposits modified the functional behavior of normal adult mouse myocytes, supporting the notion that similar consequences may occur on human myocytes.

Protein degradation characteristically occurs with myocardial aging. Senile systemic amyloidosis occurs in 25% of individuals aged ≥80 years. Amyloid-like deposits are described in nondiseased individuals between the sixth and ninth decades of life. In this report, aggregates were also markedly pronounced in relatively younger patients at early stages of HF, excluding age and disease stage accounting for protein misfolding in our iDCM population. On the other hand, the presence of protein aggregates, which are common in advanced age, may suggest that iDCM may be considered an early senility of the heart, similar to AD, in which the common senile dementia occurs at an early age.

We then evaluated whether the aggregates simply represent a tombstone of discarded material or relate to the disease. Protein aggregates are composed of misfolded proteins at various maturation stages, from monomeric peptides to intermediate oligomers and complex fibrillar structures. Interme-
mediate oligomers have been shown to be present in the brain of patients with AD and, in neurons, to be associated with cell toxicity.31,32 Here we showed that intermediate oligomers are also present in the myocardium of patients with iDCM, with a distribution similar to that observed in the brain of patients with AD.17 We also demonstrated for the first time that, on cardiomyocytes, oligomers promote an increase in systolic [Ca\(^{2+}\)] that may lead to cell dysfunction and death.33–35 An increase in cytosolic Ca\(^{2+}\) in the failing heart is coupled with Ca\(^{2+}\) depletion in the sarcoplasmic reticulum, which, in turn, favors protein misfolding.26,36,37 In the failing heart, Ca\(^{2+}\) dishomeostasis and contractile dysfunction are well-known abnormalities and are characterized by changes in excitation-contraction coupling proteins, with SERCA2a playing a key role.37

Several proteins involved in the control of Ca\(^{2+}\) cycling have been shown to be substrates of presenilin/γ-secretase.11 PS has been shown recently to interact with SERCA2b, attenuating its function when PS is downregulated.24 Importantly, PS1 also coimmunoprecipitates with SERCA2a in our iDCM cases, suggesting that PS1 may also depress SERCA2a, thereby contributing to the observed alterations in Ca\(^{2+}\) homeostasis and myocyte performance in HF. Sequencing of PSEN1 and PSEN2 led to the identification of 26 DNA variants, including 2 novel PSEN1 gene regulatory region variants, 92delC and 21G>A, in our iDCM patient sample. These 2 previously unknown variants significantly reduced mRNA and protein expression of PS1, indicating loss of function of the genetic variants. Thus, these variants likely influence Ca\(^{2+}\) homeostasis by altering SERCA2a function.

In our cases of sporadic iDCM, 2 PSEN missense mutations (PSEN1 D333G and PSEN2 R62H), previously described in familial DCM,10 were detected in addition to the variants in the PSEN promoter. A PSEN1 missense poly-
morphism E318G was also found. This mutation causes amino acid changes from polar acidic glutamic acid or aspartic acid to nonpolar neutral glycine, similar to the PSEN1 D333G mutation. E318G and D333G are located 15 amino acids apart within a “hot spot” in a large hydrophilic cytoplasmic loop between transmembrane domains 6 and 7.

This region contains critical functional domains that are processed by endoproteolysis and are essential for the interaction between PS1 and other proteins. The PSEN2 R62H mutation identified in patients 1 and 17 is located in a hot spot of the NH2 terminus and has been associated with AD and breast cancer. PSEN2 R62H enhances PS2

### Table 2. Sequence Variant Frequency Detected by DNA Sequencing Analyses of PSEN1 Gene in iDCM Patients

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<th>Gene</th>
<th>Variant</th>
<th>UCSC Genome Sequence Database (36.1)</th>
<th>SNP ID if Available</th>
<th>Minor Allele Frequency</th>
<th>Genotype Frequency (Variant/20 DCM Patients)</th>
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Values in parentheses are percentages. UCSC indicates University of California–Santa Cruz; SNP, single-nucleotide polymorphism; and ID, identification.
degradation, reduces PS2 stability, and compromises Notch function.40

The 2 presenilin missense substitutions, E318G and R62H, observed in iDCM are present in highly divergent regions of PS1 and PS2; PS1 and PS2 proteins share extensive sequence identity along their entire length with the exception of the NH2 terminal domain and the second half of the hydrophilic loop region. Thus, the E318G and R62H missense substitutions may play an important role in the pathogenesis of a subset of patients with iDCM by modulating different functions of the presenilin proteins. An important aspect is whether PSEN variants could affect amyloid precursor pro-
tein processing in the heart and determine the accumulation of amyloid aggregates. Because the sequence variants we observed are loss of function, any effect on amyloid precursor protein processing would reduce Aβ production. Aβ level was tested by immunohistochemistry in our samples, and no difference in Aβ expression was present in iDCM compared with controls.

Four of the 7 white women with iDCM possessed either a PSEN1 promoter region variant or a PSEN2 missense mutation. This gender-dependent cardiac genotype is consistent with the higher prevalence of AD in women than in men. Estrogen deficiency, which is typical in postmenopausal women, may be implicated because cardiomyocytes and fibroblasts contain estrogen receptors, and estrogens regulate the expression of specific cardiac genes.

In conclusion, we have provided the first evidence that protein misfolding is implicated in the pathogenesis of iDCM in humans. The formation of oligomeric fragments has toxic consequences on cardiomyocytes, which are mediated by alterations in Ca2+ homeostasis. Additionally, missense mutations in the PSEN1 and PSEN2 genes alter presenilin expression and may alter its interaction with proteins involved in excitation-contraction coupling. Further studies in a larger cohort and in animal models will be needed to evaluate the role and mechanisms of these sequence variants and the respective protein expression in the familial and sporadic cases compared with the nonfailing population. Furthermore, cardiac function should be assessed in AD patients and in family members unaffected by AD. The elucidation of these new pathogenetic mechanisms will facilitate the advancement of novel strategies for early diagnosis and treatment of iDCM.

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Disclosures

None.

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CHLINAL PERSPECTIVE

Heart failure is a progressive and ultimately fatal disease, which represents a leading public health problem worldwide. Despite substantial advances in the clinical management of heart failure, the only current permanent therapeutic option is heart transplantation. However, the limited supply of functional organs for transplantation and the age threshold for the surgical intervention constrain the scope of this treatment modality. The majority of cases of heart failure are ischemic in origin. The second most frequent origin of nonischemic heart failure is “idiopathic” dilated cardiomyopathy, in which no other causative events can be recognized. Recent studies highlight the recognition of the public health importance of a large number of diseases associated with defects in the ability of proteins to fold, leading to the accumulation of cytotoxic protein deposits. This group of diseases includes amyloidosis and various neurodegenerative disorders such as Alzheimer disease. Preliminary evidence suggests that idiopathic dilated cardiomyopathy may be included among these misfolding diseases. In addition, mutations in the same genes causing Alzheimer disease in a significant percentage of cases can also be at the origin of idiopathic dilated cardiomyopathy. Interestingly, a dual mechanism seems to mediate the effect of these genetic variations: (1) changes in the control of Ca2+ − handling proteins causing defect in the contractile function and (2) cell damages associated with the protein aggregation process. The incidence, prognosis, and therapeutic options for idiopathic dilated cardiomyopathy may therefore be greatly advanced by establishing a fundamental understanding of key factors leading to the disease.
Protein Aggregates and Novel Presenilin Gene Variants in Idiopathic Dilated Cardiomyopathy

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SUPPLEMENTAL MATERIAL

Immunohistochemistry:
5µm frozen sections formalin post-fixed were blocked in PBS/BSA 5% for 15 minutes and stained for 90 minutes at room temperature using anti-oligomer (A11) antibody that recognizes a generic, sequence independent epitope specific to prefibrillar amyloid oligomers (Biosource, AHB0052, 1:400). The antigen/protein complexes were visualized using fluorochrome-conjugated secondary antibody (Invitrogen, 1:1000, 45 minutes at RT) and counterstained with DAPI (Invitrogen, 1:1000). The slides were observed on a Zeiss confocal microscope. 8µm frozen sections were also fixed with buffered paraformaldehyde and pretreated with formic acid (according to the manufacturer guidelines) before exposing to anti-Aß antibody (Covance 6E10). The antigen/protein complexes were also visualized using fluorochrome-conjugated secondary antibody and counterstained with DAPI.

Immunoblotting:
The protein concentration of tissue lysate preparations were measured using the Bradford method. Immunoblotting was performed under reducing condition on a gradient gel using standard techniques. For immunoreactions, the blots were incubated with antibodies for anti-PS1 N-terminal. GADPH was used for normalization against total proteins (Zymed, 39-8600). The blots were then incubated in a solution containing HRP conjugated IgG (DAKO anti-mouse IgG, p0161, anti-rabbit IgG, 0449, 1:2000).

Electron microscopy:
Human ventricular tissue fixed with 2% glutaraldehyde was dehydrated, infiltrated with EPON-812 resin and embedded in capsules. The enclosed tissue was cut on a Reichter Ultracut E ultramicrotome in super thin sections. The sections was collected onto formvar-coated slot grid, post stained with uranyl acetate and lead citrate and viewed in a transmission electron microscope (JEM 1011 transmission electron microscope with digital acquisition of images) at 80 kV.

**Immunogold staining for electron microscopy (EM) using anti-oligomer antibodies:**
PLP fixed human heart samples were dehydrated through a graded series of ethanol and embedded in LR with resin, medium grade (EMsciences, Hatfield, PA) at 50°C overnight. Thin sections were cut on a Reichert Ultracut E ultra microtome and collected on formvar-coated gold grids. For the immunostaining the sections were blocked with 5% goat serum then incubated for 1 hr at room temperature with anti-oligomer (A11) antibody (Biosource AHB0052). The antibodies were labeled with IgG gold, 10 nM at the final concentration of 1:20 in DAKO diluent for 1 hr at room temperature. The grids were rinsed and stained on drops of 2% aqueous uranyl acetate for 5 minutes, rinsed and dried. The sections were examined at 80 kV in a JEOL 1011 TEM equipped with an ATM digital camera.

**PCR and sequencing**
Heart tissue samples for DNA analysis were obtained from iDCM patients. Lymphoblast cells lines were obtained from AD patients and non-AD controls. Genomic DNA was extracted using QIAClamp mini DNA isolation kits (Qiagen). The entire coding and 5’ promoter regions of PSEN1 and PSEN2 gene were amplified from DNA. Primers were designed on the basis of the annotated genomic sequence across each exon, including
regions of at least 20–40 bp of flanking intervening sequence. PCR was performed in 30-µl reaction volumes using DNA Engine (PTC-200) from MJ Research (now Bio-Rad). PCR products were directly sequenced on an ABI 48-capillary Prism 3730 DNA analyzers (PE Applied Biosystems). Sequence electropherograms were compared with gene sequence from GenBank (http://www.ncbi.nlm.nih.gov/, NCBI Build 36.1), the UCSC Human Genome Database (http://genome.ucsc.edu, The March 2006 Human Reference Sequence) and control samples. The allele frequency of detected variants in \( PSEN1 \) and \( PSEN2 \) gene in AD patients was screened by single-nucleotide–polymorphism (SNP) genotypes with the use of the fluorescence polarization–detected single-base extension method on a Criterion Analyst AD high-throughput fluorescence detection system (Molecular Devices). SNP data from DCM patients were also compared with allele frequency and heterozygosity for Caucasian controls from the NCBI SNP database (the CEPH collection, Utah residents with ancestry from northern and western Europe).

**Relative luciferase activity measurements:**

The promoter region spanning the 72672641-72673058 (UCSC Genome Database) was PCR amplified from the DCM patient with \( PSEN1 \) -92C/delC variant and the promoter region spanning the 72673065-72673530 was PCR amplified from the DCM patient with \( PSEN1 \) –21G>A variant. The PCR fragments were confirmed by sequence analyses and cloned into the pGL2 promoter-less Basic Luciferase Reporter Vector (Promega). Four pGL2 constructs were established containing \( PSEN1 \)-92C, -92delC and \( PSEN1 \) -21G and -21A, respectively. Human H4 naïve neuroglioma cells were cultured in Dulbecco’s
modified Eagle’s medium (high glucose) containing 9% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine. For transient transfection, H4 cells were seeded in 24-well tissue culture plate at 8x10^4 cells/well and co-transfected each appropriate construct together with constitutive Renilla construct (Promega) for controlling transfection efficiency using Lipofectamine-2000 according to manufacturer’s instructions (Invitrogen). Firefly and Renilla luciferase activities were quantified sequentially using the Dual Luciferase Assay System (Promega) in a Turner TD20-20 Luminometer. The promoter transcriptional activity was expressed as the ratio of the Firefly luciferase activity of the promoter construct to the constitutive Renilla activity and normalized using baseline Firefly luciferase activity from pGL2 promoter-less Basic Luciferase Reporter Vector alone. Results were presented as percentage change (mean±standard deviation) of variant constructs (PSEN1-92delC and -21A) in comparison with wild-type control constructs (PSEN1-92C or -21G), arbitrarily designated as 100%. Each experiment was repeated 4 times and experiment results were averaged. Statistical analysis was performed using Wilcoxon Two Sample Test.