Patterns of desmin expression in idiopathic dilated cardiomyopathy are related to the desmin mRNA and ubiquitin expression

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ABSTRACT

Desmin expression depends on desmin messenger RNA (mRNA) and ubiquitin proteasome system. This process is poorly understood in dilated cardiomyopathy. The aim of the study was to investigate whether changes of desmin mRNA and ubiquitin expression correlate with types of desmin expression in cardiomyocytes. Endomyocardial biopsy was performed in 60 patients (85% men, mean age 46±14 years) with heart failure (HF; left ventricular ejection fraction <45%). Desmin and ubiquitin expression were analysed in histological sections by immunohistochemistry and in Western blot. Desmin mRNA expression was determined by fluorescent in situ hybridization methods. In patients with weak/even desmin expression, weak/even expression of ubiquitin in the cytosol and low desmin mRNA expression in the cytosol and nuclei of cardiomyocytes were observed. Expression of ubiquitin and desmin mRNA increased along with the progression of desmin cytoskeleton remodeling. Desmin mRNA and ubiquitin were weakly expressed/absent in cardiomyocytes with low/lack of desmin expression. Variations in desmin mRNA, desmin and ubiquitin expression were associated with gradual changes in myocardial structure and clinical parameters. To conclude, changes in ubiquitin and desmin mRNA expression are related to patterns of desmin expression. An increase in the expression of ubiquitin and desmin mRNA may be a protective feature against unfavorable cell remodeling. This may reduce the adverse effects of cytoskeleton damage in the early stages of HF. Low/lack ubiquitin and/or desmin mRNA expression may be markers of end-stage HF.

Significance of this study

What is already known about this subject?

- Desmin is one of the most important cytoskeletal, intermediate filament proteins which increased expression and aggregation as well as decreased expression have been reported in various heart diseases (dilated cardiomyopathy, hypertrophic cardiomyopathy, myocarditis) and have proved crucial for the prognosis of patients with idiopathic dilated cardiomyopathy (IDCM).
- The conjugation of ubiquitin with proteins in the ubiquitination process plays an essential role in nearly all aspects of cell function, including compensatory cellular responses. An increase of myocardial ubiquitinated proteins in patients with IDCM has been reported.

What are the new findings?

- Enhanced expression of desmin mRNA and ubiquitin in cardiomyocytes appears to be a protective mechanism, compensating for the increased desmin cytoskeleton turnover in the early stages of heart failure and for the disorganization of the desmin network in the more advanced stages of heart failure.
- The absent or decreased desmin mRNA and ubiquitin expression in end-stage IDCM, with progressive decreases in desmin expression, might imply the decompensation of heart failure.
- An evaluation of the elements that play a role in desmin turnover in the context of cell structure and clinical presentation helps to shed some light on understanding the complex pathophysiological processes that regulate desmin expression in heart failure.

How might these results change the focus of research or clinical practice?

- These findings point to a previously undescribed therapeutic target that may have application in the treatment of heart failure which is often caused by disturbance of protein expression.

INTRODUCTION

Idiopathic dilated cardiomyopathy (IDCM) is characterized by a progressive increase in hemodynamic load and worsening ventricular dilatation. In the early stages of the disease, compensatory mechanisms are initiated.[1] In the end stages of IDCM, an inability to maintain these compensatory defence mechanisms is observed.

Desmin is one of the most important cytoskeletal, intermediate filament proteins.[2 3] It plays an essential structural, mechanical and

regulatory role in cardiac integrity.[4] It is a dynamic structure, which undergoes constant cycles of destruction and renewal.[5] Alterations of desmin, including changes in desmin mass and remodeling of its cytoskeleton, are known cellular compensatory mechanisms.[6–8] Increased expression and aggregation of desmin as well as decreased expression of desmin have been reported in various heart conditions (dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy, myocarditis), and have been shown to be crucial factors for the prognosis of patients with IDCM.[5 9 10] Therefore, both identifying and understanding the mechanisms that regulate desmin expression and localization in different stages of heart failure (HF) are key, in order to elucidate potential targets for therapy in the pathophysiological process of IDCM.

Desmin missense mutations in humans have previously been reported, and result in restrictive cardiomyopathy or IDCM.[9 10] Animal models with mutations of the desmin gene develop cardiomyopathy with degeneration of cardiomyocytes, fibrosis and ultrastructural defects in the cardiac muscle.[2 5 10] These changes have been shown to reduce the affected animals' life span, making them less exercise tolerant.[2 11 12] The crucial role of ubiquitin proteasome system in homeostatic protein turnover in the myocardium for proper cardiac function is widely accepted.[13] Ubiquitin, a small modifier protein, tags proteins that are either abnormal or no longer needed, for degradation via the proteasome.[14 15] The conjugation of ubiquitin with proteins in the ubiquitination process plays an essential role in nearly all aspects of cell function, including compensatory cellular responses. An increase in myocardial ubiquitinated proteins in patients with IDCM has been reported.[16] Cohen *et al* proved the possibility of desmin ubiquitination during the rapid atrophy induced by fasting.[17]

In our study, we tested the hypothesis that different levels of desmin mRNA expression, and changes in ubiquitin expression, may coexist with different patterns of desmin expression and localization in cardiomyocytes of patients with IDCM in various stages of HF. We studied the relationship between the expression of desmin, desmin mRNA and ubiquitin, as well as cardiomyocyte structural changes, biochemical and clinical parameters in patients with IDCM.

MATERIALS AND METHODS

Study population and clinical assessment

Baseline examinations were performed between January 2012 and January 2014. Patients with IDCM were enrolled prospectively and consecutively in the study. Inclusion criteria were: (1) age >18 years; (2) left ventricular ejection fraction (LVEF) <45% as assessed by echocardiography; (3) without significant coronary artery stenosis on coronary angiography (defined as the presence of any stenotic lesion with >50% reduction in lumen diameter); (4) without familial DCM and systemic diseases; (5) without myocarditis, viral genome in cardiac tissue and infiltrative DCM. Clinical, laboratory and echocardiographic assessments were performed on admission. Serum N-terminal pro-brain natriuretic peptide (NT-pro-BNP, pg/mL) concentration was measured using the Cobas e411 System (Roche Diagnostic) device, using immunoassay based on electroimmunofluorescence.

Table 1 Clinical characteristics of the study groups

Variables	Study population (n=60)
Gender, male (%)	51 (85)
Age (y)	46±14
BMI (kg/m^2)	26±5
NYHA class, n (%)	
Class I	16 (27)
Class II	27 (45)
Class III	12 (20)
Class IV	4 (7)
LVEF (%)	34±14
LVEDD (mm)	64±14
NT-pro-BNP (pg/mL)	1420 (331–2705)
Comorbidities, n (%)	
Hypertension	20 (33)
Diabetes mellitus	4 (6)
Hypercholesterolemia	6 (9)
Medication, n (%)	
ACE-I	53 (83)
ARB	6 (9)
B-blockers	52 (81)
Aldosterone antagonists	46 (72)
Diuretics	32 (50)

Data shown as mean±SD, median (with lower and upper quartiles) or number (with percentages) where appropriate.
ACE-I, ACE inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; NT-pro-BNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association.

Transthoracic echocardiography examination was carried out using a commercial diagnostic ultrasound system equipped with a 3.5 MHz transducer (iE 33; Philips Medical System, Best, The Netherlands). All echocardiographic measurements, including left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter and LVEF, were performed according to the guidelines of the European Society of Echocardiography[18] by an experienced cardiologist blinded to the patients' clinical history. LVEF was calculated using the Simpson's biplane method.[19]

Left ventricular endomyocardial biopsy was performed using a 7F bioptome (Cordis; Johnson & Johnson, New Brunswick, NJ, USA). The procedure was carried out under continuous ECG monitoring. Six tissue samples were collected from each patient. Eight hearts from donors with normal left ventricular function (that were not used for transplantation) served as controls.

The study was conducted in accordance with the Declaration of Helsinki. The characteristics of the entire study population are presented in table 1.

Histopathology

Two tissue samples from each patient were evaluated histologically with H&E, Masson's trichrome (TM), desmin and ubiquitin stains. Cardiomyocyte and nucleus hypertrophy were assessed in tissue sections stained with H&E and tissue fibrosis was assessed in tissue sections stained with TM with the use of morphometric image analysis software (CellP; Olympus, Hamburg, Germany). Tissue fibrosis was

measured, and automatically calculated as the percentage of the total defined tissue section. Images of whole sections were taken under the same lighting conditions, using the same optical settings with a ×4 objective.

Immunohistochemistry

Samples were first incubated with desmin monoclonal mouse anti-human antibody (1:50; DAKO, Glostrup, Denmark, Cat No: IR606) or ubiquitin polyclonal rabbit anti-human antibody (1:150; DAKO, Cat No: Z0458), and then with a horseradish peroxidase–conjugated goat anti-mouse antibody (En Vision System HRP; DAKO). Negative controls were obtained by omitting incubation with the primary anti-desmin or anti-ubiquitin antibodies.

Image analysis was performed using the same lighting conditions and optical settings for each sample. Immunohistochemical staining of desmin revealed 4 types: (1) type I: normal, gentle staining of desmin at Z-lines and intercalated disks, giving a regular cross-section pattern; (2) type IIA: increased, intensive staining of desmin at Z-lines and intercalated disks, giving regular cross-striation pattern; (3) type IIB: increased, intensive staining of desmin with an irregular cross-striation pattern and/or with the presence of aggregates in perinuclear areas and, occasionally, in intermyofibrillar spaces; and (4) type III: decreased or lack of desmin staining. The full classification of desmin expression in cardiomyocytes was previously published.[5] Immunohistochemical staining of ubiquitin revealed 4 types of ubiquitin expression: (1) type A: gentle, evenly scattered expression of ubiquitin in the cytoplasm of cardiomyocytes; (2) type B: increased, evenly scattered expression of ubiquitin in the cytoplasm of cardiomyocytes; (3) type C: increased, unevenly scattered expression of ubiquitin in the cytoplasm of cardiomyocytes and its increased, evenly scattered expression in nucleus; and (4) type D: weak/lack of expression of ubiquitin (figure 1). The dominant immunohistochemical pattern of desmin or ubiquitin staining in tissue sections determined the type of cytoskeletal desmin or ubiquitin expression. Two independent pathologists evaluated two sections prepared from each biopsy sample.

Immunoblotting

The total individual protein levels in the biopsy samples were analysed using Western blotting. The biopsy samples from patients were kept at –80°C, subsequently were defrost, cut into small pieces and homogenized directly in prechilled 0.05 mL of lysis buffer. Cardiac tissue protein was harvested using a cell lysis buffer (150 mmol/L sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 50 mmol/L Tris, pH 8.0) supplemented with 1× protease inhibitor cocktail (PIERCE). The samples were incubated on ice for 20 minutes with frequent tapping. Then, heart lysates were centrifuged at 14,000 ×*g* for 15 minutes to remove unsolubilized material. The protein amount in supernatants (containing total protein cell extract) was quantified using standard Bradford assay (Bio-Rad). Total protein (20 µg) was separated by 10% SDS-polyacrylamide gel electrophoresis. Western blotting was performed using: desmin monoclonal mouse anti-human antibody (1:500; DAKO, Cat No: IR606), ubiquitin polyclonal rabbit anti-human antibody (1:1000; DAKO, Cat No: Z0458), and rabbit monoclonal anti-smooth muscle actin antibody (1:100; Abcam, Cambridge, MA, USA, Cat No: ab7817).

The signal was revealed using alkaline phosphatase (AP) conjugate substrate kit (Bio-Rad, Hercules, CA, USA) after hybridization with goat anti-mouse IgG (H+L) (Bio-Rad) AP-conjugated secondary antibodies or goat anti-rabbit IgG (H+L) (Bio-Rad) AP-conjugated secondary antibodies. The membranes were scanned using VersaDoc Imaging System (Bio-Rad). Band densities were analysed using Quantity One software (Bio-Rad).

Fluorescent in situ hybridization

Two tissue samples were postfixed in 4% paraformaldehyde (PFA) (Sigma-Aldrich) in phosphate buffered saline (PBS) for 24 hours, cryoprotected with 30% sucrose (Sigma-Aldrich), and snap-frozen on dry ice. In situ hybridization was performed on 10 µm thick sections on poly-prep slides (Sigma-Aldrich). The coding sequence of human desmin mRNA in pOTB7 plasmid was purchased from imaGenes (No: IRAVp969H0179D). Sense and antisense fluorescein

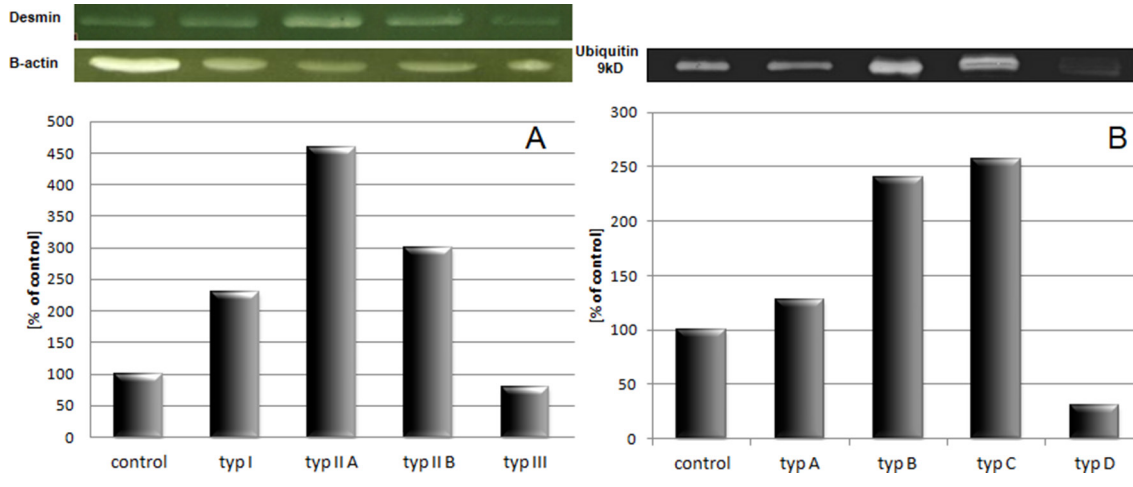

Figure 1 Western blot analyses showing: (A) desmin content in different types of desmin expression in patients with idiopathic dilated cardiomyopathy (IDCM), (B) ubiquitin content in different types of ubiquitin expression in patients with IDCM.

labeled riboprobes were generated from plasmids with SP6 or T7 polymerase sites. Specimens were fixed in 4% PFA, washed in PBS and incubated in acetylation buffer (0.1 M triethanolamine, 0.25% HCl, 0.25% acetic anhydride). Next, sections were prehybridized for 3 hours in prehybridization solution (Sigma-Aldrich), followed by overnight hybridization at 70°C in hybridization solution (Sigma-Aldrich) containing desmin sense or antisense probes. Afterwards, sections were washed in 0.2× saline-sodium citrate buffer (SSC), with the first 2 washes carried out for an hour in 70°C, followed by 5 consecutive washes for 30 minutes in room temperature (RT). Subsequently, TNB blocking solution (TSA Plus System, Perkin Elmer) was applied for an hour. Tissue samples were incubated with anti-fluorescein-POD (Roche Applied Science) 1:200 and desmin monoclonal mouse anti-human antibody (1:50; DAKO) overnight in 4°C, washed with PBST and incubated with secondary antibody, 1:1000 Alexa 488-conjugated anti-mouse IgG (Invitrogen). Hybridization signal was amplified with Cy3 TSA Plus System (Perkin Elmer). Analysis revealed different patterns of desmin mRNA expression in cardiomyocytes: (1) type 1: even but weak expression in both the cytosol and nucleus; (2) type 2: uneven but intensive expression, with more frequent localization in the nucleus than in cytosol; (3) type 3: intensive expression in the cytosol, with similar or weaker expression in the nucleus; (4) type 4: very low expression in the cytosol, and absent expression in the nucleus.

Statistical analyses

Continuous variables were expressed as mean±SD. Continuous variables with a skewed distribution were expressed as median with lower and upper quartiles. Categorical variables were expressed as percentages. Intergroup differences were tested using the Student's t-test between 2 groups, whereas analysis of variance was used for comparisons between multiple groups. Frequencies were compared using the X^2 test. Pearson's correlation coefficients were calculated between New York Heart Association (NYHA) class, NT-pro-BNP levels, LVEDD, and LVEF for each type of desmin expression. All tests were two sided with a significance level of $p<0.05$. A commercial statistical package (SPSS V.13.0; IBM) was used for all statistical analyses.

RESULTS

Histopathological analyses of desmin and ubiquitin expression

In our study population, type I desmin expression in cardiac tissue was observed in 9 (15%) patients, while types IIA, IIB and III desmin expression were observed in 23 (38%), 20 (33%) and 8 (13%) patients, respectively. Type A ubiquitin expression was present in 9 (15%) patients, while types B, C and D were present in 24 (40%), 19 (32%) and 8 (13%) patients, respectively. Desmin mRNA was evaluated in 40 patients. Type 1 desmin mRNA expression was found in 6 (15%) patients; types 2, 3, and 4 in 8 (45%), 10 (25%), and 6 (15%), respectively.

In patients with normal desmin expression (type I), immunohistochemistry revealed predominantly weak ubiquitin expression (type A) in 89% and weak desmin mRNA expression (type 1) in 100%. Ubiquitin and desmin mRNA expression increased along with the progression of desmin cytoskeleton remodeling. Patients with compensatory/adaptive an increase in desmin network (type IIA) presented an increase in ubiquitin expression in the cytoplasm of cardiomyocytes (type B) in 87% and an increase in desmin mRNA expression mainly in nucleus (type 2) in 100%. Patients with degenerative/maladaptive an increase in desmin network (desmin expression type IIB) displayed also increased expression of ubiquitin in cytoplasm and nuclei (type C) in 90% and increased expression of desmin mRNA mainly in cytosol (type 3) in 91%. An absent or only weakly expressed ubiquitin (type D) and desmin mRNA (type 4) were observed in biopsy samples collected from patients with decreased or absent desmin expression (type III) in 87% and 100%, respectively (tables 2 and 3).

Using tissue samples from patients with IDCM, double labeling fluorescent immunohistochemistry was applied to localize the immunoreactivity for desmin and desmin mRNA. This method clearly revealed the colocalization of changes in desmin and desmin mRNA (figure 2).

Content of desmin and ubiquitin in cardiomyocytes in Western blot

Differences in desmin and ubiquitin content were confirmed by Western blotting. When compared with type I desmin expression, the content of desmin increased by 100% and 30% in types IIA and IIB desmin expression, respectively, and decreased by 65% in type III of desmin expression (figure 1). The content of ubiquitin in biopsy samples from patients with IDCM increased by 91% and 101% in types B and C, respectively, and decreased by 76% in types D and A, respectively.

Cardiomyocyte diameter and fibrosis according to types of desmin and ubiquitin expression

The mean cardiomyocyte diameters were 19.5, 26.1, 28.6 and 29.4 µm in types A, B, C and D ubiquitin expression,

Table 2 Different patterns of desmin expression in biopsy samples of patients with different types of ubiquitin and desmin mRNA expression

	Ubiquitin (n/%)				Desmin mRNA (n/%)			
	Type A 9/15	Type B 24/40	Type C 19/32	Type D 8/13	Type 1 6/15	Type 2 18/45	Type 3 10/25	Type 4 6/15
Desmin (n/%)								
Type I (9/15)	8/89	1/11	0	0	6/100	0	0	0
Type IIA (23/38)	1/4	20/87	1/4	1/4	0	17/100	0	0
Type IIB (20/33)	0	2/10	18/90	0	0	1/9	10/91	0
Type III (8/13)	0	1/13	0	7/87	0	0	0	6/100

Table 3 Types of expression and localization of desmin, ubiquitin and desmin mRNA in cardiomyocytes of patients with IDCM

	Types of expression and localization			
Desmin				
Type	I	IIA	IIB	III
Localization	Cytoplasm	Cytoplasm	Cytoplasm	Cytoplasm
Expression	Even, weak	Intensive	Intensive, aggregates	Weak/absent
Ubiquitin				
Type	A	B	C	D
Localization	Cytoplasm	Cytoplasm	Cytoplasm and nucleus	Cytoplasm
Expression	Even, weak	Intensive	Intensive	Weak/absent
Desmin mRNA				
Type	1	2	3	4
Localization	Cytoplasm and nucleus	Cytoplasm and nucleus	Cytoplasm and nucleus	Cytoplasm
Expression	Even, weak	Uneven, and mainly in the nucleus	Even, intensive	Weak/absent

IDCM, idiopathic dilated cardiomyopathy.

respectively. In biopsy samples, mean fibrosis was 13.2%, 16.4%, 26.8%, and 26.9% of area slides in types A, B, C, and D ubiquitin expression, respectively (figure 3). The mean cardiomyocyte diameters were smaller in all types of desmin expression in comparison with the corresponding types of ubiquitin expression, and they were 18.0, 25.8, 29.1, and 30.9 µm in types I, IIA, IIB, and III of desmin expression, respectively. The analysis of fibrosis in different types of desmin expression also showed smaller area percentages of fibrosis, when compared with corresponding types of ubiquitin expression, and they were 12.9%, 19.3%, 24.1%, and 29.1% in types I, IIA, IIB, and III of desmin expression, respectively (figure 3).

Clinical presentation in the different types of desmin and ubiquitin expression

Clinical (NYHA class), biochemical (NT-pro-BNP level) and echocardiographic (LVEF and LVEDD) parameters were similar among patients with corresponding types of desmin, ubiquitin and mRNA expression. Statistically significant correlations were observed between the type of desmin expression and NYHA class, NT-pro-BNP, LVEF and LVEDD, while ubiquitin and desmin mRNA expression correlated significantly with NT-pro-BNP level, LVEF and LVEDD (table 4).

DISCUSSION

Despite extensive literature on the essential role of desmin in IDCM, desmin turnover in cardiomyocytes in consecutive stages of HF is not clear.[3 5] We analysed the relationship between changes in desmin mRNA expression, desmin cytoskeleton remodeling and ubiquitin expression in failing human hearts. In the present study, immunohistochemistry revealed for the first time: (1) different patterns of enhanced expression of desmin mRNA and ubiquitin in cardiomyocytes, which appeared to be a protective mechanism, compensating for the increased desmin cytoskeleton turnover (in early stages of HF) and the progression in disorganization of desmin network (in late stages of HF), and (2) the absent or decreased desmin mRNA and ubiquitin expression in end-stage IDCM, with progressive decreases in desmin expression, which might correlate with a pathophysiologically decompensated state of the human heart. An evaluation of the elements that play a role in desmin turnover, in the context of cell structure and clinical presentation, helps shed light in understanding the complex processes involved in HF.

Patterns of ubiquitin expression

Changes in ubiquitin expression have previously been described in both experimental models and in patients with IDCM.[20 21] To our knowledge, this is the first study to systematically assess the distribution and expression of ubiquitin classified according to various types (A–D), correlating with desmin and desmin mRNA expression in patients with IDCM.

Ubiquitin expression followed by ubiquitination plays both protective and adaptive roles by modifying growth and transcription factors, preventing protein aggregation, which would be harmful to the cardiomyocyte, and inhibiting apoptosis by ubiquitination of caspases.[1 13 20 22 23] These processes contribute towards the remodeling and hypertrophic growth of cardiomyocytes. Weekes *et al* indicated that total protein-ubiquitin conjugation in IDCM was 2-fold higher than that seen in ischemic heart disease samples and 5-fold greater than in control donor heart tissue.[16] Moreover, the recently discovered ligase TRIM32 suggests the possibility of post-translational modification of desmin by ubiquitin.[17 20] The colocalization and simultaneous increase in the expression of desmin and ubiquitin observed in our study may indicate desmin ubiquitination. An equal increase in both ubiquitin expression (type B) and desmin expression (type IIA) could be a compensatory mechanism in the early phases of compensated IDCM. Interestingly, increased expression of ubiquitin was found in cells with protein aggregates, as was previously described, and in cardiomyocytes without protein aggregates.[20] A significant increase in ubiquitin content was also confirmed by Western blotting.

It has been reported that, in response to cellular stresses, liable proteins are modified, and that most modified proteins easily misfold, aggregate and form intracellular inclusions, where ubiquitin accumulates.[24 25] Thus, the ubiquitin-positive granular structures observed in cardiomyocytes are considered to be aggregates of modified proteins, which

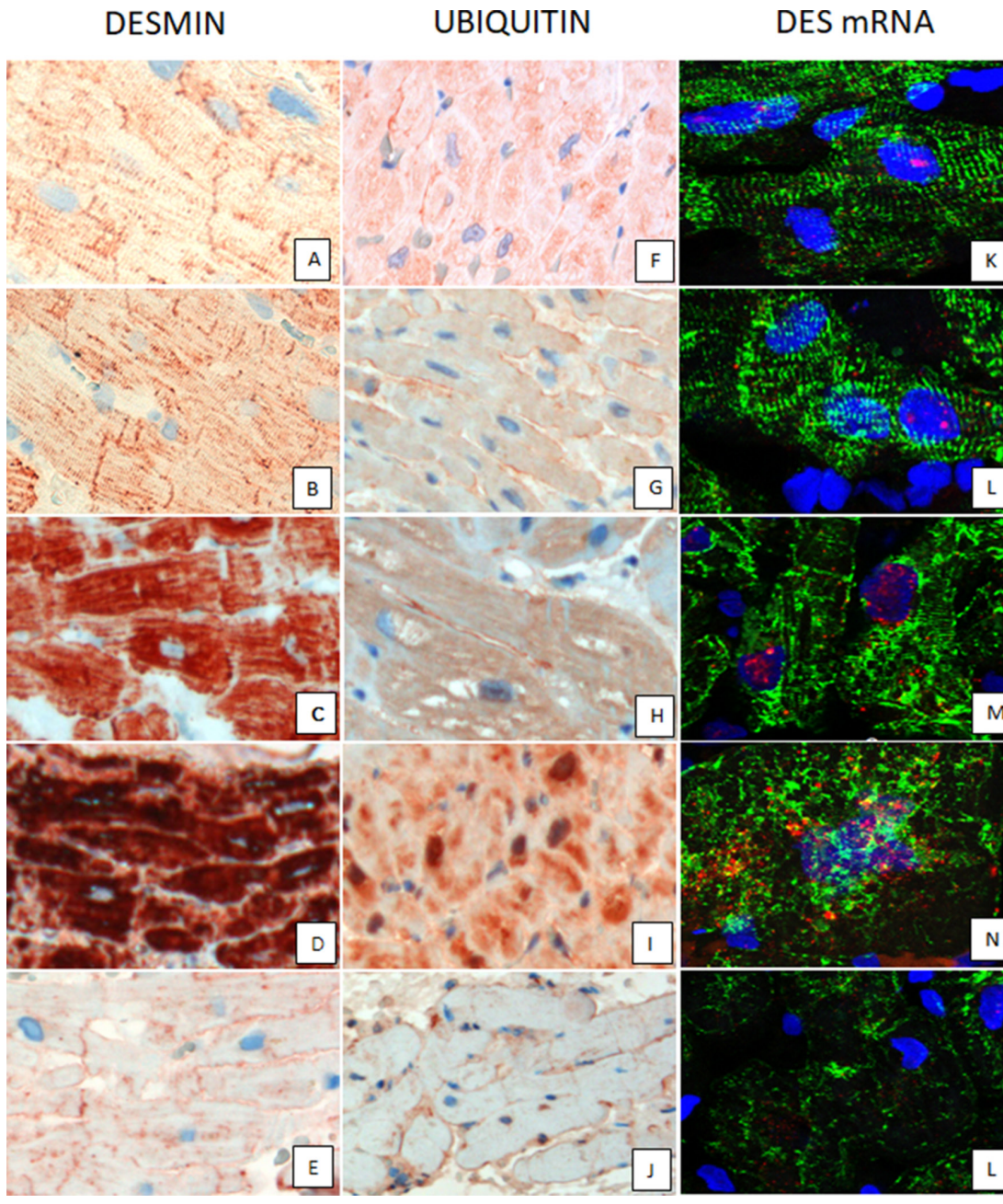

Figure 2 Immunohistochemical analyses of desmin, ubiquitin and desmin mRNA in the heart of control and in patients with idiopathic dilated cardiomyopathy (IDCM). Immunohistostainings with desmin antibodies revealed: normal, gentle staining of desmin at Z-lines and intercalated disks (A, control); normal, gentle staining of desmin at Z-lines and intercalated disks, giving a regular cross-section pattern (B, type I); increased, intensive staining of desmin at Z-lines and intercalated disks, giving regular cross-striation pattern (C, type IIA); increased, intensive staining of desmin with an irregular cross-striation pattern and/or with the presence of aggregates in perinuclear areas and, occasionally, in intermyofibrillar spaces (D, type IIB); decreased or lack of desmin staining (E, type III). Immunohistostainings with ubiquitin antibodies revealed: gentle, evenly scattered expression of ubiquitin in the cytoplasm of cardiomyocytes (F, control); gentle, evenly scattered expression of ubiquitin in the cytoplasm of cardiomyocytes (G, type A); increased, evenly scattered expression of ubiquitin in the cytoplasm of cardiomyocytes (H, type B); increased, unevenly scattered expression of ubiquitin in the cytoplasm of cardiomyocytes and its increased, evenly scattered expression in the nucleus (I, type C); weak/lack of expression of ubiquitin (J, type D) evaluated using light microscopy. Hybridization signal observed in tissue sections (K, L, M, N, O). Nuclei in each tissue section were stained with DAPI (K, L, M, N, O). Overlay demonstrated the localization of desmin (green colour) and desmin mRNA (red colour). Desmin mRNA proteins were localized in the nucleus of cardiomyocytes and very gently in the cytoplasm (K, control). In patients with DCM, desmin mRNA proteins were localized in the cytoplasm and in the nucleus of cardiomyocytes. Desmin mRNA showed: weak, but even expression in both the nucleus and cytoplasm (L, type 1); intensive expression, which was more pronounced in the nucleus than in the cytoplasm (M, type 2); intensive expression in the cytoplasm, and similar or weaker expression in the nucleus (N, type 3); absent desmin mRNA in the nucleus and very weak distribution in the cytoplasm (O, type 4).

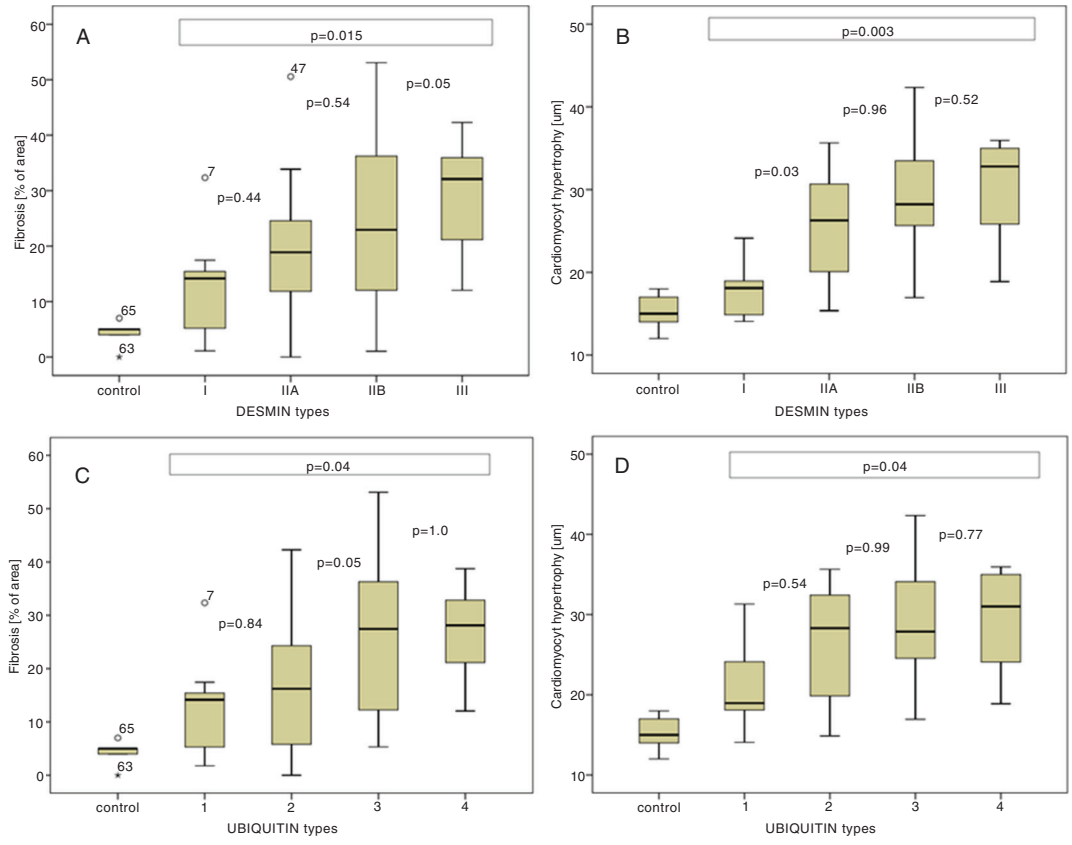

Figure 3 Box plots showing percentage of fibrous tissue area (A and C for each type of desmin and ubiquitin expression, respectively) and cardiomyocyte hypertrophy (B and D for each type of desmin and ubiquitin expression, respectively). Data displayed as mean±SD.

are ubiquitinated. Cohen *et al* suggested that the enhanced phosphorylation of desmin filaments during fasting increases their ubiquitination by Trim 32 and depolymerization and degradation.[17] In consequence, desmin disorganization and accumulation of protein aggregates, as well as the breakdown of Z-bands and thin filaments, could be observed. Furthermore, with progression of IDCM, further modifications of desmin expression could promote enhanced TRIM 32 ligase-modified degradation of the desmin cytoskeleton. This appears to facilitate the breakdown of Z-bands and thin filaments.[17] This could explain the changes observed in desmin expression (type IIB), desmin mRNA (type 3), ubiquitin expression (type C), and the contractile apparatus, which might ultimately result in cardiomyocyte degradation.[17] Desmin disorganization and accumulation of protein aggregates could overwhelm the proteasomal degradation, initiating apoptosis, autophagic myocyte loss and consequently HF progression.[14 22] Therefore, it is not surprising that in the late phase of compensated IDCM, we noticed an increase in caspase expression in numerous cardiomyocytes.

Patterns of desmin mRNA expression

We showed that desmin expression was significantly increased at the protein level and at the mRNA level, as was described previously.[3 26 27] It may be speculated that in the early compensated phase of IDCM (desmin mRNA expression—type 2, desmin—type IIA, ubiquitin—type B), the increased desmin expression is regulated by ubiquitin as was shown previously, and by desmin mRNA.[26] However, the ubiquitination of transcription factors is not the dominant process, leading to an increase in desmin mRNA, since the expression of ubiquitin in the nucleus was only routinely observed in our patients.

It is interesting that in late, compensated phase of HF, the presence of desmin mRNA was observed in the nucleus and in the cytoplasm, for most patients, including the cardiomyocytes with the intensive disorganization of desmin (type IIB) and ubiquitin expression in the nucleus and the cytoplasm (type C). Previous work on mRNA and ubiquitin expression using experimental models suggested that increased mRNA expression may be the effect of increased ubiquitination of some transcription factors.[20] The excessive scattered expression of ubiquitin in the nucleus in this phase supports this hypothesis.[28] Moreover, increased expression of desmin mRNA in the cytoplasm (type IIB), in our study, seemed to be also associated with the ubiquitination process as a consequence of increasing activation of family E3 ligases. Durairaj *et al* proved that the HECT ubiquitin ligases as well as the cullin-RING ligase CSF^{Mdm30} are connected with the transport of mRNA from the nucleus to the cytoplasm.[29] Based on our data, we suspect that increased desmin mRNA expression may be considered as an important feature of early and late phases of compensatory mechanisms against worsening HF. The possibility of regulatory influence of desmin mRNA expression in the human heart by beta-blockers, immunoadsorption and subsequent immunoglobulin substitution creates a new therapeutic option for patients with IDCM.[26]

Table 4 Echocardiographic parameters, NT-pro-BNP level and NYHA class in different types of desmin and ubiquitin expression

Type	Desmin					Ubiquitin					Desmin mRNA				
	I	IIA	IIB	III	p Values	A	B	C	D	p Values	1	2	3	4	p Values
NYHA class	1.4±0.7	1.9±0.7	2.0±0.8	2.2±0.6	0.05	1.6±0.9	1.8±0.6	2.0±0.8	2.1±0.7	0.4	1.6±0.8	2.0±0.6	2.1±0.6	2.2±0.7	0.3
NT-pro-BNP (pg/mL)	336±280	1556±657	1923±1221	1725±573	0.001	772±453	1331±765	2155±1232	1562±631	0.06	487±667	1422±993	1178±818	1770±585	0.03
LVEF (%)	48±13	36±16	28±8	27.5±7	0.001	45±10	38±11	27±7	27±7	0.004	45±14	35±16	29±7	27±7	0.03
LVEDD (mm)	59±8	61±10	66±8	67±6	0.02	58±6	62±8	66±5	67±5	0.03	58±9	62±12	66±7	66±6	0.06

p Values for statistical comparisons between desmin expression and various parameters, and between ubiquitin expression and various parameters, respectively. Data shown as mean±SD.
LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; NT-pro-BNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association.

In the decompensated phase of HF, decreased or absent desmin mRNA, desmin or ubiquitin expression was observed in our patients. It remains difficult to interpret, as there are no available data in the literature regarding desmin mRNA, desmin and ubiquitin expression in patients with IDCM. The exhaustion of compensatory mechanisms in cardiomyocytes could be the reason of absent or reduced mRNA and protein expression. The significant decrease in the number of mitochondria, changes in mitochondrial shape and localization observed in earlier studies support our suspicion.[4 5]

Desmin mRNA and ubiquitin expression versus histopathological presentation

Our study revealed that the progressive increase in desmin mRNA, desmin and ubiquitin expression in cardiomyocyte was associated with gradual changes in cardiomyocyte structure and HF progression. However, in end-stage HF, although many cardiomyocytes exhibited an absence or a decrease in desmin mRNA, desmin and ubiquitin expression, further cardiomyocyte hypertrophy and fibrosis were noticed. The significant cardiomyocyte hypertrophy and a disproportionate increase in fibrosis in early compensative phases of IDCM, as well as the significant increase in fibrosis only observed in late compensated phase of IDCM, are consistent with the elegant study by Mackiewicz *et al*.[4] They suggested that extracellular matrix remodeling seems to be secondary to primary changes in cardiomyocytes (figure 2). Moreover, disorganization of desmin rather than fibrosis per se may play an important role in the development of IDCM.[4] It is possible that both paracrine effects as well as cell-to-cell contacts participate in the crosstalk between myocytes and fibroblasts.[30] Higher myocyte diameters and more significant fibrosis observed with the different patterns of ubiquitin expression, as compared with desmin expression, may indicate that ubiquitin could be a more precise marker of myocardium remodeling. Rubin *et al* reported that the ubiquitination of growth factors is one of the most important signalling cascades, controlling cardiomyocyte hypertrophy.[31]

Desmin mRNA and ubiquitin expression versus clinical presentation

Our study showed that changes in desmin mRNA, ubiquitin and desmin expression correlated with clinical, echocardiographic and biochemical parameters. The most favorable LVEF and LVEDD measurements, the lowest NT-pro-BP levels and NYHA functional classes characterized patients with: type 1 desmin mRNA, type A ubiquitin and type I desmin expressions. In patients with increase expression of all analysed proteins and mRNA in the early and late compensated phases, we noted unfavorable and worsening parameters of LVEF, LVEDD, NT-pro-BNP and NYHA class. It appeared that these adverse parameters were related to the accumulation of proteins that initiated cell death, and cardiomyocyte degeneration.[20] The decompensated phase of IDCM was associated with the highest NYHA class, the highest NT-pro-BNP, the largest LVEDD and the lowest LVEF values. The correlation between desmin mRNA, desmin and ubiquitin expressions, and these 4 parameters emphasized the importance of these proteins in the

pathophysiology of IDCM. Changes in desmin cytoskeleton correlating with the structural cardiomyocyte features and clinical parameters have been described previously.[5] We are strongly convinced that these findings point to a previously undescribed therapeutic target that may have application in treatment of HF which is often caused by disturbance in protein expression.

CONCLUSIONS

Changes in ubiquitin and desmin mRNA expression are related to patterns of desmin expression. Increased expression of ubiquitin and desmin mRNA might be a protective feature against unfavorable cardiomyocyte remodeling, reducing the adverse effects of cytoskeleton damage in the early stages of HF. It appears that weak ubiquitin and low desmin mRNA expressions could be useful markers of end-stage HF. Our findings of concomitant expression of desmin mRNA, desmin and ubiquitin in cardiomyocytes of patients with IDCM help shed light into the pathophysiological processes regulating desmin expression in HF.

Contributors AP conceptualised and designed the study. AP, ERK and KEG conducted the study, coordinated the data collection and drafted the manuscript. AZ, LK and RJG supervised the data collection, took part in the data analysis, reviewed and revised the manuscript and approved the final version of the manuscript.

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