Clin. Lab. 2010;56:519-526

ORIGINAL ARTICLE

Are Autoantibodies Against the ß1-adrenergic Receptor Markers for Dilated Cardiomyopathy?

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SUMMARY

Background: **Dilated cardiomyopathy (DCM) is one of the leading causes of heart failure in the western world but there is still no specific and early diagnosis available. Besides a genetic predisposition and viral infections, autoimmune reactions play an important role in the pathogenesis of DCM. The ß1-adrenergic receptor (ß1-AR) has been described as the major target structure in autoimmune DCM.**
Methods: **In this study a recombinant GST-ß1-AR fusion protein comprising the second extracellular loop was generated as a target for the analysis of autoantibodies in sera from 115 patients with different heart failure diseases (41 DCM, 30 non-ischemic secondary cardiomyopathy [NISCM], 44 coronary artery disease [CAD]). Sera were collected from a non-selected population of heart failure patients in consecutive order.**
Results: **Autoantibodies against the ß1-AR were detected in 37 % of DCM, 30 % of NISCM, and 36 % of CAD patients but none of the controls were positive. Furthermore, our data show that cardiomyopathy patients with anti-ß1-AR antibodies are younger (54 years vs. 61 years [DCM], 53 years vs. 56 years [NISCM], 61 years vs. 61 years [CAD]. Regardless of diagnosis antibody-positive patients had lower EF levels (29 % vs. 32 %, $p = 0.0001$ [DCM]; 23 % vs. 25 %, $p < 0.0001$ [NISCM]; 23 % vs. 25 %, $p < 0.0001$ [CAD]) than the antibody-negative counterparts but, nevertheless, also lower NT-proBNP levels compared to antibody negative patients (567 pg/mL vs. 1296 pg/mL, $p = 0.0005$ [DCM]; 224 pg/mL vs. 1135 pg/mL, $p = 0.0002$ [NISCM]; 605 pg/mL vs. 940 pg/mL, $p = 0.0005$ [CAD]).**
Conclusions: **We conclude that DCM patients should be further characterized and differentiated by the detection of autoantibodies against β1-AR. Autoimmune DCM patients are younger compared with their non-autoimmune counterparts, possibly due to the autoimmune trigger of the disease or reflecting an early stage of the disease. Surprisingly, the autoimmune patients have worse clinical manifestations but show less excessive NT-proBNP levels. It is not clear yet, though, whether these autoantibodies have a direct impact on the NT-proBNP levels. Whether or not these data are a consequence of pathogenic antibodies has to be elucidated in further studies.**
(Clin. Lab. 2010;56:519-526)

KEY WORDS

Dilated cardiomyopathy, ß1-adrenergic receptor, autoantigen, autoimmunity, heart failure

Manuscript accepted May 24, 2010

INTRODUCTION

Dilated cardiomyopathy (DCM) is one of the most frequent causes for heart failure (HF) leading to hospitalization, increased mortality and often to heart transplantation [1-3]. Many manifestations of DCM are of unknown etiology. Beside viral infections and genetic predisposition, autoimmune reactions are discussed as major causes of this severe heart disease [4-8].

Different cardiac structures including intracellular proteins as well as transmembrane receptors have been described as structures of the autoimmune reaction [9-18]. Particularly the G-protein coupled ß1-adrenergic receptor (ß1-AR) has been described extensively as a target of autoantibodies [19-21].

Binding autoantibodies to the ß1-adrenergic receptor with a subsequent enhancement of cardiac contractility were first described in *Trypanosoma cruzi* induced Chagas' disease [22,23]. This observation prompted several groups to investigate the presence of autoantibodies against ß1-AR in sera of DCM patients [24,25]. It was shown by molecular and immunological methods that the second extracellular loop of the ß1-AR plays a prominent role as a main target for these autoantibodies [26-29]. As demonstrated in cultivated rat cardiomyocytes, these autoantibodies were able to increase the heart rate and contractility and prolong the duration of the action potential [27,30]. Furthermore, the transfer of synthetic peptides corresponding to the second extracellular loop of the ß1-AR resulted in dilated ventricles and thinner heart walls which are characteristic features of DCM [31]. In addition, rabbits immunized by synthetic ß1-AR peptides showed a desensitization of ß1-adrenergic receptors and developed a hypertrophic myocardium associated with a cardiac dysfunction [32]. Interestingly, these autoantibodies also lead to a downregulation of ß1-adrenergic receptor-mRNA and a lower susceptibility to adrenergic stimulation [33,34], a mechanism described also in other receptor associated autoimmune diseases such as Myasthenia gravis [35]. Recently, Jahns et al. succeeded in providing evidence of an autoimmune genesis of DCM by immunization of rats with GST fusion protein corresponding to the second extracellular loop (195-222) of ß1-AR. All rats with induced antibodies against ß1-AR showed a progressive left ventricular dilatation and dysfunction. Furthermore, all rats treated with these autoantibodies showed signs of DCM [36,37]. Other studies revealed that rodents immunized with a ß1-AR peptide showed clinical manifestation of DCM by decreased heart function and a left ventricular dilatation leading to the conclusion that autoantibodies may play an important role in the pathogenesis of DCM [38,39]. In addition to this striking data from animal models, the antibody mediated process of DCM can be deduced from positive data of immunoadsorption and immunomodulation [40-53].

Despite the enormous clinical impact of DCM due to its inevitable consequences in terms of progressive HF, there are only poor options for diagnosis. Therefore, in the present study consecutively recruited patients with different causes of heart failure were examined for the presence of autoantibodies against the ß1-AR using a fusion protein previously described to be involved in the pathogenesis of the disease. Cardiac patients included patients with dilated cardiomyopathy (DCM), non-ischemic secondary cardiomyopathy (NISCM), and coronary artery disease (CAD). Furthermore, the autoimmune response was evaluated in the context of clinical (age, body-mass-index (BMI), left ventricular end diastolic diameter (LVED), and ejection fraction (EF)) and non-immunological data. Particularly, the correlation between the presence of autoantibodies against the ß1-AR and the NT-proBNP, an established marker for heart failure, was elucidated.

MATERIALS AND METHODS

Patients

One hundred-fifteen consecutive heart failure (HF) patients (20 females, mean age 59 yrs; 95 males, mean age 58 yrs) recruited from the heart failure clinic of the University Hospital Hamburg Eppendorf (Germany) were included in this study. All patients gave informed written consent for blood analysis. Blood samples (10 mL of whole blood) were taken in the morning hours after an overnight fast and were collected during routine, clinically necessary blood drawing. All laboratory analyses were performed blinded without knowledge of patients' diagnoses. Control samples from patients without heart failure were also analyzed: normal blood donors (n = 34, 18 females and 16 males) and samples from patients with different autoimmune diseases (n = 38; 20 patients with systemic lupus erythematosus, 18 patients with Wegener's granulomatosis). The Hamburg ethics committee approved this study.

Clinical diagnosis

According to WHO classification primary dilated cardiomyopathy is characterized by left ventricular dilatation and insufficient contraction [54]. While patients with non-ischemic secondary dilated cardiomyopathy (NISCM, n = 30) were identified as having known triggers, e.g. valvular disease, arterial hypertension, alcohol abuse, patients with primary dilated cardiomyopathy (DCM, n = 41) were characterized by exclusion of the above mentioned conditions. Patients with coronary artery disease (CAD, n = 44) had at least one high grade stenosis (> 70 %) of one major coronary vessel or had definite myocardial infarction in the past. Patients with infiltrative cardiomyopathy (sarcoidosis, amyloidosis, etc.) were excluded from this study. Clinical data were collected from patients' sheets. Left ventricular end-diastolic diameters (LVED) and left ventricular ejections fraction (EF) were measured by echocardiography.

Generation of the recombinant GST-ß1-AR fusion protein from E. coli

The cDNA encoding the second extracellular loop (amino acid position 197-222) of the ß1-AR was cloned and expressed as a GST-fusion protein in E. coli. DNA sequences of the ß1-AR were retrieved from the DNA data bank (http://www.ncbi.nlm.gov/Entrez/) using the most recent accession number NM_000684. Synthesis of oligonucleotides for PCR-amplification including appropriate restriction sites was done by Applied Biosystems (Darmstadt, Germany). Specific cDNA was

amplified from a cardiomyocyte cDNA library (Invitrogen, Carlsbad, CA, USA) by PCR and the cDNA was introduced in a linearized prokaryotic expression vector (pGEX-6P-1). Successful insertion of cDNA was confirmed by DNA sequencing analysis using a modified dideoxymethod [55]. The fusion protein GST-ß1-AR-II was overexpressed in E. coli and purified by affinity chromatography on Glutathione Sepharose 4B columns. Protein concentrations were determined using the method of Bradford (Biorad, Hercules, CA, USA).

Immunoblot analysis of anti-ß1-AR-autoantibodies

The purified GST-ß1-AR-II fusion protein was transferred onto nitrocellulose membranes. Autoantibodies were analyzed in 1:100 dilution of human serum. After three washing steps goat-anti-human IgG conjugated with alkaline phosphatase was added. Immunoblot staining was done by 5 - 10 minute incubation in fresh NBT/BCIP-solution.

NT-proBNP measurement

For the quantitative determination of NT-proBNP in the same serum samples as for immunoblot analyses a Electro-Chemi-Luminescent-Immune-Assay was used on a Modular Analytics E170 (Roche Diagnostics, Penzberg, Germany).

Statistics

Significance was tested using the Wilcoxon signed rank Test (GraphPad Prism version 5). A p-value < 0.05 was assumed as being statistically significant.

RESULTS

Cloning and over-expression of recombinant GST-ß1-AR

Exploiting the previously published ß1-AR-cDNA sequence, reverse transcription polymerase chain reaction was applied on total RNA derived from the human keratinocyte cell line A431. The 78 bp PCR product comprising the ß1-AR-cDNA corresponding to the second extracellular loop was cloned into pGEX-6P-1. The correct insertion of the cDNA was confirmed by restriction analysis (Figure 1) and DNA sequencing. Recombinant GST-ß1-AR-II was expressed in E. coli and isolated from the cytoplasmic supernatant after lysis of the bacteria.

As shown in Figure 2 recombinant full-length GST-ß1-AR-II was detected by a monoclonal anti-GST antibody. Comparison of the migration position with recombinant GST demonstrated the presence of the entire fusion protein. The immune reactivity was demonstrated by staining of recombinant full-length GST-ß1-AR-II after incubation with a serum from a representative DCM patient, whereas the GST moiety was not detected (Figure 2, lane 3 in panel A and B).

Both DCM and CAD sera contain autoantibodies against recombinant GST-ß1-AR

By immunoblot analysis autoantibodies against recombinant GST-ß1-AR-II were detected in 37 % of DCM (15/41) and 30 % of NISCM (9/30). CAD patients were autoantibody positive in 36 % of CAD (16/44) (Table 1). Autoantibodies to GST-ß1-AR-II were neither detected in sera of patients with SLE, Graves' disease, Wegener's granulomatosis nor in sera of healthy blood donors.

Antibody positive DCM patients are younger than seronegative DCM

DCM patients with a positive autoantibody response were on average seven years younger than seronegative DCM patients (54 versus 61 years). In contrast, in NISCM patients the difference in age was only three years (53 versus 56 years). Interestingly, there was no age difference between autoantibody positive and negative CAD patients (Table 2).

Antibody positive HF patients show lower EF than seronegative patients

The ejection fraction (EF), as a central parameter for the functionality of the heart, was significantly lower in HF patients with positive autoantibody response. While seropositive DCM patients showed an EF of 29 %, seronegative DCM patients had an EF of 32 % ($p = 0.0001$). Seropositive NISCM and CAD showed an EF of 23 % versus the seronegative patients of both NISCM and CAD having an EF of 25 % ($p < 0.0001$) (Table 2). In contrast, no difference was observed in LVED in all cardiac patients. Also, there was no association between the BMI and any of the disease entities.

Antibody positive HF patients show lower NT-proBNP concentrations

All patients had increased NT-proBNP levels above the normal value of 150 pg/mL. Moreover, patients, independent of the diagnosis, with an autoantibody response against ß1-AR-II had significantly lower concentrations of NT-proBNP compared with the seronegative equivalents.

While the highest difference was observed in NISCM where the autoantibody positive subgroup reached only 20 % of seronegative NISCM patients, the difference to the seronegative counterparts observed in DCM antibody positive patients was 42 % and even 64 % in CAD positive patients (Table 2).

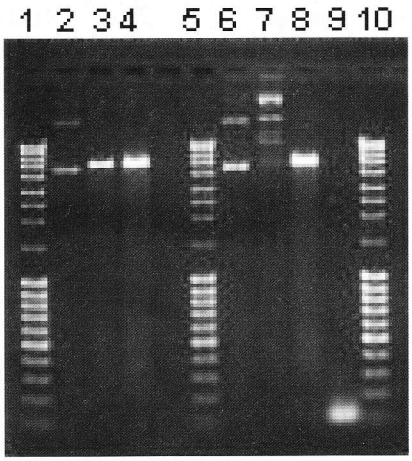

Figure 1. Agarose gel electrophoresis of cloned ß1-AR-II cDNA. Restriction analysis and PCR-analysis of the recombinant expression vector pGEX-6P-1-141. Lane 1, 5, 10: MWM (MassRuler DNA Ladder, Mix, Fermentas); Lane 2: pGEX-6P-1, non-restricted; Lane 3: pGEX-6P-1 after restriction with Eco RI; Lane 4: pGEX-6P-1 after restriction with Bam HI/ Xho I; Lane 6: pGEX-6P-1-141, non-restricted; Lane 7: pGEX-6P-1-141 Eco RI; Lane 8: pGEX-6P-1-141 after restriction with Bam HI/Xho I; Lane 9: 141-cDNA after PCR amplification with pGEX-6P-1-141 as template.

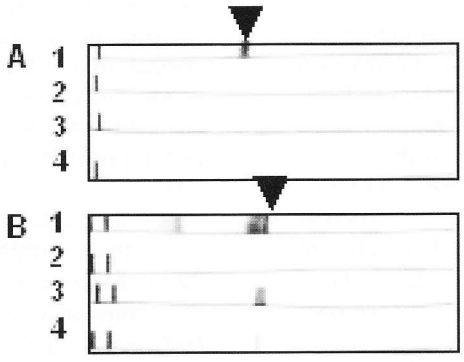

Figure 2. Representative immunoblot analysis of recombinant GST-β1AR-II fusion protein. Recombinant GST (panel A) and GST-β1AR-II (panel B) were immobilized on nitrocellulose membranes and stained with Goat-anti-GST-Antibody (Lane 1), serum of a normal healthy blood donor (Lane 2), rabbit antiserum against β1AR-II (Lane 3), and a representative DCM-patient (Lane 4). Arrows show the location of reaction with GST and GST-β1AR-II, respectively.

Table 1. Antibody reactivity against recombinant GST-ß1-AR-II.

	Total	Positive	%
DCM	41	15	37
NISCM	30	9	30
CAD	44	16	36
AID	38	0	0
NHC	34	0	0

DCM, idiopathic dilated cardiomyopathy; NISCM, non-ischemic dilated cardiomyopathy; CAD, coronary artery disease; AID, autoimmune disease; NHC, normal healthy controls.

Table 2. Age, BMI, and cardiac parameters of heart failure patients.

		Age [yr]	BMI [kg/m^2]	LVED [mm]	EF [%]	NT-proBNP [pg/ml]
DCM	positive	54	27	68	29	567
	negative	61	26	69	32	1296
NISCM	positive	53	27	69	23	224
	negative	56	26	69	25	1135
CAD	positive	61	25	70	23	605
	negative	61	25	68	25	940

DISCUSSION

This study shows that autoantibodies against the ß1-adrenergic receptor are specific markers for patients with autoimmune heart failure including dilated cardiomyopathy. Therefore, this assay may be used for the further characterization and differentiation of DCM.

This was demonstrated by immunoblot analyses using a recombinant GST-fusion protein derived from *E. coli* comprising the second extracellular loop of ß1-adrenergic receptor. For the detection of autoantibodies the second extracellular loop of the ß1-AR was isolated as a heterologously expressed GST fusion protein derived from *E.coli*. Thirty-four percent of patients with HF of different origin were identified by this assay, whereas none of the control sera (from other autoimmune patients and normal healthy blood donors) were positive. In this regard this assay was highly specific for HF. The sensitivity of 34 % was in the same range as described previously by others [56,57]. However, this assay was not able to distinguish between DCM and CAD patients. This observation was also described previously by other authors, who reported findings of autoantibodies against ß1-adrenergic receptor in patients with myocarditis, in patients with malignant essential hypertension or malignant secondary hypertension, in ICM patients [59,60,61] or even in healthy blood donors [59,62].

Interestingly, in this study sera from both DCM and CAD patients reacted in the same order of magnitude with recombinant GST-ß1-AR-II which is remarkable because antibodies against ß1-AR are thought to play a pathogenic role in the development of DCM. In numerous studies it was shown that rodents immunized with synthetic or recombinant peptides corresponding to the second extracellular loop of ß1-adrenergic receptor developed signs and symptoms of DCM [31,36-39,58]. In addition to these animal models, the biological activity of anti-ß1-Abs was assessed by a novel FRET assay by which 55 DCM patients and 22 ICM patients were detected positively [63].

Our result that autoantibodies against the ß1-adrenergic receptor were detected in DCM as well as in NISCM and CAD patients raised the question whether these patients with CAD and NISCM might also suffer from a not yet detected autoimmune DCM. Owing to the fact that DCM is diagnosed by exclusion of other cardiac diseases it is conceivable that patients who were initially diagnosed as having a CAD may, in addition, also have an autoimmune DCM.

In this context it should be noted that antibody positive DCM patients were younger than antibody negative DCM patients (54 versus 61 years). In contrast, a difference in age was seen neither in NISCM nor CAD patients. The higher mean age in CAD patients may be explained by the circumstance that higher age itself is supposed to be one of the high risk factors leading to CAD. This may be due to the long-term consequences of other risk factors such as smoking, arterial hypertension or high levels of LDL-cholesterol.

Furthermore, antibody positive HF patients were shown to have a lower EF than seronegative patients. The correlation between worse EF and autoantibody detection fits with the results of other studies and underscores a potential autoimmune mechanism of DCM. The lower EF as a marker for a distinctive heart failure in autoantibody positive patients might indicate that autoantibodies against ß1-AR may boost the clinical onset of the disease. The association between EF and antibody response could also be demonstrated by the results of immunoadsorption therapies, where the EF was improved after the antibody depletion [40-52]. Even considering the observation that positivity for anti-ß1-antibodies may be a non-specific phenomenon occurring in heart failure by general immune activation, it is remarkable that immunoadsorption relieves characteristic symptoms of DCM such as the improvement of EF [64]. The clinical impact of this test is to be seen in this context, enabling the selection of a subpopulation of patients with confirmed DCM, who may benefit from an immunoadsorption therapy.

Surprisingly, no differences were observed in LVED between the different HF subgroups. Also, BMI had no impact on immune reaction against ß1-AR. However, age was associated with the prevalence of autoantibodies: seropositive patients were younger than antibody negative patients. Provided that antibodies play a pivotal role in pathogenesis of DCM and taking into account that the onset of autoimmune diseases often occurs between the third and fifth decade of life, the mean age of seropositive DCM patients of 54 years might point towards an autoimmune cause of DCM.

Moreover, the level of NT-proBNP, a neurohumoral marker which correlates very well with cardiac function and prognosis, showed a negative correlation with the autoimmune response [65,66]. All seropositive cardiac patients showed a NT-proBNP levels up to 80 % lower than the seronegative patients even though the NT-proBNP levels are still above the reference values (< 150 pg/ml). Prospective studies are necessary to elucidate these findings.

In conclusion, the present study demonstrates that both DCM and CAD patients have an autoimmune response to ß1-AR. However, these disease entities cannot be distinguished by this assay. In patients with confirmed DCM, this test may serve to select an autoantibody-positive subgroup, which might benefit from an immunosuppressive or immunomodulating therapy including immunoadsorption. Finally, we conclude that our test may be used to support the diagnosis of autoimmune DCM. It should be mentioned though that this assay is not intended to be used as a screening assay due to its sensitivity of 37 %. Further studies will be aimed at determining the role of autoantibodies in disease expression. It will also need to be shown if the extent of the autoantibody response to ß1-AR, particularly IgG3, correlates with the onset and course of these diseases.

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