Autoantibodies to cardiac troponin I in patients with idiopathic dilated and ischemic cardiomyopathy

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Abstract

Background: Progressive dilatation and functional compromise of heart function is attributed to a variety of pathogenic mechanisms. Experimental data suggests that autoantibodies could promote myocardial damage by inducing either inflammation or alternatively, augmentation of Ca\textsuperscript{2+} currents or activation of surface receptors on cardiomyocytes. Cardiac troponin I (cTpnI) is an essential protein component of the contractile heart. Herein, we studied the presence and functional properties of autoantibodies to cTpnI in patients with idiopathic dilated cardiomyopathy (DCM) and ischemic cardiomyopathy (ICM).

Methods and results: Anti-cTpnI antibody ELISA was established for assessment of sera obtained from 33 patients with ICM, 32 with DCM and 42 healthy subjects. Binding specificity of purified cTpnI-reactive IgG fractions from patients with ICM and DCM was confirmed by competitive inhibition studies employing fluid-phase cTpnI. The effect of IgG preparations on intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]i) transients was tested in cultured neonatal rat ventricular myocytes (NRVM).

Six of the 33 ICM patients (18.2\%) and 5 of the 32 DCM patients (15.6\%) had positive anti-cTpnI antibody titer as compared to none in the healthy subjects. Purified IgG from positive patients appeared specific to cTpnI. IgG preparations reactive with cTpnI did not exhibit measurable effects on [Ca\textsuperscript{2+}]i transients in cultured NRVM nor did they bind the respective cells by direct immunofluorescence.

Conclusion: IgG antibodies to TpnI are increased in a significant number of patients with both ICM and idiopathic DCM, yet it appears that these autoantibodies cannot bind heart muscle cells or influence [Ca\textsuperscript{2+}]i transients.

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1. Introduction

Dilated cardiomyopathy (DCM) is characterized by dilatation and impaired contraction of the left ventricle [1]. It maybe idiopathic, familial/genetic, viral, immune, alcoholic/toxic, or associated with recognized cardiovascular disease in which the degree of myocardial dysfunction is not explained by the extent of ischemic damage. Histology is nonspecific. Presentation is usually with heart failure, which is often progressive. Arrhythmias, thromboembolism, and sudden death are common and may occur at any stage.

Several lines of data may point to the involvement of autoantibodies in the pathogenesis of DCM: (1) A variety of autoantibodies against cardiac cellular proteins have been identified, including G-protein-linked receptors, myosin, mitochondrial proteins, actin, tubulin, heat shock proteins, and the sarcoplasmic reticulum ATPase [2–8]. The pathophysiological relevance of these autoantibodies, however, has not been resolved. (2) Experimental animal studies show
that injection of antibodies or alternatively immunization against peptides corresponding to muscarinic cholinergic or adrenergic receptors could result in cardiac dysfunction [9–11]. (3) If cardiac autoantibodies do play a role either in initiating DCM or in contributing to the progressive functional deterioration of the myocardium, their removal would be expected to lead to disease stabilization or improvement. Dörfel et al. [12] demonstrated clinical and hemodynamic improvement after a short course of immunoadsorption that led to a decline in immunoglobulin levels. Staudt et al. [13] recently reported that 29 out 45 treated congestive heart failure (CHF) patients responded well (left ventricular ejection fraction (LVEF) increased from 20.8±1.0% to 30.5±1.0%; cardiac index, CI, increased from 2.2±0.1 to 3.1±0.1 l/min/m²) within 3 months. It should be emphasized however, that primary endpoints of efficacy were not achieved in these studies.

This collective set of observations may suggest that autoantibodies could potentially be involved in the pathogenesis of DCM either by direct binding to sarcolemmal, mitochondrial or surface proteins or alternatively through induction of myocarditis.

Cardiac troponin I (cTnI) is a member of the troponin complex, responsible for regulating the contraction of cardiac muscle. Mutations in the gene encoding cTnI have been found in familial hypertrophic cardiomyopathy and DCM [14,15]. In the diastolic phase, cTnI binds to actin and inhibits the binding of actin to myosin. Ca²⁺ influx induces the interaction of Ca²⁺ and cardiac troponin C, which releases cTnI from actin and facilitates interaction between actin and myosin with subsequent contraction [16].

In a recent study, Okazaki et al. [17] showed elegantly, that antibodies from PD-1 mice that exhibit DCM are reactive with troponin I. They further went on to prove that these antibodies augmented voltage-dependent Ca²⁺ current of cardiomyocytes as much as 1.4-fold and were found by immunofluorescence to bind to the surface of cardiomyocytes. To date, there has been no report of autoantibodies to cTnI in human subjects nor has there been a possible role attributed for these antibodies in the possible induction of DCM. In the current study we tested the hypothesis that patients with DCM have increased levels of anti-cTnI autoantibodies, and studied their effects on [Ca²⁺]ᵢ transients in ventricular myocytes in vitro.

2. Methods

2.1. Patients

Institutional ethics committee approved the study and informed consent was obtained from all patients. CHF patients were recruited from the local outpatient clinic. Thirty-four had DCM confirmed by a recent coronary angiography, 33 had ICM evident by multi vessel coronary stenosis and previous percutaneous coronary interventions. Patients were defined as having ICM if they had previous documentation of myocardial infarctions and multivessel coronary artery disease and ejection fraction of less than 40%. Patients with DCM were defined as having EF of <40% and normal coronary arteries. Patients with left ventricular aneurysm were excluded. The control group consisted of 42 healthy participants.

2.2. Assessment of serum anti-cTnI antibody levels by ELISA

Plates were coated overnight at 4 °C with human cTnI (1 µg/ml). Next, plates were washed 3 times with a solution containing 0.05% Tween 20 in PBS at pH=7.4, and then blocked with 1.5% BSA in PBS for 2 h at room temperature. After another series of washings, samples of diluted serum 1:50 (in diluent solution 0.15% BSA in TBS) from patients and controls were added to the plates and incubated on a shaker for 2 h at room temperature. Following washings, alkaline phosphate conjugated detection antibody (rabbit anti human IgG) in a diluent solution was added to the plates for 1 h at room temperature. Next, the plates were washed and the substrate pNPP in NaHCO₃+MgCl₂ at pH=9.6 was added for 30 min. Absorbance was determined using an ELISA plate reader at 450 nm.

2.3. Purification of human IgG

IgG from cTnI-positive and negative sera was purified by a protein G-sepharose column (Pharmacia, Uppsala, Sweden).

2.4. Competitive inhibition studies

The concentration of anti-cTnI IgG giving 50% maximal binding in the anti-cTnI ELISA was determined. The anti-cTnI IgG at the predetermined concentration was pre-incubated with equal volumes of cTnI or human serum albumin (HSA) at varying concentrations for 1 h at room temperature. The mixtures were transferred in 100 µl aliquots into cTnI-coated wells and bound antibodies were detected by absorbance using an ELISA-plate reader at 450 nm.

2.5. Preparation and isolation of neonatal rat ventricular myocytes (NRVM) cultures

NRVM cultures were prepared from ventricles of 1- to 2-day-old Sprague-Dawley rats and experiments performed on cultures 4–6 days after plating [18].

2.6. Immunofluorescence studies of IgG binding to cardiomyocytes

NRVM were fixed, permeabilized and sequentially incubated with 5% BSA in PBS for 30 min at RT, with
stained by fluorescein isothiocyanate conjugated affinity purified goat anti-human IgG and examined under a fluorescence microscope.

2.7. Measurement of [Ca\(^{2+}\)]\(_i\) transients

NRVM cultures firing spontaneously were loaded for 25 min at room temperature (24 °C to 25 °C) with fura 2-AM (Molecular Probes), at a final concentration of 5 μmol/l, in a 1:1 mixture of Tyrode’s solution and dissociation solution containing 2% bovine albumin as described [19]. Excess fura 2 was removed by rinsing twice with Tyrode’s solution. Myocytes were then transferred to a nonfluorescent chamber mounted on the stage of an inverted microscope (Diaphot 300, Nikon) and visualized with a ×40 oil immersion Neofluor objective. The chamber was perfused with Tyrode’s solution at a rate of 1 ml/min. Experiments were performed at 37 °C. Fura 2 fluorescence was measured using a dual-wavelength system (Delta-Scan, Photon Technology Intl.). Briefly, light emitted from a xenon arc lamp was fed in parallel into two independent monochromators to obtain quasimonochromatic light beams of two different wavelengths exciting the cell at 340 and 380 nm. Either a 340- or 380-nm wavelength was switched by a rotating chopper disk at a frequency enabling ratio measurements at a rate of 150/s. The two separate monochromator outputs were collected by the ends of a bifurcated quartz fiber optic bundle. The emitted fluorescence (510 nm) was collected by the microscope optics, passed through an interference filter, and detected by a photomultiplier tube (710 PMT Photon Counting Detection System, Photon Technology Intl). Raw data were stored for off-line analysis by Felix software (Photon Technology Intl) as 340- and 380-nm counts and as the following ratio: \[ R = \frac{F_{340}}{F_{380}} \] where \( F_{340} \) and \( F_{380} \) indicate fluorescence at 340 and 380 nm, respectively. For scaling the fluorescence ratio, cell-derived autofluorescence and non-cell fluorescence were subtracted from the measured fluorescence. In these experiments, myocytes were stimulated at 1.0 Hz using platinum wires embedded in the walls of the perfusion chamber.

\[ [Ca^{2+}]_i \] transients were initially measured in NRVM cultures superfused with Tyrode’s solution (Control, \( t=0 \) min). Thereafter, the regular Tyrode’s was replaced with Tyrode’s containing the antibodies (10 μg/ml), and \([Ca^{2+}]_i\) transients measured every 5 min for 30 min. Only spontaneously beating cultures were chosen.

2.8. Statistical analysis

Normality was assessed using Kolmogorov–Smirnov test. Results were expressed as mean±S.D., and differences between groups were assayed by a one-way ANOVA test. Differences between means were considered significant at \( p<0.05 \). Data were analyzed using SigmaStat statistical software package version 3.0.

3. Results

The clinical characteristics of the studied patients are provided in Table 1. No differences were evident between the ICM and DCM with regard to age, sex, New York Heart Association Class (NYHA), ejection fraction (EF) or use of medications.

Initially, we investigated the presence of IgG autoantibodies to cTpnI in patients with ischemic and idiopathic cardiomyopathy in comparison with healthy subjects. Mean levels of IgG autoantibodies to cTpnI were significantly increased in patients with ICM (mean optical density (OD) of 0.61±0.06) and DCM (0.54±0.04) in comparison with healthy subjects (0.37±0.02; \( p<0.001 \) for both comparisons).

To define ‘positivity’ for cTpnI, we set the mean optical density value obtained for the healthy subjects plus three standard deviations. Employing this threshold level, we found that five patients from the 32 having DCM (15.6%) and 6 patients from the 33 having ICM (18.2%) had anti-cTpnI autoantibodies. None of the healthy subjects was positive for anti-cTpnI antibodies (Fig. 1).

Next, we set out to study the possible cell-binding and functional properties of anti-cTpnI antibodies. For this purpose, we purified on a protein G column, all 11 positive fractions as well as 5 cTpnI-negative preparations from ICM and DCM and tested their binding to solid phase immobilized cTpnI. As could be observed in the representative example, ‘positive’ IgG preparations exhibited a dose dependent binding to cTpnI coated on the ELISA plates, from which the concentrations yielding 50% of the maximal binding were determined as 0.8 and 1.2 μg/ml for DCM and ICM preparations, respectively.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>Dilated CMP</th>
<th>Ischemic CMP</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=42)</td>
<td>(n=32)</td>
<td>(n=33)</td>
<td></td>
</tr>
<tr>
<td>Gender, males</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>males</td>
<td>13 (31%)</td>
<td>22 (69%)</td>
<td>27 (82%)</td>
<td>NS</td>
</tr>
<tr>
<td>Age, years (mean)</td>
<td>33.7</td>
<td>63.3±2.3</td>
<td>71.2±1.8</td>
<td></td>
</tr>
<tr>
<td>Weight, kg (mean)</td>
<td>–</td>
<td>79±3.3</td>
<td>83.8±4.6</td>
<td></td>
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<tr>
<td>Hemoglobin, g/dl</td>
<td>–</td>
<td>13.4±0.28</td>
<td>13±0.3</td>
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<tr>
<td>NYHA class (mean)</td>
<td>1</td>
<td>2.34±0.16</td>
<td>2.57±0.13</td>
<td></td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>–</td>
<td>29.1±1.2</td>
<td>29.1±2.2</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>18 (43%)</td>
<td>14 (44%)</td>
<td>14 (42%)</td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0</td>
<td>8 (25%)</td>
<td>13 (39%)</td>
<td></td>
</tr>
</tbody>
</table>

**Medications**

|                |                  |             |             |              |
| Aspirin        | 22 (52%)         | 14 (44%)    | 25 (76%)    | \( p=0.01 \) |
| ACE inhibitors | 0                | 25 (78%)    | 24 (73%)    |              |
| Furosemide     | 0                | 26 (81%)    | 30 (91%)    |              |
| Spironolactone | 0                | 21 (66%)    | 20 (61%)    |              |
| Nitrates       | 0                | 8 (25%)     | 13 (39%)    |              |
| Erythropoietin | 0                | 3 (9%)      | 5 (15%)     |              |
| Digitalis      | 0                | 10 (31%)    | 9 (27%)     |              |
| Warfarin       | 0                | 17 (53%)    | 7 (21%)     | \( p=0.01 \) |
| Statins        | 0                | 14 (44%)    | 24 (73%)    |              |
| Beta-blockers  | 22 (52%)         | 19 (59%)    | 22 (67%)    |              |
| Calcium channel blockers | 0           | 11 (34%)    | 4 (12%)     | \( p=0.04 \) |

ACE=angiotensin-converting enzyme. NYHA=New York Heart Association. The \( p \) values relate to differences in comparison with healthy controls.
binding to cTpnI was determined. Next, we validated the specificity of these IgG preparations to cTpnI. As can be seen in the provided example, binding of IgG anti-cTpnI to coated cTpnI, was inhibited in a dose dependent manner, by fluid phase cTpnI but not by an irrelevant human protein (human serum albumin) (Fig. 2).

We then investigated whether IgG anti-cTpnI bind directly cultured NRVM as has been shown for antibodies recovered from PD-1 mice [17]. However, employing different concentrations of IgG, we could not detect binding to fixed cardiomyocytes in any of the preparations reactive with cTpnI.

We then set out to study the possibility that human autoantibodies to cTpnI alter [Ca^{2+}]_i transients in NRVM. Employing several IgG preparations that exhibited specific reactivity to cTpnI, we failed to detect any effect on [Ca^{2+}]_i transients characteristics at any of the IgG concentrations tested (0.1–25 μg/ml) (Fig. 3).

4. Discussion

In this study, we demonstrate for the first time, the presence of antibodies reactive with human cardiac troponin I in patients with DCM. These antibodies were found to reside in the sera of ~18% of patients with ischemic DCM, and ~15% of the patients with idiopathic DCM and none of the healthy subjects. These antibodies appear specific as inferred from the competitive inhibition studies showing that fluid phase cardiac troponin I was capable of inhibiting the binding of purified ‘positive’ IgG fractions reactive with cTpnI to the respective protein immobilized on solid phase surface.

Autoantibodies to sarcolemmal, mitochondrial and surface proteins have been described in patients with DCM [1–8]. However, evidence suggesting that these autoantibodies are capable of inducing or accelerating myocardial damage in humans, is lacking. There are several potential mechanisms by which autoantibodies can induce myocardial damage [20,21]. Thus, myocardial inflammation has been suggested to trigger autoimmune myocarditis, whereas activation of surface alpha and beta adrenergic receptors has been proposed to lead to chronic cardiac stimulation [22].

The recent finding by Okazaki et al. [17], showing that in DCM prone PD-1 mice, antibodies to cTpnI trigger myocardial damage, possibly by augmentation of L-type Ca^{2+} currents, has prompted us to investigate the possible presence of these antibodies in DCM patients. As shown, detectable IgG autoantibodies to cTpnI were found in some of the patients with ischemic and idiopathic DCM. This is indeed the first demonstration of anti-cTpnI autoantibodies in human subjects. However, immunofluorescence studies failed to demonstrate binding of these antibodies to NRVM. Furthermore, none of the purified antibody preparations reactive with cTpnI appeared to influence the [Ca^{2+}]_i transients in cultured NRVM. Several explanations can be offered for the observed results: Autoantibodies to cTpnI are not involved in the pathogenesis of myocardial damage in humans with DCM. These results are supported by the lack of binding of purified IgG anti-cTpnI fractions with cultured NRVM and the lack of effect on [Ca^{2+}]_i transients in cultured NRVM. Moreover, it would be expected that autoantibodies to cTpnI would be higher in patients with idiopathic than...
with DCM, where an obvious reason for compromised myocardium exists; this was not the case, as similar percentage of patients with idiopathic and ischemic DCM exhibited antibodies to cTpnI. It should be realized however that in patients with myocardial dysfunction, the appearance of antibodies could represent a non-specific immune response regardless of the etiology. Moreover, we have not tested the presence of a possible infectious trigger in patients with idiopathic DCM, that could potentially have induced humoral cardiodepressant effects.

An alternative explanation for the lack of antibody effects on Ca$^{2+}$ currents and binding to cultured cardiomyocytes was that our IgG preparations contained a relatively small fraction of potentially 'pathogenic' cTpnI autoantibodies. Affinity purification through a cTpnI-column would have provided a more concentrated preparation, yet the expected yield of such a column was extremely low to provide sufficient amounts to be employed in the in vitro assays. Moreover, it should be emphasized that anti-cTpnI antibodies were reactive with solid phase bound human cTpnI, yet tested on rat cardiomyocytes; the lack of complete sequence preservation between humans and rats may have been responsible for the lack of high affinity binding required for intact and potent functional properties evident by the in vitro assays. Unfortunately, we could not obtain rat cTpnI to resolve this issue.

Our finding of increased levels of cTpnI autoantibodies in patients with ischemic CM is apparently surprising. We assume that ischemia releases cTpnI to the systemic circulation and the protein may be modified and lead to a respective autoimmune reaction. Consistent with this hypothesis, we have recently observed that in patients with a prior myocardial infarction, cellular immunity to an additional cardiac protein – myosin – was evident [23]. Thus, ischemic events could be triggers of autoimmune-like responses that may turn out to be of pathogenic significance.

In conclusion, we describe here for the first time, the presence of IgG autoantibodies to cTpnI in patients with idiopathic and ischemic DCM. Although we failed to demonstrate binding of these antibodies to cultured cardiomyocytes and effects on Ca$^{2+}$ currents, it would nevertheless be interesting to further explore possible mechanisms of cTpnI antibody mediated damage to the myocardium.

References


