Expedited Publications

Troponin I Gene Expression During Human Cardiac Development and in End-Stage Heart Failure


Recent reports have demonstrated the presence of two isoforms of troponin I in the human fetal heart, namely, cardiac troponin I and slow skeletal muscle troponin I. Structural and physiological considerations indicate that these isoforms would confer differing contractile properties on the myocardium, particularly on the phosphorylation-mediated regulation of contractility by adrenergic agonists. We have investigated the developmental expression of these isoforms in the human heart from 9 weeks of gestation to 9 months of postnatal life, using Western blots revealed with troponin I antibodies to detect troponin protein isoforms and Northern blots to detect the corresponding mRNAs. The results show the following: 1) Slow skeletal muscle troponin I is the predominant isoform throughout fetal life. 2) After birth, the slow skeletal isoform is lost, with cardiac troponin I being the only isoform detectable by 9 months of postnatal development. 3) The protein isoforms and their corresponding mRNAs follow the same pattern of accumulation, suggesting that the transition in troponin expression is regulated at the level of gene transcription. The developmental transition in troponin I isoform content has implications for contractility of the fetal and postnatal myocardium. We further analyzed right and left ventricular muscle samples from 17 hearts in end-stage heart failure resulting from pulmonary hypertension, ischemic heart disease, or dilated cardiomyopathy. Cardiac troponin I mRNA remained abundant in each case, and slow skeletal muscle troponin I mRNA was not detectable in any of sample. We conclude that alterations in troponin I isoform content do not therefore contribute to the altered contractile characteristics of the adult failing ventricle. (Circulation Research 1993;72:932–938)

KEY WORDS • troponin • gene expression • cardiac development • end-stage heart failure

The troponin complex is located on the thin filament of striated muscle and controls the interaction of thick and thin filaments in response to alterations in intracellular Ca$^{2+}$ concentrations. It is composed of three protein components: troponin I (TnI), which acts as an inhibitory subunit, troponin C (TnC), the calcium binding subunit, and troponin T (TnT), which is involved in the attachment of the complex to the thin filament. Each of these subunits exists as a number of different isoforms that are associated with different muscle types (i.e., fast and slow skeletal muscle and cardiac muscle).

In the case of troponin I, three isoforms have been identified that are the products of three separate genes.6–15 In adult muscle, these isoforms are partitioned between fast skeletal muscle fibers (TnIf), slow skeletal muscle fibers (TnIs), and cardiac muscle (TnIc).

An important feature of the cardiac isoform as compared with isoforms of fast and slow skeletal muscle is that it carries an extended N-terminal sequence containing serine residues that are phosphorylated in response to adrenergic stimulation of the heart. Phosphorylation of TnIc has an influence on the affinity of the troponin complex for Ca$^{2+}$ and, as a consequence, acts to alter the contractile characteristics of the heart.16–19 This mechanism forms part of the phosphorylation-mediated control of cardiac contractility by adrenergic agonists. Troponin isoform transitions have been documented for the developing heart in both rodents and chicken,9,11,19,21 and this has been proposed as a molecular mechanism underlying alterations in neonatal physiology of the heart.22–24 In the fetal heart, the predominant TnI protein is that of the skeletal muscle isoform TnIs; in the rat, transition to TnIc occurs at the time of birth. Recent reports have suggested that similar transitions occur in humans, although only limited data based on a small number of fetal samples are available.

We present here a developmental analysis of TnI expression in the human heart from 9 weeks of gestation...
to 9 months of postnatal development analyzed quantitatively at both protein and mRNA levels. The results show that both TnIc and TnIs are detectable throughout fetal development but that TnIc is the predominant isoform at all fetal stages. Transition to TnIc alone, the adult phenotype, occurs after birth, and in the analysis presented here, TnIc was undetectable by 9 months of postnatal development. Because certain genes that are normally expressed during fetal cardiac growth are reexpressed in end-stage heart failure and hypertrophy, we examined ventricular samples from a series of 17 hearts in end-stage heart failure for their pattern of TnI expression. The results show that TnIc mRNA is undetectable in these samples. Therefore, reexpression of TnIc cannot be proposed as a molecular mechanism for the altered contractile characteristics associated with the failing adult ventricle.

Materials and Methods

Tissue Collection

Fetal and postnatal cardiac and skeletal muscle was obtained from elective terminations and at autopsy and stored in liquid nitrogen before use. Samples of late fetal (≥20 weeks) and postnatal heart were of ventricular muscle only. Early fetal samples were principally ventricular muscle, although contamination with atrial muscle could not be excluded. All samples were judged to be from nonpathological tissue: fetal samples had no known developmental or chromosomal abnormalities, and postnatal death was from cases without cardiovascular complications. The age of fetal samples was determined by anatomic measurement and is given as calculated gestational age derived by reference to standard growth curves. Adult pathological samples were obtained from hearts in end-stage heart failure taken at the time of transplantation.

Protein Preparation and Western Blot Analysis

Total protein extracts were prepared by pulverizing tissue while frozen, followed by homogenization in 1% sodium dodecyl sulfate. Protein concentrations were determined, and 50-μg samples were run on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. After electrophoresis, gels were equilibrated in 25 mM Tris base/192 mM glycine/20% methanol, and proteins were electroblotted onto nitrocellulose filters. Resulting Western blots were stained with previously characterized antibody (called 42/25) diluted 1/3,000 in phosphate-buffered saline containing 0.1% Tween 20. Bound antibody was revealed either by peroxidase-linked antibody (Dakopatts) or using the ECL light detection system (Amersham).

RNA Preparation and Northern Blot Analysis

Total cellular RNA was prepared from tissues using guanidine thiocyanate extraction. RNA from adult skeletal muscle, smooth muscle (stomach), and liver was purchased from Cambridge Biochemicals. RNA concentrations were determined by optical density measurement, and 20 μg was loaded onto 1.5% agarose-formaldehyde gels by standard techniques. After electrophoresis, RNA was revealed by ethidium bromide/ultraviolet photography to assess integrity and loading and was subsequently transferred to Hybond N+ (Amersham) membranes. 32P-labeled probe for the specific detection of TnI mRNAs was prepared by random priming of previously characterized cDNA fragments: In the case of TnIc, a 150-bp Sac I fragment containing only 3' noncoding sequence was derived from the published cDNA. In the case of TnIs, a 470-bp partial cDNA clone (pCTI-3) containing 3' coding and noncoding sequence was used. To verify equal loading of RNA between lanes, Northern blots were also hybridized with a gyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA clone. Labeling of cDNA fragments and hybridization conditions were standard.

Results

Accumulation of TnI Isoforms During Cardiac Development

To determine the temporal pattern of TnI expression during normal cardiac development, we analyzed total protein extracts from a series of fetal and postnatal time points. TnI proteins were detected using antibody 42/25, which has been shown to detect all known isoforms of TnI in cardiac and skeletal muscle, namely TnIf, TnIc, and TnIc, in chickens, rats, and humans. Figure 1 summarizes the results of this analysis. Two TnI proteins are detected in both fetal and neonatal heart samples, and these are indistinguishable, on the basis of their electrophoretic mobility and antibody reactivity, from those of adult cardiac muscle (TnIc) and slow skeletal muscle (TnIs), as has been previously described. Both proteins are detected throughout
Accumulation of adult tissues estimated from their band of hybridization was demonstrated to be gene specific. To verify their specificity under the conditions used here, we first hybridized to a Northern blot of RNAs derived from a variety of adult tissues (Figure 3). When the TnIc probe is used, a single band of approximately 1,200 nucleotides corresponding to TnIc is detected in adult cardiac muscle only, and when the Tnls probe is used, a single band of approximately 1,050 nucleotides is detected in adult skeletal muscle only. The size of these mRNAs was estimated from their migration relative to 18S and 28S ribosomal RNAs. Hybridization with a TnIf-specific probe failed to detect mRNA in any of the cardiac samples (fetal, adult, and pathological) analyzed here (data not shown). The results demonstrate the specificity of hybridization and confirm the restricted distribution of Tnls and TnIc mRNA expression in adult muscle tissues.

To determine the pattern of TnI mRNA accumulation during fetal and postnatal cardiac development, the same series of hearts used for protein studies were analyzed by Northern blot hybridization. A total of seven independent Northern blots were analyzed, the results of which are illustrated in Figure 4. TnIc mRNA is detectable throughout fetal life at a low level and rapidly increases after birth. On Northern blots containing adult cardiac RNA, no significant difference in intensity was detectable between adult and 9-month (38-week) postnatal tracks (data not shown), suggesting that the 9-month value may represent the maximum level of expression. In contrast, the level of Tnls mRNA remains high throughout fetal life, even though there is variation between the early fetal samples. The level of Tnls mRNA drops rapidly after birth and is undetectable in the 9-month postnatal heart analyzed here. To quantify the accumulation of TnIc and Tnls mRNAs, the results of each Northern blot were analyzed by scanning densitometry. To account for minor variations in loading, transfer, and detection between tracks, the intensity of hybridization with GAPDH was determined, and the values for Tnls and TnIc were corrected relative to this. The results of this analysis are summarized in Figure 5. Comparison of the optical density of Northern blots hybridized with both probes shows the relative level of Tnls mRNA to be fivefold to 10-fold greater than that of TnIc during fetal development when corrected to account for length of the hybridizing probe, its specific activity, and length of autoradiographic exposure.

TnI Gene Expression in End-Stage Heart Failure

We have previously described the analysis of a series of end-stage heart failure samples taken at the time of transplantation. A total of 34 samples of left and right
ventricular wall from 17 patients with end-stage heart failure resulting from primary pulmonary hypertension (five patients), dilated cardiomyopathy (eight patients), or ischemic heart disease (four patients) were analyzed.

For levels of several mRNAs including those encoding the ryanodine receptor (RYR2), calcium uptake pump (Ca\(^{2+}\)-ATPase SERCA2), phospholamban, caldesmus, and the atrial natriuretic peptide (ANP) (see Table 1). All samples contained significant levels of ANP mRNA and levels of RYR2, Ca\(^{2+}\)-ATPase SERCA2, and phospholamban that were coordinately decreased in proportion to the severity of heart failure as assessed by the level of ANP mRNA. Here we analyzed the same series of heart samples for TnIc and TnIs mRNA. The results, illustrated in Figure 6, clearly show that TnIc mRNA is a major transcript in the failing heart. No TnIs mRNA was detectable in any of the pathological samples, even on prolonged exposure times. The same 120-bp 3' noncoding sequence probe was used in these experiments as for the analysis of cardiac development. To ensure that low-level expression was not present, we also carried out hybridization with a 550-bp Pst I probe.

Table 1. Summary of Data From 17 Patients With End-Stage Heart Failure

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Diagnosis</th>
<th>LV ANP mRNA* (arbitrary unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>PPH</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>PPH</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>PPH</td>
<td>3.7</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>PPH</td>
<td>3.2</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>PPH</td>
<td>29.4</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>IHD</td>
<td>3.0</td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>IHD</td>
<td>4.9</td>
</tr>
<tr>
<td>8</td>
<td>59</td>
<td>IHD</td>
<td>91.4</td>
</tr>
<tr>
<td>9</td>
<td>27</td>
<td>IHD</td>
<td>74.7</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>DCM</td>
<td>3.2</td>
</tr>
<tr>
<td>11</td>
<td>49</td>
<td>DCM</td>
<td>4.9</td>
</tr>
<tr>
<td>12</td>
<td>51</td>
<td>DCM</td>
<td>15.2</td>
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<td>63</td>
<td>DCM</td>
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</tr>
<tr>
<td>17</td>
<td>9</td>
<td>DCM</td>
<td>6.8</td>
</tr>
</tbody>
</table>

LV, left ventricular; ANP, atrial natriuretic peptide; PPH, primary pulmonary hypertension; IHD, ischemic heart disease; DCM, dilated cardiomyopathy.

*Derived from Reference 34.
Figure 6. Analysis of troponin I (TnI) mRNA in end-stage heart failure. A total of 34 heart samples of left ventricular (LV) or right ventricular (RV) muscle derived from 17 explanted hearts were analyzed for cardiac TnI (TnIc) and slow skeletal muscle TnI (TnIs) mRNA accumulation by Northern blot hybridization. The results illustrated are from six hearts in end-stage heart failure resulting from primary pulmonary hypertension (PPH, patients 3 and 4 in Table 1), ischemic heart disease (IHD, patients 4 and 5), or dilated cardiomyopathy (DCM, patients 6 and 7).

Fragment of cDNA, pHMTnI-S,8 comprised of the 3’ coding and 3’ noncoding sequence to increase the sensitivity of detection. No TnIc mRNA was detectable with this probe when using prolonged exposure times, although weak cross hybridization to TnIc mRNA became evident. The mRNAs for TnIc and TnIs are of different sizes (see Figure 3) and are easily distinguishable on this basis. We conclude that TnIc mRNA is not expressed in the samples analyzed here and that it is not therefore a feature of ventricular muscle in end-stage heart failure. In the absence of suitable matched controls, it is not possible to determine whether the absolute level of TnIc mRNA is altered in end-stage heart failure. However, comparison with the level of TnIc mRNA in the 9-month postnatal sample suggests that no gross alteration in TnIc mRNA abundance occurs. Minor variation in the level of TnIc mRNA was apparent between samples in this series but showed no significant correlation with disease group, level of ANP mRNA, or left and right ventricular samples (data not shown).

Discussion

We have demonstrated that both TnIc and TnIs are expressed in the human ventricle throughout fetal development but that at all fetal stages TnIs remains the predominant isoform. Switching to the expression of TnIc alone, the phenotype of adult myocardium,12,25 occurs after birth and, in the study reported here, is complete by 9 months of postnatal development. Accurate definition of the timing of this postnatal transition is hampered by difficulties in obtaining suitable tissue samples. The data presented here indicate that TnIs protein and mRNA are absent by 9 months of postnatal development, although the limited number of postnatal samples precludes precision of this point. Whatever the exact timing of the switch from TnIs to TnIc, it is probable that this developmental transition in troponin gene expression has functional implications on the contractility of the developing myocardium and raises questions as to the mechanisms regulating expression of these genes. The extended N-terminal sequence of cardiac TnI contains serine residues that, in the rabbit, are selectively phosphorylated when hearts are perfused with adrenergic agonists16,35 or when purified TnI is treated with cAMP-dependent protein kinase.36,37 Phosphorylation at this site alters the calcium binding characteristics of the troponin complex, through subunit interaction with TnC, resulting in a shift in the Ca2+/force curve18 and an increased rate of relaxation.17 The transition in expression from TnIs to TnIc during neonatal life may account in part for the differing response of fetal and neonatal myocardium to β-adrenergic stimulation.22–24,38,39 Recent evidence also suggests that the relative insensitivity of neonatal myocardial activity to acidic pH is due to the presence of TnIs in the troponin complex.23,24

Certain genes that are expressed during normal fetal cardiac growth are reexpressed during pathological cardiac hypertrophy and in end-stage heart failure.26 Given both the developmental changes in isoform composition during development and the functional importance in regulating muscle contraction, the troponin complex forms a potential site for alterations associated with adult heart failure. In the analysis presented here, TnIs mRNA was not detectable in any of the 17 explanted hearts in end-stage heart failure resulting from dilated cardiomyopathy, ischemic heart disease, or primary pulmonary hypertension. We conclude that there is no qualitative change in TnI isoform expression associated with end-stage heart failure and that alterations in TnI isoform content cannot therefore be invoked as an underlying mechanism for the altered characteristics of contractility associated with the failing ventricle. Cumulative evidence suggests that the same is true for TnC expression, which is unaltered during development and remains constant in the pathological heart.40 In contrast, alterations in TnT expression may be associated with adult heart failure in humans, because the presence of a TnT isoform that is normally present during fetal cardiac development has been reported in the adult pathological heart.41

Our data cannot exclude the possibility that heart disease in infants has an influence on TnI gene expression during neonatal and early postnatal growth. The youngest pathological sample was derived from a 3-year-old child with end-stage heart failure resulting from primary pulmonary hypertension (see Table 1). As with all the pathological samples analyzed, no mRNA for TnIs was detected in either left or right ventricular samples from this individual, indicating that TnIs is not reexpressed in end-stage heart failure even at this early stage of postnatal development. In contrast, a recent report based on four hearts with congenital malformations12 found that TnIs mRNA was detectable up to 2 years after birth. Therefore, it is possible that congenital malformations result in a retarded postnatal TnI transition. It should be noted that therapeutic intervention may itself influence troponin expression, given that cardiac gene expression can be altered by a variety of pharmacological agents (e.g., see Reference 42). Fur-
ther experimental evidence is now required to clarify the exact timing of the postnatal TnI switch, the effect of congenital and postnatal disease, and the influence of therapeutic intervention on this process.

In the case of both TnIs and TnIc, the level of protein and the corresponding mRNA follow similar kinetics of accumulation during development of the ventricular myocardium. For TnIc, levels of both mRNA and protein remain low during fetal life and rapidly increase after birth. In the case of TnIs, both mRNA and protein are abundant throughout fetal life and both decrease rapidly during postnatal development. These patterns indicate that TnI isoform accumulation in ventricular myocardium is probably governed largely by the abundance of the corresponding mRNAs and is highly suggestive of regulation at the level of gene transcription. This contrasts with the situation in the rat, where TnIc mRNA is detected in the fetal ventricle throughout development but TnIc protein is only detected in late fetal stages. Most components of the contractile apparatus show coordinate accumulation of mRNA and corresponding protein in both cardiac and skeletal muscle (e.g., see Reference 42), and in the case of both actin and myosin expression in the heart, this has been directly demonstrated to be due to changes in transcriptional activity of the corresponding genes. The molecular mechanisms that may influence the expression of the troponin genes remain to be determined. Potential factors include hormonal and neural influences that are known to alter during neonatal and postnatal growth. Thyroid hormone levels influence the transcriptional activity of a variety of genes including those encoding cardiac myosin, and studies on euthyroid and hyperthyroid rats have demonstrated that thyroid status has a quantitative effect on TnIc accumulation in the neonatal heart. Preliminary data suggest, however, that thyroid levels do not affect TnI expression (e.g., see Reference 9). During skeletal muscle development, neural contact plays an important role in the development of the mature phenotype, and denervation or cross innervation between fast and slow skeletal muscle types has a profound effect on isoform transitions (e.g., see Reference 46). Evidence from work carried out in vitro, where embryonic chick cardiac myocytes were cultured in the presence or absence of either sympathetic nerves or nerve extracts, suggests that the developmental transition from TnIs to TnIc requires nerve-derived factors. The influence of cardiac innervation during postnatal cardiac development in vivo remains to be determined.

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