Functional analysis of human cardiac troponin by the in vitro motility assay: comparison of adult, foetal and failing hearts

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Abstract

Objective: Human cardiac development and heart failure are associated with altered troponin isoform expression and phosphorylation. As the functional effects of these changes in troponin are unknown, we isolated troponin from human foetal, normal adult and failing adult hearts and investigated their regulatory function. Methods: Human cardiac troponin was assayed for regulatory function by in vitro motility assay and for protein content by SDS PAGE and immunoblotting. Results: Human cardiac troponin regulated movement of actin–tropomyosin filaments over a bed of immobilised heavy meromyosin. At pCa 9, troponin from foetal and adult hearts reduced the fraction of filaments moving from 90% to less than 15% with a modest (25–30%) decrease in velocity. At pCa 5, troponin from normal adult hearts increased filament velocity by up to 47±3% with no change in the fraction of filaments moving. Foetal troponin increased velocity by only 4±6% and the effect of troponin from failing hearts was between these values at 31±5%. Foetal hearts showed different troponin I and T isoform expression compared with adult hearts. No differences in troponin isoform expression were demonstrated between normal and failing adult hearts. Conclusions: Functioning troponin and tropomyosin may be isolated from human heart and their properties investigated by in vitro motility assay. Both functional and isoform expression differences exist between foetal and adult cardiac troponin. The regulatory function of troponin from adults with end stage heart failure is different from normal adult troponin. These data suggest a role for altered troponin function in human cardiac development and heart failure.

Keywords: Contractile apparatus; Contractile function; Heart failure; Developmental biology; Cardiomyopathy

1. Introduction

Cardiac development and cardiac failure are associated with changes in myocardial thin filament protein expression and phosphorylation [1]. In man, cardiac development is associated with changes in troponin I and troponin T isoform expression [2–7] while heart failure has been associated with reversion of troponin T expression towards the foetal pattern [2,8,9] and reduced phosphorylation of troponin I [10,11].

Data from animal experiments have clearly shown that changes in troponin which are seen during human cardiac development and heart failure could result in significant functional effects [12–19]. Currently, no functional data is available on human foetal cardiac thin filament proteins and functional data on adult human cardiac troponin are scant, conflicting and derived from indirect experiments. Changes in cardiac troponin T isoform expression has been found to correlate with maximum myofibrillar ATPase in failing ventricles [2] and indicators of disease severity in patients with congenital heart disease [8]. However, changes in the Ca$^{2+}$-sensitivity of isometric force in failing heart myofibrils appeared to be related to modulation of troponin I phosphorylation [11] and not to troponin T isoform expression. It is therefore important to directly characterise and compare cardiac troponin function in human foetal, normal adult and failing adult heart.

The purpose of this study was to develop a method to isolate functionally competent troponin and tropomyosin from human myocardium and to investigate troponin

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regulation of actin–tropomyosin filaments by in vitro motility assay. The in vitro motility assay allows us to study the behaviour of individual thin filaments moving over immobilised myosin. Filament movement may be characterised by two parameters which seem to be related to important aspects of the crossbridge cycle [20]. The fraction of filaments that are moving appears to be a measure of the population undergoing weak to strong actin–myosin binding transitions and the velocity is related to the crossbridge cycling rate. Skeletal muscle troponin–tropomyosin can control these parameters independently in vitro. A 30–42.5% saturated cut contained the crude extract was fractionated by ammonium sulphate precipitation powder preparation and protein extraction. The measure of the population undergoing weak to strong E-64, leupeptin and pepstatin A were used throughout ether powder preparation and protein extraction. The extract was fractionated by ammonium sulphate precipitation. A 30–42.5% saturated cut contained the crude troponin fraction and the 65% saturated pellet contained tropomyosin. Troponin from control and test hearts (foetal or failing adult) were isolated in parallel and assayed in parallel within three days of isolation. Tropomyosin was further purified by two successive precipitations at pH 4.5 followed by chromatography on DEAE Fast-Flow in 20 mM Tris–HCl pH 8.0, 10 mM 2-mercaptoethanol, 0–0.5 M NaCl gradient. The human cardiac tropomyosin was exclusively αα-isoform.

2. Methods

2.1. Human myocardial samples

Adult myocardium was obtained from patients with end-stage heart failure undergoing orthoptic cardiac transplantation and patients with normal hearts who were rejected as heart donors on technical grounds. Hearts classified as normal were obtained from patients with no history of cardiac disease, a normal cardiac examination, normal ECG and normal ventricular function on echocardiography performed within 24 h of heart explantation. Myocardium was stored on ice for a maximum of 60 min prior to being frozen in liquid nitrogen where it was stored until analysis. Foetal myocardium was obtained from Dr L Wong, MRC Foetal Tissue Bank, RPMS, London, UK, from morphologically normal foetuses having undergone termination and was stored at –80°C until analysis. Ethical approval was obtained in each institution and the investigation conformed with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997;35:2–3).

2.2. Protein preparation

Rabbit skeletal muscle actin and heavy meromyosin (HMM) were prepared by standard protocols [22]. HMM was pre-spun in the presence of actin and ATP to sediment rigor heads as previously described [20]. Human cardiac ether powder was prepared from 2 g of myocardium according to the protocol described by Potter [23]. Left ventricular myocardium was used for adult troponin and tropomyosin extraction. Both ventricles were used for foetal troponin extraction. Ether powders were stored at –80°C for up to 8 weeks. Troponin and tropomyosin were extracted from ether powder with 2×10 vols of 1 M KCl, 20 mM TES pH 7.0 and 15 mM β-mercaptoethanol. 2 μg/ml of each of the protease inhibitors chymostatin, E-64, leupeptin and pepstatin A were used throughout ether powder preparation and protein extraction. The extract was fractionated by ammonium sulphate precipitation. A 30–42.5% saturated cut contained the crude troponin fraction and the 65% saturated pellet contained tropomyosin. Troponin from control and test hearts (foetal or failing adult) were isolated in parallel and assayed in parallel within three days of isolation. Tropomyosin was further purified by two successive precipitations at pH 4.5 followed by chromatography on DEAE Fast-Flow in 20 mM Tris–HCl pH 8.0, 10 mM 2-mercaptoethanol, 0–0.5 M NaCl gradient. The human cardiac tropomyosin was exclusively αα-isoform.

2.3. In vitro motility assay

In vitro motility assays were performed as described previously using 100 μg/ml skeletal muscle HMM immobilised on siliconized coated cover glasses [20,21,24]. Rabbit skeletal muscle actin was labelled with rhodamine phalloidin (ϕ) [25]. Then, 100 nM actin-ϕ, 200 nM human cardiac troponin and crude human cardiac troponin (20–160 μg/ml) were premixed and then diluted to a working concentration of 10 nM actin-ϕ in 50 mM KCl, 25 mM imidazole–HCl pH 7.4, 4 mM MgCl₂ and 5 mM DTT immediately prior to infusion into the motility cell. Control assays were carried out with actin-ϕ filaments and actin-ϕ–tropomyosin filaments at experimental concentrations. Filament movement in 1 mM MgATP and 0.5% methyl cellulose at 28°C was observed with a fluorescence microscope (Zeiss AxioLab x64/1.4 NA Planapochromat lens, DAGE-SIT68 Camera) and analysed to determine fraction of filaments moving and velocity of motile filaments using an automatic tracking program [26]. Experiments only proceeded if the initial fraction of motile actin-ϕ filaments was greater than 0.80 and if tropomyosin–troponin reduced the fraction of motile filaments to below 0.20 at pCa 9. To obtain the best consistency of results each experiment included measurements of actin-ϕ actin-ϕ tropomyosin, actin-ϕ tropomyosin–troponin–control (normal adult) and actin-ϕ tropomyosin–troponin-test (foetal or failing adult) to facilitate comparisons where only the troponin varied.

2.4. Statistical analysis

The fraction of motile filaments is shown in absolute values. Actin-ϕ tropomyosin–troponin filament velocity is expressed relative to the velocity of actin-ϕ tropomyosin
filaments measured in the same experiment. Results are expressed as mean±standard error of the mean (S.E.M.). Each experiment (n) is the independent analysis of a different human adult heart or batch of pooled foetal hearts. Filament movement vectors are known to be normally distributed [26]. Data were compared by Student’s t-test.

3. Results

Approximately 100 μg of crude cardiac troponin was obtained from every gram of human heart muscle. Samples of troponin from four non-failing hearts, six failing hearts and two batches (n=20) of foetal hearts were studied. SDS-PAGE analysis of purified human cardiac tropomyosin demonstrated a single band of α-tropomyosin (Fig. 1). Analysis of the crude cardiac troponin samples demonstrated that all troponin subunits (T, I and C) were present and occurred in consistent proportions in all preparations. Identifiable troponin species made up the majority of proteins in the samples, in particular there was no tropomyosin contamination and very little actin; 70±3% of total protein quantity in the crude troponin extract was troponin. Western blotting with anti-cardiac troponin T showed a single band for all adult samples which co-migrated with the troponin T3 standard. An additional band with lower mobility was seen in the foetal specimens and was presumably troponin T1 [2]. Western blotting with anti-troponin I antibody detected a predominant protein band in foetal specimens which had faster mobility than the adult cardiac troponin I standard and was presumably slow skeletal troponin I. Two bands were detected by anti-troponin I antibody in the adult specimens. The band with lower mobility co-migrated with cardiac troponin I while the higher mobility band co-migrated with foetal troponin I. This was likely to be degraded troponin I rather than slow skeletal troponin I since expression of slow skeletal troponin I has not previously been reported in normal or failing adult myocardium [3,4,18]. The relative quantity of this fragment was similar in normal and failing heart troponin samples.

Thin filaments were reconstituted by mixing 100 nM actin with tropomyosin and troponin followed by ten-fold dilution. Sedimentation experiments showed that tropomyosin and troponin I, C and T were bound to actin under the conditions of motility assay (Fig. 1B). The band immediately below troponin I, which we suggested was degraded troponin I was present in reduced quantity in the sedimented thin filaments.

When 20 nM of human cardiac α-tropomyosin was mixed with actin-β filaments the fraction of filaments which were motile remained high and filament speed increased by 20±3% over actin-β filaments. The fraction of motile actin-β tropomyosin filaments and their speed were insensitive to alterations in calcium concentration.

The effect of all the human cardiac troponin samples on actin-β–α-cardiac tropomyosin filament motility was the same qualitatively as we have reported for skeletal muscle troponin (Fig. 2); the primary effect of troponin in relaxing conditions (pCa 9) is to reduce the fraction of filaments motile to about 10% whilst reducing the speed of moving filaments only slightly [20]. The observation of actin-β–α-tropomyosin regulation by only 2–16 μg/ml of human cardiac troponin indicates strong affinity of troponin for actin–tropomyosin filaments as in vivo which is confirmed by the sedimentation experiments (Fig. 1B). The effects on filament movement reached a maximum within the concentration range of troponin investigated (Fig. 3). Thus functional properties were independent of troponin concentration and could be compared between different troponin samples.

At pCa 9 addition of troponin to actin-β–tropomyosin resulted in a large decrease in the fraction of filaments motile and a modest decrease in the filament speed (Fig. 2). The effect of troponin from non-failing, failing and foetal hearts was indistinguishable (Fig. 3A,C). The effect of troponin reached a maximum at 8 μg/ml. The fraction of filaments that were motile was 0.11±0.03 compared with 0.85±0.02 in the absence of troponin. The speed of filaments that were motile decreased by 29±4% compared with actin-β–tropomyosin control.

At pCa 5 human cardiac troponin caused an increase in actin-β–tropomyosin filament speed, reaching a maximum at approximately 8 μg/ml troponin, while maintaining the high proportion of motile filaments (Figs. 2 and 3B,D).

It was clear that the magnitude of the increase in speed depended upon the origin of the troponin. Troponin from normal adult hearts caused a mean increase in speed of 47±5% over the actin-β–α-tropomyosin filament control at 8 μg/ml compared with a 4±6% increase in speed caused by foetal cardiac troponin or skeletal muscle troponin (Table 1, Fig. 3). All the cardiac troponin samples extracted from adults with end-stage heart failure gave speed increases that were intermediate between normal adult and foetal actin-β–α-tropomyosin–troponin filaments. There was no detectable correlation between disease type or severity and filament speed in the samples analysed.

4. Discussion

It has been established that the isoform expression pattern of cardiac regulatory proteins changes in development from foetus to adult [2–7] and that in failing hearts the expression pattern may revert towards the foetal phenotype [2,8,27]. However, very little information is available as to whether such protein changes are associated with any functional alteration in the regulatory mechanism other than in small animal studies [14–19]. We have been able, for the first time, to isolate human cardiac troponin...
Fig. 1. SDS PAGE of human cardiac troponin and tropomyosin preparations. Panel A. Coomassie blue stained SDS PAGE analysis of troponin prepared from two normal adult hearts, two failing adult hearts and a batch of 20 pooled foetal hearts. Human cardiac tropomyosin was prepared from normal adult heart. Troponin standards are recombinant expressed human sequence troponin subunits. Panel B. Analysis of the pellets following sedimentation of actin–tropomyosin–troponin mixtures under in vitro motility assay conditions. Actin pellet 100 nM: actin 100 nM, 200 nM tropomyosin, 40 μg/ml troponin. Actin pellets 10 nm: the same mixture sedimented after ten-fold dilution in 50 mM KCl, 25 mM imidazole–HCl pH 7.4, 4 mM MgCl₂ and 5 mM DTT at pCa9 and pCa5.

and tropomyosin in a fully functional state and have investigated their functional properties by means of the in vitro motility assay. This assay enables us to measure the Ca²⁺-dependent control of movement of single actin filaments over a bed of HMM. Analysis of the movement gives us a number of motility parameters which seem to be related to important aspects of the contractile cycle, such as cross bridge recruitment (fraction of filaments motile) and rate of cross bridge cycling (filament speed) [20,24].

4.1. Tropomyosin

In the in vitro motility assay both human cardiac tropomyosin and human cardiac troponin have properties which are quantitatively different from skeletal muscle troponin and tropomyosin (Table 1). In particular human cardiac tropomyosin, which we found to be exclusively αα-isofrom, increased the speed of actin-β filaments, in common with the recombinant chicken and human αα-ser-
Fig. 2. Regulation of actin–tropomyosin filament movement by human troponin. The movement of actin–human tropomyosin filaments over immobilised skeletal muscle heavy meromyosin was analysed and the results are shown in the form of frequency histograms. Addition of human troponin caused the filaments to progressively stop moving in pCa 9 buffer. At pCa 5 the effect of troponin was to increase the speed of the filaments.

**αα-tropomyosin previously investigated [21]. In contrast, skeletal muscle αβ-tropomyosin did not increase filament speed under comparable conditions. The difference seems to be a consequence of the equilibrium constant between on and off states of actin–tropomyosin being greater for α-tropomyosin. This may be experimentally demonstrated by adding NEM S-1, a chemically modified myosin which can switch actin–tropomyosin filaments to the fully on state in the presence of ATP. NEM S-1 addition did not increase the speed of actin–αα-tropomyosin filaments, indicating that they were already fully on, whereas it did increase the speed of αβ-tropomyosin filaments up to the level of αα-tropomyosin-containing filaments [24,28].**

4.2. Troponin

All the cardiac troponin–tropomyosin combinations we tested showed a similar inhibition of filament motility at pCa 9 (major decrease in motile fraction, smaller decrease in filament speed), which approached saturation. This response was also very similar to the inhibition previously observed with rabbit skeletal muscle troponin and αβ-tropomyosin or recombinant ala-ser-αα-tropomyosin [20,21,24] indicating that the human cardiac troponin and tropomyosin preparations were functionally competent. The only parameter which differed between troponin from foetal, normal and failing hearts was actin-β–tropo-
Fig. 3. Comparison of the effects of human foetal, normal adult and failing adult cardiac troponin on actin–tropomyosin filament movement. The upper panels show the effects of human cardiac troponin on filament speed at pCa 9 (A) and pCa 5 (B). The lower panels show the effects of human cardiac troponin on the fraction of filaments which are motile at pCa 9 (C) and pCa 5 (D). Actin–tropomyosin–troponin filament speed is given as a proportion of the actin–tropomyosin speed to eliminate day to day changes in the speed of actin–tropomyosin filaments over HMM (mean speed of actin–tropomyosin filaments was 4.1±0.1 μm/s, n=24). The fraction of filaments which are motile is given as an absolute value. Results are expressed as mean±S.E.M. from different patients or heart batches in the case of foetal results (normal adult n=4; failing adult n=6; foetal n=2). * p<0.05 for comparison by Student’s t-test at the same concentration.

The difference in filament velocity can be confidently attributed to troponin as all other experimental variables were controlled. The normal adult human cardiac troponin–tropomyosin combination gave a remarkable 47±5% increase in speed over actin–tropomyosin alone, which is 2-fold greater than the largest previously reported effect (rabbit skeletal troponin–tropomyosin, Table 1). Foetal human cardiac troponin gave a very different effect, with only a 4±6% increase in speed at pCa 5. The properties of foetal cardiac troponin were similar to rabbit skeletal muscle troponin (Table 1); this may be due to the presence of the slow skeletal troponin I isoform rather than cardiac troponin I in the human foetal heart [3,4,18].

4.3. Troponin from failing hearts

There was a significant functional difference between troponin from normal compared with failing adult hearts. Cardiac troponin from failing hearts gave a smaller increase in filament speed at pCa 5 than normal cardiac troponin (Fig. 3). There was no difference in the fraction

Table 1
Effects of tropomyosin and troponin on actin–filament speeda

<table>
<thead>
<tr>
<th>Type of troponin</th>
<th>Type of tropomyosin</th>
<th>Alα-ser-tropomyosin</th>
<th>Human cardiac tropomyosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal</td>
<td>20±2 a,d</td>
<td>5±1b</td>
<td>2±2</td>
</tr>
<tr>
<td>Adult cardiac</td>
<td>17±3c</td>
<td>47±3d</td>
<td>47±5e</td>
</tr>
<tr>
<td>Foetal cardiac</td>
<td>4±6</td>
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a The table shows the percentage increase in filament speed±standard errors caused by troponin at pCa 5 for various combinations of troponin and tropomyosin.
bp<0.05 compared with other tropomyosin species by Student’s t-test.
ec<0.001 compared with other troponin species by Student’s t-test.
d Data from Ref. [21].
of motile filaments. The association of heart failure with lower filament speed in the motility assay is consistent with the finding of depressed maximum Ca\(^{2+}\)-dependent actomyosin MgATPase activity in human failing heart preparations [27,29,30] and may lead to lower speed of unloaded shortening in intact muscle. Our results are consistent with a partial reversion of troponin function to foetal phenotype in end-stage heart failure. SDS-PAGE (Fig. 1) does not indicate any systematic difference between normal and failing heart troponin isoforms, however, altered phosphorylation of troponin subunits remains a possible explanation for the functional difference in failing hearts [10,11].

Many changes take place in subcellular processes in heart failure [31,32] and the sequence of these changes in human disease is unknown. At present we do not know whether the alteration in troponin function is a cause of progression to heart failure or a compensatory mechanism. Recent studies on familial hypertrophic cardiomyopathy indicate that mutations in troponin T or tropomyosin which cause small changes in in vitro contractile performance [21,33] are associated with, and presumably cause, clinically important hypertrophy and contractile dysfunction [34]. The large difference in troponin function, which we have demonstrated in failing hearts, would be expected to produce a chronic reduction in cardiac contractility which might eventually produce heart failure.

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References


