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Focused Issue on K_{ATP} channels

DNA immunizations with M_2 muscarinic and β_1 adrenergic receptor coding plasmids impair cardiac function in mice

Luis E.D. Giménez [a,b], Ciria C.Q. Hernández [b], Elisabete C. Mattos [c], Izaira Tincani Brandão [d], Bianca Olivieri [e], Roberto P. Campelo [a], Tânia Araújo-Jorge [e], Célio Lopes Silva [d], Antônio C. Campos de Carvalho [b], Eleonora Kurtenbach [a,*]

[a] *Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, Universidade Federal de Rio de Janeiro, Centro de Ciências da Saúde, Cidade Universitária, 21941-590, Rio de Janeiro, RJ, Brazil*
[b] *Instituto de Biofísica Carlos Chagas Filho, Universidade Federal de Rio de Janeiro, 21949-900, RJ, Brazil*
[c] *Ecodata Exames Médicos Ltda., Rio de Janeiro, 22020-120, RJ, Brazil*
[d] *Departamento de Bioquímica e Imunologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Avenida Bandeirantes 3900, Ribeirão Preto, SP, Brazil*
[e] *Departamento de Ultra-estrutura e Biologia Celular, Instituto Oswaldo Cruz, Rio de Janeiro, 21045-900, RJ, Brazil*

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Abstract

Autoimmune mediated myocardial damage is likely to be a pathogenic mechanism for acquired dilated cardiomyopathies. Evidence confirms that autoantibodies that bind to M_2 muscarinic (M_2AChR) and β_1 adrenergic receptors (β_1AR) are present in idiopathic dilated cardiomyopathy and Chagasic patients' sera. To elucidate the role of these antibodies in cardiac functional impairment, we used a murine model immunized with plasmids encoding the M_2AChR or β_1AR via gene-gun bombardment. Anti-M_2AChR and β_1AR antibodies were detected over the course of 37 weeks. These antibodies were directed to the second extracellular loop (el2) of both receptors and the third intracellular loop (il3) of the M_2AChR. Peak antibody titers from weeks 2 to 5 against M_2AChR-el2 and β_1AR-el2 as well as elevated titers against M_2AChR-il3 were detected. Anti-M_2AChR-il3 and anti-β_1AR-el2 antibodies were predominant in IgG1 subclass immunoglobulins, suggesting a T-helper-2 biased lymphocyte response. Heart morphology and function was assessed by echocardiography over the course of 42 weeks. Data showed progressive decrease in left ventricular (LV) wall thickness and LV mass that was mostly evident for β_1AR-immunized mice albeit a small change in LV dimensions. Fractional shortening was altered and values of 41%, 37% and 48% were observed at week 42 for the M_2AChR, β_1AR and control groups respectively. In support of autonomic deregulation, a twofold increase in M_2AChR and a similar decrease in β_1AR density were observed in radioligand saturation assays for both experimental groups. Histological analysis revealed myofibril disarray and fibrosis, pointing towards remodeling as a consequence of the long-term presence of anti-receptor antibodies.

Keywords: Dilated cardiomyopathy; Autoimmunity; Autoantibody; M_2 muscarinic acetylcholine receptor; β_1 adrenergic receptor; DNA immunization; Small animal echocardiography

1. Introduction

Acquired dilated cardiomyopathies (DCM) constitute a major cause of heart failure and transplantation in developed countries [1]. Following this trend, the burden of DCM in less developed countries has persistently increased due to acute cardiovascular morbidity such us ischemic heart disease [2]. Idiopathic dilated cardiomyopathy (IDC) and chronic Chagas' disease (CCD) are both acquired forms of DCM for which enterovirus and the parasitic protozoan *Trypanosoma cruzi* were identified as etiologic agents [3]. However, the pathogenic mechanisms that determine the evolution from the acute myocarditis frequently observed in the initial stages of IDC and CCD to DCM remain unclear [4].

An open question resides in the difficulties of establishing a causative relationship between the persistence of viral genomes or *T. cruzi* amastigotes in the myocardium and the

* Corresponding author. Tel.: +55-21-256-26753; fax: 55-21-227-08647.
E-mail address: kurten@bioqmed.ufrj.br (E. Kurtenbach).

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process of heart remodeling and dysfunction described in IDC and CCD patients [3,5]. Thus lack of evidence supporting the hypothesis of direct infectious agent action as the sole determinant of chronic myocardial lesions led to the proposal of aberrant cellular and humoral immune responses as coadjutants in the progression of initial stage myocarditis to chronic DCM [5,6].

In support of this hypothesis, humoral abnormalities were observed in patients with IDC and CCD [3,5]. Antibodies to heart muscle components, such as anti-sarcolemmal and anti-myosin alpha and beta heavy chains, against the mitochondrial adenine nucleotide translocator and against cardiac membrane receptors such as the β_1 adrenergic receptor (β_1AR) and the M_2 muscarinic acetylcholine receptor (M_2AChR) were described [7]. Of all the serologic markers of inflammatory heart disease described, only the anti-mitochondrial and the anti-cardiac membrane receptor antibodies showed relevant functional activity [6,8].

Antibodies with adrenergic (positive chronotropic effect in cultured spontaneously beating rat cardiomyocytes) or muscarinic agonistic (negative chronotropic effect and impairment of L type Ca^{2+} currents in isolated cardiomyocytes) functional activities were detected in the sera of IDC and CCD patients [9–12]. The antibody binding properties to these receptors were also characterized [9,12]. The epitopes of these anti-receptor antibodies were mapped using short overlapping peptides in the context of neutralization experiments in spontaneously beating cardiomyocyte cultures. In these experiments, dominant epitopes were identified in the first and second extracellular loops (el1, el2) of the β_1AR [13]. Roughly 80% of IDC patient sera showed binding to β_1AR-el1 or el2 [13]. The observed incidence of anti-M_2AChR antibodies in IDC patients was 36–50% and in this case, the dominant epitope was mapped to the el2 [11]. On the other hand, the reported occurrence of anti-M_2AChR antibodies in CCD patients was significantly higher than the values reported for IDC [14] and recognition of alternative epitopes on the third intracellular loop (il3) by antibodies from sera of different stage CCD patients that correlate with disease progression was also reported [15].

Previous reports [16–19] dealt with the establishment of animal models of autoimmune cardiomyopathy. Elevated antibody titers were obtained from the sera of these animals by immunizing against synthetic peptides corresponding to the el2 of the human β_1AR and M_2AChR [16,18,19]. In these studies, the rabbit raised antibodies recognized in situ myocardial adrenergic receptors and inhibited muscarinic antagonist binding [16,19]. Moreover, the anti-β_1AR-el2 antibodies had a positive chronotropic effect and the anti-M_2AChR-el2 antibodies had a negative chronotropic effect on rat cardiomyocytes in culture as seen for affinity-purified antibodies from IDC patients [16,18].

The limitations associated to these studies reside in the short-lived humoral responses and the elevated cost of continued synthetic peptide immunizations [20]. Thus, we explored a different approach to immunize our animals, employing the helium-driven dermal bombardment of gold particles coated with plasmid DNA coding for the M_2AChR and β_1AR proteins. Immune autoreactive responses should be enhanced by this immunization procedure, representing an attractive alternative for the establishment of an autoimmune model of DCM [20].

In this study, BALB/c mice were immunized with plasmid DNA coding for the human M_2AChR or β_1AR. To determine the effect of the immunizations, heart function was directly assessed by real-time echocardiographic imaging and receptor antibody titer profiling. Histopathological analysis to assess tissue architecture and radioligand binding assays to determine heart muscarinic and adrenergic receptor expression were also performed.

2. Materials and methods

2.1. DNA Plasmids

The pcDNA3-hM_2 construct coding the human M_2AChR sequence was prepared by subcloning a 1950 bp AvrII restriction fragment from the pcD-hM_2 plasmid that originally included the M_2 muscarinic receptor sequence (from Dr. Edward Hulme, NIMR, London, UK) into the XbaI unique site in the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA, USA). The pBC12BI empty plasmid and the pBC12BI-hβ_1AR plasmid coding for the human β_1AR were from Dr. Bryan R. Cullen and Dr. Robert J. Lefkowitz respectively (Duke University Medical Center, Durham, NC, USA). The plasmids were grown in DH5α *E. coli* strains and purified with Qiagen Giga Kits (Qiagen, Chatsworth, CA, USA) following the manufacturer's instructions.

The el2 from the mouse and the human M_2AChR and β_1AR share a 100% sequence identity. The overall identity between the human and mouse M_2AChR is 92% and 70% among the β_1AR from both species. These high identities allowed for the use of human receptor-coding plasmids in this study.

2.2. Animals and immunizations

Male BALB/c mice, 7 weeks old and weighing 20.4 ± 4.6 g were randomly assigned to a control group immunized either with the pcDNA3.1 or the pBC12BI plasmid (n = 19), to a group immunized with the pcDNA3-hM_2 plasmid construct (n = 18, M_2AChR group) and to a group immunized with the pBC12BI-hβ_1AR plasmid (n = 19, β_1AR group). The animals from each group were individually identified and kept at the faculty animal facility. All animal procedures were carried out in accordance with the guidelines established by the Fiocruz Committee for Ethics for the Care and Use of Laboratory Animals (Resolution 24 2/95).

Immunizations were performed using a helium-driven gene gun (Bio-Rad, Hercules, CA, USA). DNA-coated particles were prepared by combining 25 mg of 1.6 μm gold microcarriers and 100 μl of 0.05 mol/l spermidine with plasmid DNA