Transgenic Modeling of a Cardiac Troponin I Mutation Linked to Familial Hypertrophic Cardiomyopathy

Jeanne James, Yan Zhang, Hanna Osinska, Atsushi Sanbe, Raisa Klevitsky, Timothy E. Hewett, Jeffrey Robbins

Abstract—Multiple mutations in cardiac troponin I (cTnI) have been associated with familial hypertrophic cardiomyopathy. Two mutations are located in the cTnI inhibitory domain, a highly negatively charged region that alternately binds to either actin or troponin C, depending on the intracellular concentration of calcium. This region is critical to the inhibition of actin-myosin crossbridge formation when intracellular calcium is low. We modeled one of the inhibitory domain mutations, arginine145→glycine (TnI146Gly in the mouse sequence), by cardiac-specific expression of the mutated protein in transgenic mice. Multiple lines were generated with varying degrees of expression to establish a dose relationship; the severity of phenotype could be correlated directly with transgene expression levels. Transgenic mice overexpressing wild-type cTnI were generated as controls and analyzed in parallel with the TnI146Gly animals. The control mice showed no abnormalities, indicating that the phenotype of TnI146Gly was not simply an artifact of transgenesis. In contrast, TnI146Gly mice showed cardiomyocyte disarray and interstitial fibrosis and suffered premature death. The functional alterations that seem to be responsible for the development of cardiac disease include increased skinned fiber sensitivity to calcium and, at the whole organ level, hypercontractility with diastolic dysfunction. Severe affected lines develop a pathology similar to human familial hypertrophic cardiomyopathy but within a dramatically shortened time frame. These data establish the causality of this mutation for cardiac disease, provide an animal model for understanding the resultant pathogenic structure-function relationships, and highlight the differences in phenotype severity of the troponin mutations between human and mouse hearts. (Circ Res. 2000;87:805–811.)

Key Words: hypertrophic cardiomyopathy mouse cardiac troponin I sarcomere

Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant disease of the sarcomere with well over 100 associated mutations in 9 sarcomeric proteins, including cardiac troponin I (cTnI).1–2 cTnI is a small peptide (210 residues) that, together with troponin C and troponin T, forms the troponin complex. In striated muscle, this complex, which is associated with the thin filament, forms the major calcium sensor and, thus is responsible for controlling the thick-thin filament interactions that result in the contractile cycle. Troponin I is bound to cardiac troponin C (cTnC) in an antiparallel fashion such that its amino terminus associates with the carboxyl terminus of cTnC. cTnI differs from the slow skeletal isoform of TnI by a 32 amino acid extension at the cTnI amino terminus. Two serine residues that are substrates for protein kinase A (PKA) lie within this extension.3,4 cTnI is also sensitive to protein kinase C phosphorylation at serines 43 and 45 and threonine 143.5 The effect of cTnI phosphorylation is primarily a downward modulation of cardiac contractility, either by decreased ATPase activity, which is mediated by PKC, or by increased binding of cTnI to the thin filament, mediated by PKA. A truncation of cTnI (cTnI1–193) has been associated with myocardial stunning.6,7 and measuring the serum level of cTnI has become the standard of care in the diagnosis of myocardial injury.8–12 The so-called inhibitory region of cTnI is an 11 amino acid motif that is evolutionarily conserved (Figure 1). This region alternatively binds to either the thin filament or cTnC, depending on the intracellular concentration of calcium ([Ca2+]i). High [Ca2+]i values promote Ca2+ binding to cTnC, facilitating a shift of the inhibitory region of cTnI from the thin filament to cTnC. This produces positional changes in the actin-tropomyosin conformation and promotes the transition from weakly bound to strongly bound actin-myosin crossbridges, leading to ATP hydrolysis.13 During diastole, when [Ca2+]i is low, the inhibitory region of cTnI remains bound to the thin filament, suppressing the power stroke. Therefore, cTnI is a critical element, transmitting the systolic and diastolic variations in [Ca2+]i, to the sarcomere.

Mutations that can cause FHC have been defined in 3 different domains of cTnI: 2 mutations in the inhibitory domain, 1 in the C-terminal domain, and 4 in the protein’s distal region. Patients with the inhibitory region mutations or one of the distal mutations (L206Q) develop the characteristic ventricular hypertrophy of FHC. In contrast, patients carrying...
Figure 1. TG constructs. Full-length mouse α-MHC promoter was used to drive expression of wt mouse cTnI cDNA. The sequence of the cTnI<sup>146Gly</sup> mutation in the inhibitory domain and the location of the FLAG tag in cTnI wt are shown. Light gray boxes indicate the α-MHC promoter exons.

the other cTnI mutations develop hypertrophy only at the cardiac apex.<sup>1,2</sup> To begin exploring the functional significance of a single amino acid mutation in the inhibitory region of cTnI, we generated transgenic (TG) mice that express the R145G (R146G in the mouse sequence) mutation. This mutation replaces a positively charged arginine with an uncharged, polar glycine residue. The consequences of the mutation were studied by generating transgenic (TG) mice that express the wt transcript at 5-fold the endogenous level. In particular, compared with endogenous cTnI RNA. Line 52 is a multiple lines of TG transgenic mice (Figure 1). To rule out any phenotype attributable to overexpression of TnI, lines carrying the wt cDNA were made, and one was selected for analysis (line 52) whose cTnI RNA level exceeded that of the cTnI<sup>146Gly</sup> lines used. A total of 4 lines carrying the TnI<sup>146Gly</sup> transgene were analyzed. Two of these, lines 135 and 141, could not be studied in detail because of the paucity and early death of TG offspring. In fact, the founders died prematurely and had grossly abnormal hearts (data not shown). The remaining 2 lines (121 and 133) were selected for analysis. RNA overexpression levels for lines 121 and 133 were 1.2-fold and 3.5-fold higher, respectively, compared with endogenous cTnI RNA. Line 52 expressed the wt transcript at 5-fold the endogenous level. In previous experiments using the α-MHC promoter to drive expression of TG contractile transcripts, levels of transgene expression varied by 1- to 15-fold<sup>16,18,23–25</sup> and sometimes more. Thus, we found that TG expression may not have survived to birth. Supporting this hypothesis, mice from the highest expressing line, 133 (with 3.5-fold greater expression relative to the endogenous transcript), died between 13 and 17 days after birth and had markedly abnormal hearts.

Western blot analysis of ventricular myofibrils was performed using a cTnI-specific affinity purified polyclonal antibody (Research Diagnostics, Inc) and a peroxidase-conjugated donkey antigoat IgG secondary antibody (Jackson ImmunoResearch Laboratories). Five micrograms of myofibrils was subjected to SDS-PAGE, transferred to polyvinylidene fluoride membrane (BioRad), and incubated with primary antibody (1:5000) and secondary antibody (1:10000). Immunofluorescence was analyzed with a STORM 820 imager (Molecular Dynamics). The percentage of TG replacement was calculated as FLAG-tagged cTnI/FLAG-tagged cTnI + endogenous cTnI. The percentage of TG protein replacement was plotted as a function of transcript overexpression.

Structural analyses at the light and electron microscopy levels were performed as described.<sup>21</sup> Using 2 to 4 mice of mixed gender, multiple sections were cut and >50 fields were observed. To assess the pattern of the FLAG-tagged cTnI incorporation into the sarcomere, isolated cardiomyocytes<sup>22</sup> were examined by confocal microscopy with anti-FLAG M2 monoclonal antibody (Sigma), M.O.M. biotinylated antimouse IgG, and Texas Red Avidin D (Vector Laboratories).

Functional assays included skinned fibers analysis, described in detail elsewhere.<sup>23,24</sup> Skinning was carried out essentially as described by Strang et al,<sup>24</sup> who noted previously in these preparations that the myofibril proteins are not phosphorylated. All experiments were performed using a commercially available apparatus (Scientific Instruments). Strip tension (mN/mm²) was determined by dividing force by fiber cross-sectional area (calculated from widths measured at the major axis). Isolated working heart function was also determined, as previously described.<sup>25</sup>

Results

Generation of Mice Carrying Troponin I Transgenes

Three constructs were used to generate multiple lines of TG mice (Figure 1). To rule out any phenotype attributable to overexpression of TnI, lines carrying the wt cDNA were made, and one was selected for analysis (line 52) whose cTnI RNA level exceeded that of the cTnI<sup>146Gly</sup> lines used. A total of 4 lines carrying the TnI<sup>146Gly</sup> transgene were analyzed. Two of these, lines 135 and 141, could not be studied in detail because of the paucity and early death of TG offspring. In fact, the founders died prematurely and had grossly abnormal hearts (data not shown). The remaining 2 lines (121 and 133) were selected for analysis. RNA overexpression levels for lines 121 and 133 were 1.2-fold and 3.5-fold higher, respectively, compared with endogenous cTnI RNA. Line 52 expressed the wt transcript at 5-fold the endogenous level. In previous experiments using the α-MHC promoter to drive expression of TG contractile transcripts, levels of transgene expression varied by 1- to 15-fold<sup>16,18,23–25</sup> and sometimes more. Thus, the relatively low and narrow range of TG expression in the cTnI<sup>146Gly</sup> lines suggested that potential founders with high TG expression may not have survived to birth. Supporting this hypothesis, mice from the highest expressing line, 133 (with 3.5-fold greater expression relative to the endogenous transcript), died between 13 and 17 days after birth and had markedly abnormal hearts.

Despite a relatively narrow range of expression, differences in morbidity and mortality presented. Line 121 had no overt phenotype in early adulthood, although females stressed by pregnancy did develop cardiac pathology (see below). However, life expectancy in this line did not differ appreciably from normal animals (Figure 2). Expression of wt cTnI at

Materials and Methods

Transgene Construction

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee. Three TG constructs were used. The wild-type (wt) cDNA for mouse cTnI was obtained by reverse transcription–polymerase chain reaction using total RNA isolated from mouse cardiac ventricle as template. The 5′ primer contained a portion of the cTnI 5′ untranslated region (5′-GGGGAGGAGACAGCAGTATATTTAG-3′), whereas the 3′ primer was made complementary to the 3′ untranslated region (5′-GAGGTTTATTCTGCAGGGACGTCC-3′). The wt cDNA was subcloned into pBluescript and sequenced in full. The TnI<sup>146Gly</sup> mutation was created using standard polymerase chain reaction-based methods. A third cDNA was generated that included a FLAG tag<sup>14,15</sup> inserted at the amino terminus just distal to the translational start site of the wt cDNA. The 3′ cDNAs (TnI<sup>146Gly</sup>, wt, and wt-tagged) were separately subcloned into the mouse α-MHC promoter (Figure 1), and the purified DNA was used to generate multiple lines of TG mice.<sup>16</sup>

Phenotype Analyses

Except where noted, 3 to 6 animals of mixed gender were used for our studies after preliminary experiments showed no gender differences. For assessment of cTnI<sup>146Gly</sup> and cTnI wt transcript size, Northern blot analysis was performed using 7.5 μg of total RNA, as described. Expression levels were determined by RNA dot blot analysis with a 32P-labeled oligonucleotide (5′-GGCGTGTTGGCTCG-GTGGCATAAGCCTCGTAGTGCCAGGAGGAGCGGCGTGGA - CAGG-3′). Molecular markers of hypertrophy were examined by RNA dot blot analysis using 32P-labeled oligonucleotides, as described.<sup>18–20</sup> Two to three TG and non-TG (NTG) mice from each line were analyzed, and statistically significant differences were determined by unpaired Student’s t test, with significance defined as P<0.05.
5-fold the level of the endogenous message (line 52) resulted in a cohort of mice with normal survival (Figure 2A). Breeding line 121 TG mice to one another doubled transgene expression to 2.4-fold and resulted in death of the animals carrying a double dose of the transgene at 18 to 26 days. Transgenic mice from line 133, with 3.5-fold overexpression at the transcript level, did not survive past 17 days. Although some aspects of a hypertrophic response were observed at the molecular level, no increases in the ventricle or body weights were detected in the adult cTnI146Gly animals (Figure 2B).

RNA and Protein Expression in TG Animals

We wished to determine whether animals displaying early lethality showed any abnormalities in RNA expression in the heart, reminiscent of the altered patterns that have been observed in other models of cardiac disease.20 At the RNA level, line-133 mice at 10 days after birth (3 to 7 days before death) exhibited aspects of the expression pattern observed in adult hearts undergoing a hypertrophic response at the molecular level. Relative to NTG animals, atrial natriuretic factor and α-skeletal actin transcripts were elevated, whereas SERCA2 and phospholamban transcripts were decreased (Figure 3). Line 121 and the wt cTnI-expressing line 52 were analyzed at 4 to 5 months of age. Line 121 only showed upregulation of atrial natriuretic factor, consistent with the milder phenotype, whereas the animals that expressed the wt TnI transgene did not differ from NTG animals.

The changes observed in contractile protein isoform expression, although striking, were not consistent with the resultant mortality. We wished to see if any major changes in the contractile apparatus had occurred as a result of cTnI146Gly expression. Previous experiments in which sarcomeric proteins were expressed showed a linear relationship between the level of TG RNA expression and the degree of protein replacement.18 Thus, by comparing the level of TG overexpression determined by RNA dot blot analysis to the percentage of replacement of endogenous cTnI by tagged cTnI (determined by Western blotting), we were able to generate an equation describing the relationship of RNA expression to TG protein replacement (Figures 4B and 4C). No unincorporated FLAG-tagged cTnI was detected by confocal microscopy (Figure 4A).
indicating that Western blot analysis of myofibrillar protein accumulation accurately represents the amount of TG protein present in cardiomyocytes. Assuming that FLAG-tagged cTnI, cTnI146Gly, and cTnI(wt) incorporate into myofibrils with equal efficiency, we estimated that the cTnI complement of line 133, with the highest level of cTnI146Gly TG overexpression, consisted of ~50% of mutant protein, whereas lines 121 and 52 had 40% and 60% replacement, respectively, of endogenous cTnI with TG cTnI. (Figure 4C).

At 18 days of gestation, the TG and NTG ventricles of the highest expressor, line 133, were indistinguishable from one another, a result consistent with the lack of TG expression in the ventricle at this developmental stage (Figure 5A). However, by 10 days after birth the ventricles demonstrated cardiomyocyte disarray, interstitial and perivascular fibrosis, and nuclear degeneration (Figure 5B). This line could not be examined at later stages because of death at 13 to 17 days. Male and nulliparous female TG mice from the lower-expressing line 121, had no discernible pathology at any age (data not shown). However, 13-month postpartum females showed cardiomyocyte hypertrophy as well as patchy areas of interstitial and perivascular fibrosis (Figure 5C). Line-52 TG mice (wt overexpressors) were indistinguishable from NTG littermates (data not shown). Thus, the effects of cTnI146Gly on the myocardium seem to be dose-dependent such that the highest expressing line showed accelerated pathological changes and a decreased life expectancy, whereas line 121 demonstrated abnormalities only after the physiological stress of pregnancy.

Ultrastructural changes in lines 121, 133, and 52 were documented (Figure 6). In comparison with age-matched NTG littermates (Figure 6A), 9-day-old line-133 TG mice

**Figure 4.** Estimation of cTnI146Gly transgenic protein replacement. A total of 2 or 3 mice of mixed gender per line were analyzed. Line 133 was analyzed at 10 days after birth; all other lines were analyzed at 4 weeks after birth. A, Western blot showing the total amount of cTnI in the mutant lines. B, Western blot depicting the relative amounts of endogenous cTnI and FLAG-tagged cTnI. The addition of the FLAG tag results in slowed mobility by SDS-PAGE analysis. C, Graph and equation describing the relationship of cTnI RNA and protein replacement. D, Confocal image of an isolated cardiomyocyte from the highest expressing cTnI/FLAG line. E, FLAG-tagged cTnI replacement levels. C, interpolated cTnI146Gly replacement levels.

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had abnormally short sarcomeres (≈1 to 1.5 μm), with wide Z bands and no visible I bands (Figure 6B). Sarcomeric organization was disturbed in many areas and completely lacking at isolated foci. The sarcoplasmic reticulum (SR) was swollen and disorganized, with disruption of the normal spatial relationship between the sarcomeres and SR. The mitochondrial ultrastructure and distribution was likewise abnormal. A representative field from a line-121 parous female revealed features of hypoxia in some cardiomyocytes, as evidenced by swollen mitochondria and large gaps between the SR, mitochondria, and sarcomeres. The interstitium contained small amounts of collagen fibers (Figure 6C). Line 52 could not be distinguished from NTG littermates (data not shown).

We hypothesized that cTnI146Gly expression would have direct effects on both fiber mechanics and kinetics. The pCa-force relationship was determined using dephosphorylated skinned papillary muscle strips from cTnI146Gly line 133 at 10 days after birth. A significant increase in Ca2+ sensitivity compared with NTG littermate controls was observed, and maximum tension (P0) was significantly depressed (Figure 7A). There were no differences detected in unloaded shortening velocity, maximum shortening velocity, or maximum relative power (data not shown). We also studied the less affected animals, line 121, at 6 weeks of age and again found increased Ca2+ sensitivity and significantly decreased P0 (Figure 7B). As with line 133, line 121 did not have alterations in unloaded shortening velocity, maximum shortening velocity, or maximum relative power (data not shown). Line 52 was indistinguishable from NTG animals (Figure 7B).

To examine the consequences of cTnI146Gly on whole heart function, lines 121 and 52 were studied using the isolated working heart preparation18 (Table). Line 133 could not be analyzed because of early death. Line 52 did not differ significantly from NTG littermate controls. However, the striking differences in both +dP/dt and −dP/dt in line 121 was surprising given the otherwise mild manifestations of cardiac pathology at the molecular and microscopic levels. Contractility was significantly enhanced, whereas relaxation was impaired. The time constant of relaxation, τ, a load-independent measure of diastolic function, was markedly prolonged in TG mice from line 121. Thus, expression of cTnI146Gly in the mouse heart results in enhanced systolic function but compromised diastolic function.

**Discussion**

There are few data concerning the relative morbidity and mortality of the cTnI mutations in humans. Three of the 7 mutations (R145G, R145Q, and L206Q) resulted in a typical hypertrophic cardiomyopathy, 1 (K183del) caused apical hypertrophy in one family member and typical hypertrophy in another, and 3 (R162W, S199N, and G203S) caused apical hypertrophy resulting in a spade-shaped ventricle.1,2 In 9 family members carrying S199N, 2 experienced sudden death in the sixth decade, 6 others had varying symptoms (arrhythmia or left ventricular dysfunction), and 1 was asymptomatic at 21 years of age.2

Our work describes the effects of the cTnI146Gly mutation in mice. Consistent with the presentation of many of the pathological features of typical FHC, the mice exhibited myofibrillar disarray, and interstitial fibrosis; some of the molecular markers of cardiac hypertrophy are activated although no overt hypertrophy, as measured by increases in ventricle mass/body mass were observed. At the whole organ level, contractile function is enhanced but relaxation is compromised. Because cTnI binds to both cTnC and actin, it seems likely that the primary etiology of cardiac dysfunction in the cTnI146Gly mice lies in altered cTnI interactions with cTnC, actin, or both. Certainly, different cTnI isoforms can modulate the effective cross bridge detachment rate.26 The binding of the inhibitory domain of cTnI to actin inhibits actomyosin ATPase activity, depresses cross-bridge cycling, and prevents contraction. The inhibition is released as cTnI binding shifts from actin to cTnC, an action favored by an increase in [Ca2+], during systole, when Ca2+ binds to cTnC. This alters the tertiary structure of cTnC and exposes a hydrophobic pocket in the amino terminus of protein. It is here that the inhibitory region of cTnI binds, allowing movement of tropomyosin on actin, exposing the myosin binding site, and promoting contraction. Augmented contractility and depressed relaxation in the working heart are consistent with in vitro data obtained using purified, recombinant human cTnI. The decreases observed in maximal tension, although inherently contradictory with the hypothesis that the switch is in the “on” position, may well be accounted for by the myocyte dropout that we observed in these animals (Figure 5 and data not shown). Functional abnormalities, which included a reduced ability to inhibit the actin-tropomyosin–activated ATPase as well as an increase in Ca2+ sensitivity,27 suggest that cTnI146Gly either does not interact appropriately with actin or perhaps binds strongly to cTnC. The net effect would be lack of ATPase inhibition, in essence leaving the molecular switch in the “on” position. Decreased ATPase inhibition attributable to the R145G mutation supports this hypothesis.27,28

This model shows an accelerated disease course, with very early death noted at 13 to 17 days after birth in mice having ≈50% TG cTnI replacement. The original report of the R145G mutation provides scant clinical information regarding patient age at onset of symptoms, severity of hypertrophy, or life expectancy but did not emphasize a particularly malignant course.1 This raises the question of why mice expressing this mutation seem to be more susceptible to cardiac dysfunction than humans. The answer may lie in the intrinsic chronotropic demands placed on the mouse heart, which must rapidly cycle through systole and diastole. The molecular switch between contraction and relaxation depends on precise molecular cooperation between thin and thick filament proteins, with the troponin complex serving as a molecular clutch. Intuitively, it would seem reasonable that as the heart cycles through systole and diastole at increasingly rapid rates, the margin of error for compromised protein interactions becomes ever smaller. The pathological outcome of an abnormal molecular switch would thus be more pro-
nounced as intrinsic heart rate increases, with the most severe effects occurring at the shortest cycle lengths.

Sensitivity of the mouse heart to abnormal troponin proteins has been demonstrated in several experimental models. Targeted ablation of cTnI was temporarily compensated by expression of the neonatal (slow skeletal) isoform of TnI. Transgenic mice have also been used to model FHC-associated mutations in cardiac troponin T (cTnT). Human patients develop little or no ventricular hypertrophy, yet have a high incidence of sudden death. When a cTnT mutation was transgenically expressed in mice, animals with 5% replacement of endogenous cTnT with mutated cTnT had normal life spans. When these developed diastolic dysfunction, decreased ventricular mass, decreased sensitivity to calcium, and ventricular enlargement.30 Transgenic mice have also been used to model cTnI 146Gly mutation in the human disease. Recently, a transgenic rabbit expressing the β-MHC R403Q mutation was created.33 The rabbits accurately reproduced human FHC, demonstrating, in addition to the typical histological features of the disease, increased ventricular mass, preserved contractile function, and abnormal relaxation. The differences in phenotype between the mouse and rabbit experiments are not surprising given the distinctive biochemical properties of fast α-MHC and slow β-MHC. Our ability to specifically express transgenes in the rabbit heart has progressed rapidly,34 and these mutations can be generated in the larger animals whose contractile apparatus more closely resembles that of the human. These experiments are underway.

Figure 7. pCa-force relationship curves of skinned fibers from ventricular papillary muscle. Fiber kinetics, including the pCA-force relationships, unloaded and maximum shortening velocity, and maximum relative power, were determined as described in Materials and Methods. Data were analyzed by one way ANOVA using Statview software (SAS Institute, Inc). The number of mixed-gender mice and the P0 (maximal tension expressed as mN/mm²) is indicated. A, Line-133 TG and NTG at 9 days after birth. B, Line-52, line-121, and NTG controls at 6 weeks after birth.

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