Developmental Changes in the Expression of Rabbit Left Ventricular Troponin T

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We examined cardiac troponin T (TnT) isoform expression in rabbit left ventricular myocardium at three different stages of postnatal development. Using sodium dodecyl sulfate gel electrophoresis (PAGE), we resolved five isoforms: TnT$_1$, TnT$_2$, TnT$_3$, TnT$_4$, and TnT$_5$. TnT$_1$ had the slowest electrophoretic mobility and TnT$_5$ the fastest. The predominant isoforms were TnT$_2$, TnT$_3$, and TnT$_4$. The relative amounts of TnT$_1$, TnT$_2$, TnT$_3$, and TnT$_4$ were examined in myocardium from three age-groups: 3 days (Group 1), 21–22 days (Group 2), and 99–109 days (Group 3). The amount of TnT$_1$, relative to the total amount of TnT (determined by the ratio of the areas under the densitometric curves) decreased significantly ($p<0.01$) with age from 42 ± 4% in Group 1 to 25 ± 3% in Group 3. In contrast, the relative amount of TnT$_4$ increased with age from 23 ± 2% in Group 1 to 33 ± 4% in Group 3 ($p<0.01$). The relative amounts of the other two isoforms changed biphasically with development: TnT$_2$ decreased from Group 1 to Group 2 and increased from Group 2 to Group 3. TnT$_3$, a minor isoform, increased from Group 1 to Group 2 and decreased from Group 2 to Group 3. These developmental changes in troponin T expression may account for some of the maturational changes observed in the physiological and biochemical properties of the myocardium. (Circulation Research 1988;63:742–747)

Troponin T (TnT) binds the troponin complex to tropomyosin and is essential in the interaction of TnT, troponin I (TnI), and tropo-nin C (TnC), which confers on the myofilaments their sensitivity to calcium.$^1$ In avian fast skeletal muscle, multiple TnT isoforms are present, and with development, both their expression and the sensitivity of the myofilaments to calcium change.$^{2,3}$ In the mammal, five TnT isoforms have been common in fast skeletal muscle.$^4$ This expression, which is believed to occur through differential RNA splicing,$^5$ is correlated with the sensitivity of the skeletal muscle fibers to calcium.$^6$ Mammalian ventricular myocardium has recently been found to contain more than one isoform of TnT. Gusev et al$^7$ found two isoforms in bovine myocardium, while Pearlstone et al.$^8$ using peptide sequence analysis, found at least two major isoforms in rabbit ventricular myocardium. This peptide analysis also demonstrated that rabbit cardiac TnT and rabbit skeletal TnT differed so sufficiently that they most likely have different genes. In a study that assessed the functional effects of the two bovine cardiac TnT isoforms, myofibrillar ATPase activity was found to depend on the isoform of TnT present.$^9$ In chicken cardiac muscle, TnT expression has been shown to vary with development: one form of TnT mRNA was more predominant in early embryonic life, another more predominant in the adult.$^{10}$

This is the first study that examines whether, in the mammalian heart, TnT isoform expression changes with development. We found that, like rabbit fast skeletal muscle,$^4$ rabbit cardiac ventricle expresses five isoforms of TnT. The relative expression of the three major cardiac TnT isoforms was found to change significantly with development.

**Materials and Methods**

New Zealand White rabbits were examined at three different postnatal ages: Group 1, 3 days old ($n = 8$); Group 2, 21–22 days old ($n = 6$); and Group 3, 99–109 days old ($n = 5$). Each rabbit came from a different litter. The rabbits were anesthetized with sodium pentobarbital (350 mg/kg body weight i.p.). The hearts were removed and washed in Krebs-Henseleit solution (KH) (pH 7.4). The left ventricular free wall was dissected from the heart and placed in a dish cooled on ice.
Preparation of Samples for Developmental Comparisons

Protease inhibitors were placed in all buffers (0.5 μg/ml antipain, 0.5 μg/ml chymostatin, 0.5 μg/ml pepstatin, 2.0 μg/ml α-macroglobulin, 100 units/ml trasylol, 0.5 mM phenylmethylsulfonyl fluoride, and 2.0 μg/ml leupeptin). Pieces of myocardium, approximately 1 mm³, were cut from the central portion of the free wall and immediately placed in 0.6 ml of skinning solution (75 mM KC₂H₃O₂, 15 mM KPO₄, 5 mM K₂EGTA, 5 mM MgCl₂, 5 mM K₂ATP, 0.5% Triton X-100, and 15–20 mM MOPS, pH 7) chilled on ice. After 5–10 minutes, the tissue was washed twice in KH and placed in 200 μl of sample buffer (2.0% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 6.8], 20% glycerol, 0.0125% bromophenol blue, and 1.0% β-mercaptoethanol). The sample was then frozen at −80° C until the proteins were examined by SDS polyacrylamide gel electrophoresis (SDS-PAGE), which followed the Laemmli approach and used a 3.3% stacking gel and a 1.1% bis-acrylamide concentration.¹¹

Electrophoretic mobility of the left ventricular proteins was examined by allowing them to migrate through an 8% acrylamide separating gel. Standards were run on each gel, including the TnT standard purified from rabbit heart (see below) and canine cardiac actin (kindly provided by Dr. R. John Solaro, University of Illinois at Chicago). Gels were silver-stained and photographed.¹²

Positive transparencies made from the gels were scanned with an LKB Ultroscan laser densitometer (Bromma, Sweden). The relative amounts of the TnT isoforms and actin were determined by integrating the areas under the densitometric curves. A previous study of the silver stain technique demonstrated that optical density had a linear relation to protein mass from 2 to 70 ng.¹² We have found that when excessive loading is avoided, variations in the protein load do not affect the proportions of the isoforms. The amount of each isoform was expressed as a percentage of total TnT. The contribution of TnT₅ to the total TnT content was not examined because this minor isoform migrated at a rate so close to that of actin that it could not be separated easily from actin on an 8% gel (see Figure 1). The data from the three age-groups were examined statistically using analysis of variance. The arcsine transformation was applied to the data to approximate a normal distribution.

Preparation of Thin Filament Proteins

Standards were prepared from rabbit ventricles (n = 12, 6 weeks to 3 months of age). All procedures were performed at 4° C to avoid proteolysis.¹³ TnT and TnI were prepared (Figure 1) by conventional fractionation.¹³ This procedure included ammonium sulfate extraction, CM Sephadex chromatography, and DEAE Sephadex chromatography. Tropomyosin (Figure 1) was obtained as a by-product of the preparation of troponin¹³ by dialyzing the pellet from the 42.5–60% ammonium sulfate extraction into a buffer solution containing 100 mM KCl, 50 mM Tris, and 0.1 mM dithiothreitol, pH 8.0. The protein was loaded on a DEAE Sephadex column, equilibrated with buffer and eluted with a gradient of 100–500 mM KCl. The isolation steps were monitored by SDS-PAGE. The standards were stored at −80° C.

Results

TnT, purified from rabbit ventricular myocardium, was separated by SDS-PAGE into five isoforms: TnT₁, TnT₂, TnT₃, TnT₄, and TnT₅ (see Figure 1). These isoforms migrated faster than actin
and slower than tropomyosin and TnI (Figure 1), with TnT4 having the slowest mobility and TnT2 the fastest. TnT2, TnT3, and TnT4 were the predominant forms in the purified standard and in the ventricular myocardium. With the molecular weight standards as a reference (Figure 2), Mr of the TnT isoforms ranged around 39,000 daltons.

**TnT Isoform Expression During Development**

At the three stages of development examined, the left ventricle contained proteins with electrophoretic mobilities similar to purified TnT standard (Figure 2). The isoforms TnT2, TnT3, TnT4, and TnT5, were readily identified at each age. The changes with development of the major isoforms, TnT2, TnT3, and TnT4, are evident in Figure 2. The densitometric scans of three representative lanes (Figure 3) further illustrate these changes.

The amounts of TnT2, TnT3, TnT4, and TnT5, expressed as percentages of the total TnT content, changed with development (see Figure 4 and Table 1). TnT2 decreased, TnT3 decreased then increased, TnT4 increased, and TnT5 increased then decreased with age.

Analysis of variance showed significant F values across development (p < 0.002). Paired comparisons using Tukey’s HSD test showed that differences between all pairs of means were significant at the 0.05 level. The following differences between pairs were significant at the 0.01 level: TnT2, groups 1 and 2, groups 1 and 3, groups 2 and 3; TnT3, groups 1 and 3, groups 2 and 3; and TnT5, groups 1 and 2, groups 1 and 3, groups 2 and 3; TnT5, groups 1 and 2.

**Discussion**

Our results provide the first evidence that expression of mammalian cardiac TnT, an essential molecule in the control of contraction and relaxation, changes significantly with maturation. In rabbit fast skeletal muscle, where five TnT isoforms are commonly found, the sensitivity of the myofilaments to calcium was found to be related to the TnT isoform content of the single fiber. Thus, it is reasonable to suspect that the developmental changes in cardiac TnT isoform expression observed in this study could similarly result in changes in the sensitivity of the cardiac myofilaments to calcium. Such a change would contribute to the developmental changes in myocardial contractility.

Our results strongly support our finding five isoforms of TnT in rabbit cardiac muscle. We used conventional procedures for cardiac TnT purification in this study to resolve the five isoforms. Pearlstone et al., using a modified purification procedure, found at least two major isoforms of TnT in rabbit myocardium. Our observation of five TnT isoforms in rabbit myocardium is consistent with the expression of multiple isoforms reported in studies of other striated muscles. These studies reported five or more TnT isoforms in rabbit and chicken fast skeletal muscle, two TnT isoforms in bovine cardiac muscle, and two TnT mRNA in chicken cardiac muscle.
FIGURE 3. Comparison of rabbit left ventricular troponin T isoforms (TnT₂, TnT₃, TnT₄, and TnT₅) at three ages. Densitometric scans of three lanes of an 8% polyacrylamide gel in the region to which actin and the TnT isoforms migrated: Upper panel, 3-day-old; middle panel, 21-day-old; lower panel, 99-day-old. Developmental changes in proportions of TnT isoforms for all hearts are illustrated graphically in Figure 4 and presented in Table 1.

In a separate, ongoing immunological and molecular investigation of the TnT isoforms of rabbit myocardium, we have raised a polyclonal antibody to the TnT isoform TnT₄. Immunoblots demonstrate that this antibody recognizes the five bands characterized as TnT isoforms in this report. This study will be the subject of another communication.

We have performed two-dimensional gel electrophoresis on rabbit ventricular myocardium in a separate study and reported five protein spots that comigrated with the purified TnT, each exhibiting two isoelectric points. That study and the present one provide an Mᵣ estimate for the TnT isoforms between 38,500 and 39,500 daltons. The two bovine cardiac TnT isoforms have a comparable Mᵣ, as estimated by gel electrophoresis. The Mᵣ of rabbit cardiac TnT obtained from amino acid sequence is 32,881 daltons. The difference between the Mᵣ, obtained from the amino acid sequence and that estimated using gel electrophoresis is similar to the difference observed for the Mᵣ of skeletal muscle TnT using these two approaches.

The developmental differences in TnT expression observed in this study most likely reflect changes in the composition of the thin filament rather than in a cytosolic pool of TnT, composed of several isoform types available for rapid insertion into the thin filaments, for example, in response to a given physiological stimulus. In fact, since the myocardium was detergent skinned and washed before being placed in the SDS sample buffer, a hypothetical pool of unbound TnT would have been lost during membrane extraction, leaving behind only the isoforms contained in the thin filaments.

Developmental changes in mammalian cardiac TnT isoform expression could explain the effects of maturation on myofibrillar ATPase activity described by others. Solaro et al found that at pH 7.0, the myofibrillar ATPase activity was the same in the adult and the perinatal canine myocardium; on the other hand, at low pH, the ATPase activity was decreased in the adult but not in the perinatal myocardium. (A resistance to acidosis would be advantageous for survival during the perinatal period when episodes of acidosis are common.) Since myosin ATPase was the same at the two ages studied and TnC had the same properties, Solaro et al concluded that changes in the isoform expression of other thin filament regulatory proteins must occur to explain these differences. They have also found...
recently that the myofibrils from 4-day-old rabbit heart bind less calcium than myofibrils from the 22-day-old and adult heart.\textsuperscript{18} PAGE analysis indicated that this maturational increase was not related to TnC, but was likely to be caused by differences in the distribution of TnT isoforms.\textsuperscript{18} The developmental regulation of TnT isoform expression could account for the differences in myofibrillar ATPase and in myofibril calcium binding.

The maturational increase in cardiac contractility\textsuperscript{14} could be related to the changes in TnT isoform expression. These changes during mammalian development warrant studies that attempt to correlate the changes in the composition of myofilament proteins to the sensitivity of the myofilaments to calcium. If TnT expression changes in response to the maturational increase in myocardial workload, thus altering the physiological properties of the myofilaments, it is tempting to speculate that a change in TnT isoform expression due to a genetic lesion may cause certain forms of cardiomyopathy.\textsuperscript{19}

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**TABLE 1. Proportions of Troponin T\textsubscript{2}, T\textsubscript{3}, T\textsubscript{4}, and T\textsubscript{5} Present in Left Ventricular Myocardium at the Three Stages of Development**

<table>
<thead>
<tr>
<th>Group</th>
<th>Troponin T\textsubscript{2}</th>
<th>Troponin T\textsubscript{3}</th>
<th>Troponin T\textsubscript{4}</th>
<th>Troponin T\textsubscript{5}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>[3 days old, (n=8)]</td>
<td>42 ± 4%</td>
<td>30 ± 4%</td>
<td>23 ± 2%</td>
</tr>
<tr>
<td>Group 2</td>
<td>[21–22 days old, (n=6)]</td>
<td>38 ± 6%</td>
<td>27 ± 4%</td>
<td>29 ± 4%</td>
</tr>
<tr>
<td>Group 3</td>
<td>[99–109 days old, (n=5)]</td>
<td>25 ± 3%</td>
<td>35 ± 4%</td>
<td>33 ± 4%</td>
</tr>
</tbody>
</table>
standards from rabbit ventricles. The authors are grateful to Dr. R. John Solaro and to Pat Walker who provided the actin standard and helpful information.

References

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