Immunoabsorption and subsequent immunoglobulin substitution decreases myocardial gene expression of desmin in dilated cardiomyopathy

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Abstract Cardiac autoantibodies play a pathogenic role in dilated cardiomyopathy (DCM). Removal of antibodies by immunoabsorption (IA) induces hemodynamic improvement in DCM patients. The present study investigated the effects of IA on myocardial gene expression of the inter-

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mediate cytoskeletal filament desmin, which is upregulated in heart failure. RNA was isolated from five explanted non-failing hearts and five explanted failing hearts of DCM patients, and myocardial gene expression of desmin was estimated by real-time polymerase chain reaction (PCR). In a case-control study in six DCM patients (LVEF < 40%,

NYHA II–III), IA and subsequent IgG substitution were performed at monthly intervals until month 3. Endomyocardial biopsies (EMBs) were obtained before and after IA (after 3–6 months). From six DCM patients without IA therapy (controls), EMBs were also obtained over a comparable time interval. Expression of the desmin gene was analyzed in these EMBs by real-time PCR. In failing explanted hearts, expression of desmin was significantly increased (0.88 ± 0.12 vs 0.45 ± 0.15 in non-failing hearts, $P < 0.05$). After IA, myocardial gene expression of desmin was significantly decreased (from 0.26 ± 0.05 [baseline] to 0.14 ± 0.04 [$P < 0.05$] vs baseline and controls). Removal of antibodies by IA modulates myocardial gene expression of desmin in DCM patients.

Keywords Dilated cardiomyopathy · Immunoadsorption · Myocardial gene expression · Desmin

Introduction

Dilated cardiomyopathy (DCM) is a chronic myocardial disease characterized by progressive depression of myocardial contractile function and by ventricular dilatation [1]. It is one of the most common myocardial diseases leading to heart transplantation. Disturbances in humoral immunity play an important role in cardiac dysfunction of DCM patients. A number of autoantibodies against cardiac cell proteins have been identified in DCM: e.g., antibodies against the cardiac beta-1 adrenergic receptor, contractile proteins, mitochondrial proteins and the muscarinic acetylcholine receptor-2 [2–7]. Experimental and clinical data indicate that cardiac antibodies play an active role in the pathogenesis of DCM and may contribute to cardiac dysfunction. Animal models have shown that DCM can be triggered by autoantibodies [8–11]. Recent clinical studies indicate that cardiac antibodies may play an active role in the pathogenesis and progression of the disease in humans: removal of antibodies by immunoadsorption (IA) induces hemodynamic improvement and ameliorates myocardial inflammation in patients with DCM [12–17]. Elimination of cardiodepressant antibodies may be one explanation for the beneficial hemodynamic effects of IA in patients with DCM [15–17].

Previous data indicate that the cytoskeleton, together with contractile proteins, is a major site of the cellular damage and impairment of myocyte function in DCM [18–21]. Desmin belongs to the intermediate filaments and is exclusively expressed in muscle cells [22]. An increase in myocardial cytoskeletal proteins, including desmin, has been described in myocyte hypertrophy and failing hearts [18–21]. Moreover, myocardial desmin expression accelerates during transition from compensated ventricular hypertrophy to heart

failure in an experimental model of cardiac hypertrophy [19]. In addition, there is inverse correlation between myocardial desmin expression and left ventricular ejection fraction of DCM patients [23].

Although the hemodynamic effects of IA have been well described its influence on gene expression remains to be elucidated. To ascertain DCM-associated changes in myocardial gene expression, we measured expression of desmin in myocardial samples obtained from explanted failing and non-failing human hearts. Furthermore, in a separate case-control study, we measured the effects of IA on gene expression of desmin in endomyocardial biopsies (EMBs) of DCM patients.

Materials and methods

Myocardial tissue samples from explanted hearts

Human myocardial samples were obtained, as described before, from the left ventricular free wall of five explanted non-failing hearts that were not used for cardiac transplantation and of five explanted failing hearts of DCM patients [24]. The non-failing hearts were obtained from potential donors without evidence of heart disease in their medical histories. Echocardiography showed normal fractional shortening and no evidence of regional wall-motion abnormalities or valve disease. Valves were taken and used for human homografts. Myocardium was used for experimental purposes. Patients died from intracerebral hemorrhage or head injury. The failing hearts were obtained during heart transplantation from patients suffering from end-stage heart failure due to DCM. In these patients, significant stenoses of the coronary arteries were excluded by coronary angiography. Therapy of patients suffering from DCM consisted of digitalis, diuretics, nitrates, β -blockers, and angiotensin-converting enzyme inhibitors. Tissue samples were immediately snap-frozen in liquid nitrogen.

Immunoadsorption (IA)

In a case-control study, DCM patients ($n=6$) were treated with Ig-Therasorb (PlasmaSelect, Teterow, Germany), an anti-IgG immunoadsorber, at monthly intervals until month 3, as described previously [13]. After every final IA session, the patients received 0.5 g/kg polyclonal IgG (Venimmun-N®) for safety reasons to restore IgG plasma levels and to reduce the risk of infection after immunoglobulin depletion. EMBs (five to eight) were obtained from all patients from the interventricular septum of the right ventricle at baseline before the study began (<7 days), and after 3–6 months. From age-matched, comparable DCM patients who were treated only conservatively with stable oral medication for heart failure (control group, $n=6$), EMBs were obtained from the interventricular septum of the right

ventricle at the same time intervals (baseline and after 3–6 months) for clinical diagnostic reasons. Patients demonstrated persistent cardiac heart-failure symptoms and dysfunction despite stable heart-failure therapy.

Oral medical treatment (β -blockers, ACE inhibitors, and diuretics) of both groups of DCM patients was comparable and stable for more than 3 months before the study and throughout the study period. All patients demonstrated left ventricular dysfunction (LVEF < 40%, as assessed by two-dimensional echocardiography), and symptoms of chronic heart failure (New York Heart Association [NYHA] functional classes II–III). Coronary heart disease was excluded by angiography. Patients were excluded if they had suffered from active infectious diseases, cancer, chronic alcoholism, or heart failure due to known origins (e.g., primary valvular disease). Echocardiography was performed at baseline and after 3 months. LVEF was measured according to the Simpson rule.

RNA isolation

Total RNA was isolated from human non-failing and failing heart samples and human EMBs by use of a Qiagen RNeasy micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA digestion was included. RNA quantity was determined by A260 measurement. All 260/280 nm ratios were above 1.8.

cDNA synthesis and amplification

Limitation in the amount of RNA from EMBs necessitated a preamplification technique. We chose polymerase chain reaction (PCR)-based amplification using the SMART-PCR Synthesis Kit and the Advantage 2 PCR Kit (Clontech, San Jose, CA), which is known to amplify high, medium, and low transcripts in a representative manner, and to provide full-length cDNA [25]. Total RNA was reverse-transcribed using the SMART-PCR Synthesis Kit, according to the manufacturer's protocol. For PCR-based cDNA amplification, we subsequently employed an Advantage 2 PCR Kit according to the manufacturer's protocol. To avoid saturation, we determined the optimal number of PCR cycles to ensure that the cDNA would remain in the exponential phase of amplification by real-time PCR and by electrophoresis. The PCR conditions were 95°C for 1 min, followed by 21 cycles with 95°C for 15 s, 65°C for 30 s, and 68°C for 3 min, under the conditions in which linearity of the amplification was confirmed. The resulting preamplified cDNA was purified using the Qiaquick PCR Purification Kit (Qiagen, Hilden, Germany).

Relative mRNA quantitation

Using amplified 5 ng cDNA of human heart samples and EMBs, we performed quantitation by using an ABI Prism

7700 sequence detection system (TaqMan®) using the 5'-nuclease activity of TaqDNA polymerase to generate real-time DNA analysis. Amplifications were generated at 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing, and synthesis (1 min, 60°C), with use of the TaqMan® universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany). All PCR products include an intron–exon breakpoint to avoid amplification of DNA contamination. The primers (human desmin forward 5'-CGGACGTGGATGCAGCTACT-3', human desmin reverse 5'-CCTCTTCATGCACTTTCTTAAGGAA-3', human desmin probe TAMRA/FAM 5'-CCTGGA GCGCAGAATTGAATCTCTCAAC, and commercially available human GAPDH-primers from Applied Biosystems) were employed in a final concentration of 400 nM. The PCR-reaction products were analyzed on agarose gels stained with ethidium bromide. Before the normalization of the expression of the target gene (desmin) to an internal standard gene [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] to accomplish comparative quantitation (ΔC_T), we verified the comparable dose-dependent linear amplification of both genes. The TaqMan assay was repeated three times, with each assay containing duplicate reactions, and with each assay including an independent amplification of the probes.

Statistics

Results are expressed by mean $\pm$ SEM. The paired Wilcoxon test was applied to detect changes in gene expression before and after IA. Changes in gene expression in failing hearts vs non-failing hearts were analyzed by the Mann–Whitney *U* test. Significance was assessed at the $P < 0.05$ level.

Ethics

Written consent was obtained from each patient, and the protocol was approved by the local Hospital Ethics Committee. The case-control study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Furthermore, the Ethics Committee likewise approved the use of samples of explanted hearts for scientific purposes.

Results

Gene expression of desmin in explanted hearts

To study disease-associated changes in desmin gene expression, RNA was isolated from five explanted non-failing hearts and five explanted failing hearts from DCM patients. Expression of desmin which was normalized to GAPDH was significantly elevated in failing explanted hearts [0.88 ± 0.12 vs 0.45 ± 0.15 (non-failing hearts, $P < 0.05$)], (Fig. 1).

Fig. 1 Desmin gene expression in explanted failing and non-failing hearts. The RNA of explanted non-failing donor hearts ($n=5$) and DCM hearts ($n=5$) was isolated and reverse-transcribed. The gene expression of desmin was quantified relative to GAPDH by real-time PCR (* $P < 0.05$ vs non-failing hearts)

The effects of immunoadsorption on myocardial desmin gene expression of DCM patients

In both groups at baseline, the following data were comparable: age, gender, LVEF, oral medication at baseline, and NYHA-classification (Table 1). In the IA group, LVEF increased significantly from $27.67 \pm 2.71\%$ to $34.00 \pm 2.40\%$ ($P < 0.05$), whereas the LVEF of the control group did not significantly change ($32.17 \pm 1.36\%$ to $32.33 \pm 1.54\%$, n. s.).

At baseline and after 3 months, changes of desmin gene expression were measured in EMBs of DCM patients treated with IA, and of DCM patients without IA treatment. At baseline, expression of desmin did not differ significantly between the control group and IA group. In the control group, gene expression of desmin increased during the study period (from 0.29 ± 0.03 to 0.42 ± 0.05 n.s. [Fig. 2a]). In contrast, desmin expression significantly decreased in the IA group after 3–6 months [from 0.26 ± 0.05 to 0.14 ± 0.04 , $P < 0.05$ vs baseline and controls (Fig. 2b)].

Table 1 Baseline characteristics of study population

Characteristics	Control group ($n=6$)	IA group ($n=6$)
Age	45.3 ± 4.2	41.5 ± 6.3
Gender (male/female)	5/1	6/0
LVEF (%) ^a	32.17 ± 1.36	27.67 ± 2.71
NYHA functional class (II/III)	2/4	1/5
Medication		
β -blocker	6	6
ACE-inhibitor	6	6
Diuretics	6	6

^a LVEF Left ventricular ejection fraction

^b NYHA New York Heart Association

Discussion

The cytoskeleton is a well-organized structure which consists of microtubules, actin filaments, and intermediate filaments such as desmin that is exclusively expressed in muscle cells [22]. Changes within the cardiac cytoskeleton, including the loss of contractile filaments and increase in cytoskeletal proteins—which leads to desmin disorganization and impairment of contractile function—are part of the pathological alteration occurring in heart failure and DCM [18, 20, 21, 23]. In addition to its involvement in cellular stability, the cytoskeleton is also responsible for signal transmission from cellular membrane to the nucleus.

The essential role of desmin for cardiac integrity has been investigated in animal models with null mutations of the desmin gene. At birth, no anatomical defects are apparent in mice lacking desmin [22]. However, after birth, these mice develop cardiomyopathy, skeletal myopathy, and smooth muscle dysfunction. These disorders reduce their

Fig. 2 Changes in desmin gene expression during IA. At baseline and after 3 months, the RNA of endomyocardial biopsies of DCM patients without IA (control group, $n=6$; **a**) and with IA (IA, $n=6$; **b**) was isolated and reverse-transcribed. The gene expression of desmin was quantified relative to GAPDH by real-time PCR (* $P < 0.05$ vs baseline, # $P < 0.05$ vs controls)

life span and make them less tolerant of exercise [22]. Furthermore, in animal models, null mutation of the desmin gene gives rise to ultrastructural defects in cardiac muscle [26], degeneration of cardiomyocytes, accumulation of macrophages, and fibrosis [27]. Mice lacking desmin develop cardiomyocyte hypertrophy and age-dependent loss of cardiac performance, with chamber dilatation and compromised systolic function resembling cardiomyopathy [28]. On the other hand, desmin rescue mice with cardiomyocyte-specific desmin expression display a wild-type cardiac phenotype [29]. These findings indicate that desmin accumulation is not the cause of myocardial disease; on the contrary, they demonstrate the cytoprotective effects of the protein and its essential role for cardiac integrity. The important functional and structural role of desmin in cardiac tissue is documented by several different hereditary desmin missense mutations in humans, resulting in restrictive cardiomyopathy [30] or DCM [31]. The pathogenic properties of desmin-mutation were shown in a mouse model for desminopathy as well; transgenic mice with a 7-amino acid deletion (D7-des) exhibited structural defects of the myocardium that resembled human desmin-related cardiomyopathy with aberrant intrasarcoplasmic and electron-dense granular filamentous aggregates that were desmin-positive [32].

Abnormalities of the humoral immune system are present in patients with DCM, and various antibodies have been identified in DCM [2–7]. Animal models further indicate that under certain pathological conditions, DCM can be triggered by autoantibodies. A recent experimental study provided direct evidence that antibodies against the β_1 -receptor can induce DCM [9]. BALB/c mice, deficient for the immune-regulatory PD-1 receptor, generate autoantibodies against a sarcolemmal protein [10], which was identified as cardiac troponin I [11]. These autoantibodies induce cardiac dysfunction resembling DCM.

Recent studies demonstrated that IA removes functional active antibodies from the plasma of patients with DCM. These antibodies induce a negative inotropic effect in isolated rat cardiomyocytes through depression of calcium transients; they are furthermore detectable in the majority of DCM patients [15, 17]. Recent *in vitro* data indicate that these antibodies belong to the IgG-3 subclass [16]. Elimination of antibodies by IA results in hemodynamic improvement and a reduction in myocardial inflammation [12–17]. The IgG, which was substituted for safety reasons after IA [13] may additionally influence the immune system through various mechanisms [33]—and can possibly have beneficial effects in patients with heart failure [34].

In this study, we investigated the effects of IA on the level of gene expression. Our findings show that in accordance with published data, desmin is upregulated in failing DCM hearts [18, 23]. The present study did not ascertain the

function of desmin in DCM and heart failure. Whereas this protein plays an essential role for cardiac integrity [22, 26–29, 32], the consequences of desmin upregulation in DCM are still unknown. However, the inverse correlation between the amount of myocardial desmin and the ejection fraction in DCM patients [23] may establish desmin as a valuable myocardial marker for cardiac dysfunction. Our study shows for the first time that specific immunomodulatory therapy—i.e., removal of antibodies by immunoadsorption and subsequent IgG substitution—induces changes in myocardial gene expression of DCM patients. Parallel to the increase in LVEF, expression of desmin was significantly reduced, indicating improvement of left ventricular function.

Medical therapy may modulate desmin gene expression as well. As was shown recently, initiation of β -blocker therapy induces changes in myocardial expression of various genes in patients with DCM [35]. However, our patients had received stable oral medication for more than 3 months before the study and during the study period. Hence, the present study excluded the possibility that an alteration of gene expression was induced or modulated by medical therapy or that it reflects the natural course of disease. This conclusion is supported by the fact that patients of the control group with similar clinical characteristics and similar medication demonstrated no change in desmin transcription.

Study limitations

The present case-control study analyzed changes of myocardial gene expression of desmin after IA therapy. It would be of interest to ascertain the influence of IA on the complex pattern of myocardial gene expression by performance of expression profiling for a broad spectrum of other genes known to be involved in development of heart failure. This, however, would be out of the focus of this pilot study that encompassed only a small number of DCM patients. Large-scale studies should be designed to evaluate changes in myocardial gene expression on a genome-wide scale. Furthermore, as only a small amount of myocardial tissue was obtained for ethical reasons, assessment of the influence of IA on protein expression was not feasible.

Conclusions

Findings of the present study indicate that production of cardiac autoantibodies is involved in DCM-associated changes in myocardial gene expression, and in turn, that removal of these antibodies by IA may reverse some changes in myocardial gene expression—as shown for the intermediate cytoskeletal filament desmin. Ongoing studies will analyze the influence of immunoadsorption on myocardial expression of further genes.

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