Nonmyofilament-associated troponin T fragments induce apoptosis

Jeong EM, Wang X, Xu K, Hossain MM, Jin JP. Nonmyofilament-associated troponin T fragments induce apoptosis. Am J Physiol Heart Circ Physiol 297: H283–H292, 2009. First published April 24, 2009; doi:10.1152/ajpheart.01200.2008.—Troponin T (TnT) is a striated muscle-specific protein and an abundant component of the myofilaments. Nonmyofilament-associated TnT is rapidly degraded in myocytes, implying an importance in the maintenance of the cellular environment. However, if the level of nonmyofilament-associated TnT or TnT fragments exceeds the degradation capacity, it may cause cytotoxicity. To investigate this hypothesis, we constructed bicistronic vectors to express different portions of TnT polypeptide chain, together with nonfusion green fluorescent protein as a tracer for the transfection. Cytotoxicity of the TnT fragments was studied through forced expression in C2C12 myoblasts and human embryonic kidney-293 nonmuscle cells and examination of the viability of the transfected cells. The results demonstrated that, in the absence of myofilaments, the conserved COOH-terminal and middle fragments of TnT were highly effective on inducing cell death via apoptosis, whereas the NH2-terminal variable region was not. As combined effects, the conserved COOH-terminal and middle fragments of TnT exhibited intermediate cytotoxicity. A particular significance of this finding is that peak releases of TnT or TnT fragments from decomposition of a large number of myofibrils in acute myocardial infarction may breach the cellular protection of proteolytic degradation and result in apoptosis as a potential cause for the loss of cardiomyocytes.

TROPONIN T (TNT) IS A SUBUNIT of the troponin complex in striated muscle cells, which interacts with troponin I (TnI), troponin C (TnC), and tropomyosin in the actin thin-filament-based Ca2+ regulatory system (9). The polypeptide chain of vertebrate TnT isoforms contains 233–305 amino acids. The COOH-terminal and middle regions of TnT bear the binding sites for TnI, TnC, and tropomyosin (27) and are highly conserved among cardiac, slow, and fast muscle TnTs and across species (18). In contrast, the NH2-terminal region of TnT is hypervariable and confers the size and charge differences between TnT isoforms (18). The NH2-terminal region of TnT does not bind any known myofilament proteins and may be proteolytically cleaved by \( \mu \)-calpain as a functional adaptation to myocardial stress conditions (33). The NH2-terminal truncated cardiac TnT remains associated with the myofilament and sustains the contraction of cardiac muscle (7).

Despite being an abundant myofilament protein, TnT has a relatively short half-life (3–4 days) in muscle cells (23). Overexpression of TnT in cardiac muscle of transgenic mice does not increase TnT stoichiometry, but effectively replaces the endogenous cardiac TnT (3, 14). Consistently with a rapid turnover of extra TnT, nonmyofilament-incorporated TnT or TnT fragment is rapidly degraded in myocytes (31) and in the muscle of Amish nemaline myopathy patients, in which a nonsense mutation of Glu180 in exon 11 of slow skeletal muscle TnT results in a COOH-terminal truncation (15). Therefore, the fast turnover rate of TnT reflects a high capacity of synthesis, as well as an effective proteolytic removal of surplus or damaged proteins. The excessive synthesis for an effective replacement of damaged TnT in muscle cells is plausible for the maintenance of structural integrity and function of the highly organized contractile machinery in striated muscle cells. On the other hand, the very rapid and near-complete removal (31) of nonmyofilament-associated TnT or TnT fragments may imply another critical necessity in maintaining normal cellular functions.

Experimental data have suggested a harmfulness of nonmyofilament-associated TnT and TnT fragments to cells. Despite the fact that we could readily establish stable transfected C2C12 cell lines for a TnT promoter-directed, differentiation-dependent expression of exogenous TnT (31), repeated experiments using the same procedure and conditions failed to establish even a single stable transfected clone for unregulated viral promoter-driven expression of intact TnT or TnT fragment in undifferentiated myoblasts and nonmuscle eukaryotic cells (unpublished results). For the difference in the presence or absence of myofibrils in differentiated muscle cells and undifferentiated myoblasts or nonmuscle cells, this observation suggests cytotoxicity of nonmyofilament-associated TnT or TnT fragments.

Massive releases of TnT or TnT fragments from myofibrils occur in acute myocardial infarction (21), toxic myocardial injury (26), and skeletal muscle injury (1). If the peak level of free cytosolic TnT or TnT fragments exceeds the protective capacity of the proteolytic degradation in the muscle cell, it may result in toxic effects. The cytotoxicity of TnT fragments released from myofibrils may be correlated with the observation that activation of caspase-3, a signaling molecule in the pathways that induces apoptosis in various cell types, in cardiomyocytes, cleaves cardiac TnT (5), resulting in a decreased binding to tropomyosin (2) to cause dissociation of TnT from myofibrils. Adult cardiomyocytes have lost the ability to regenerate in ischemic heart disease and other types of myocardial injuries, which is an underlying mechanism for the development of cardiomyopathy and heart failure (22). Therefore, it is of clinical importance to understand the potential effect of TnT fragments released from myofibrils on inducing apoptotic cell death, which may play a pathogenic role in cell death in acute myocardial infarction and in heart failure due to chronic losses of cardiomyocytes.

In the present study, we examined the cytotoxicity of different regions of the TnT polypeptide chain using transfective

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expression in cultured cells in the absence of neurohumoral and hemodynamic influences. The results demonstrated that the conserved COOH-terminal and middle fragments of TnT were highly cytotoxic through inducing apoptosis, whereas the NH2-terminal variable region was not. The data suggest that the apoptotic effect of nonmyofilament-associated TnT fragments may be a potential cause of cell death in myocardial injury and diseases.

MATERIALS AND METHODS

Construction of cDNA templates encoding representative fragments of TnT. The TnT polypeptide chain can be dissected into three structural domains that have been extensively studied for functions in the Ca2⁺ regulation of muscle contraction: the conserved COOH-terminal and middle regions, and the hypervariable NH2-terminal region (Fig. 1A) (18, 27). Therefore, we examined the potential cytotoxicity of TnT in engineered fragments representing the three regions. A COOH-terminal truncated slow TnT found in the Amish nemaline myopathy (31) and intact cardiac TnT were also examined for comparisons (Fig. 1B).

Using a cDNA encoding intact mouse slow skeletal muscle TnT, cloned in pAED4 plasmid (16), as template, two truncated cDNAs encoding the middle and COOH-terminal regions of TnT were constructed using polymerase chain reaction (PCR) mutagenesis. Outlined in Fig. 1C, an oligonucleotide primer was designed corresponding to the region flanking amino acids 154–160 (MsTnT-M157F: CTGTCATATGAGGCTCAT), in which an Ndel restriction

Fig. 1. Structure-function domains of troponin T (TnT) and expression vectors. A: the variable and conserved regions of TnT are illustrated with the relationships to other muscle thin-filament proteins. B: the linear maps of intact TnT (represented by HcTnT), the NH2-terminal variable region (represented by HcTnT-N68), the middle region (represented by MsTnT51met-154), and the COOH-terminal region (represented by MsTnT-T2) of TnT, together with the COOH-terminal truncated slow TnT (MsTnT1-179). The recombinant prokaryotic expression vector pAED4 (C) and bicistronic mammalian expression vector pTracer (D) encoding intact TnT and TnT fragments are illustrated in circular maps. The cloning sites and the positions of translational initiation and termination codons in the TnT cDNA inserts are indicated. TnC, troponin C; TnI, troponin I.
enzyme site (underlined) was integrated with an endogenous Met codon ATG (bold) that was used as the translational initiation codon for the expression of the COOH-terminal T2 region (amino acids Met<sub>1</sub>-Lys<sub>82</sub>). MsTnT-M157F was paired with a reverse primer pET-R in the vector sequence for PCR to amplify the T2 coding region. After modification at the ends by NdeI and EcoRI digestions, the MsTnT-T2 cDNA was unidirectionally inserted into pAED4 expression plasmid precut by the same restriction enzymes.

cDNA template encoding the middle region of mouse slow TnT (amino acids Pro<sub>52</sub>-Leu<sub>154</sub>, MsTnT<sub>51met-154</sub>) was constructed by PCR using a forward primer designed corresponding to the region encoding amino acids 49–55 (MsTnT-NDF: TCTCTATTGTC CCCGAA-GAT), which contains an artificial translational initiation Met codon ATG (bold) and an NdeI cloning site (underlined) and a reverse primer corresponding to the coding sequence for amino acids 152–159 (TnT-Ter2: AGGCACCATGAGACTACAGAGCCCT), which contains an artificial translational termination codon TAG (the antisense bases are in bold), and an NcoI cloning site (underlined). The PCR product was modified at the ends by NdeI and NcoI digestions and cloned into NdeI-EcoRI-cut pAED4 expression plasmid by two-step ligation, in which the ligation of NdeI sticky ends was followed by Klenow polymerase fill-in of the NcoI and EcoRI ends and subsequent blunt-end ligation.

A cDNA encoding full-length adult human cardiac TnT (HcTnT) was cloned by reverse transcription-coupled PCR (RT-PCR) from anonymous human cardiac mRNA using a method similar to that described previously for the cloning of cat, dog, and guinea pig cardiac TnT cDNAs (3). This investigation was determined to be exempted research by the Evanston Northwestern Healthcare Institutional Review Board.

A cDNA fragment was constructed using PCR to encode the NH<sub>2</sub>-terminal variable region of TnT (amino acids 1-68 of HcTnT, HcTnT-N68). A reverse primer was designed corresponding to the region encoding amino acids 66-73 of adult HcTnT (HcTnT-68T: GTTGGAATTCAA), which contains an artificial translational initiation codon ATG (bold) and an EcoRI cloning site (underlined). The PCR product was modified at the ends by NdeI and NcoI digests and cloned into NdeI-EcoRI-cut pAED4 expression plasmid by two-step ligation, in which the ligation of NdeI sticky ends was followed by Klenow polymerase fill-in of the NcoI and EcoRI ends and subsequent blunt-end ligation.

A cDNA encoding COOH-terminal truncated mouse slow TnT (MsTnT<sub>T2</sub>) was previously constructed in pAED4 vector (31).

The five recombinant plasmids cloned in pAED4 (Fig. 1C) were sequenced to verify the modifications and authenticity of cDNA inserts. The construction of representative TnT fragments was further confirmed by protein expression in transformed BL21(DE3)pLysS E. coli cells, as described previously (31).

SDS-polyacrylamide gel electrophoresis and Western blotting. Protein samples were homogenized in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS and heated at 80°C for 5 min. Using a Bio-Rad mini-gel system, as previously described (31), intact HcTnT and MsTnT<sub>T1-170</sub> were resolved on 14% SDS-PAGE gel with an acrylamide-bisacrylamide of 180:1, and the HcTnT-N68, MsTnT<sub>51met-154</sub> and MsTnT-T2 fragments were resolved on 15% SDS-PAGE with an acrylamide-bisacrylamide of 29:1.

Duplicate gels were processed for Western blotting using site-specific anti-TnT monoclonal antibodies (MAb) to verify the specific TnT fragments. Intact HcTnT and MsTnT<sub>T1-170</sub> fragment were detected by MAb CT3 (16). To identify the MsTnT-T2 fragment, a mouse MAb IgG (IgG<sub>1</sub>) against an epitope in the COOH-terminal region was generated by hybridoma technology through a short-term immunization, with HcTnT purified from E. coli expression, as described previously (2). The standard methods for hybridoma fusion and subcloning were described previously (17). The middle fragment of TnT (MsTnT<sub>51met-154</sub>) was identified using MAb 2C8 against a middle region epitope (15, 33). For the identification of the NH<sub>2</sub>-terminal fragment of cardiac TnT, a mouse hybridoma MAb 3G7 (IgG<sub>1</sub>) was generated as above, using immunization with HcTnT-N68 fragment expressed in E. coli and purified by ammonium sulfate precipitation and anion-exchange chromatography.

Western transfers for intact HcTnT and the MsTnT-T2, MsTnT<sub>51met-154</sub> and MsTnT<sub>T1-170</sub> fragments using a Bio-Rad Laboratory semidry electrotransfer apparatus was done using standard conditions (31). Blotting of the small and highly acidic HcTnT-N68 fragment (Table 1) was carried out using polyvinylidene difluoride membrane of 0.2-μm pore size with much reduced current and time (5 mA/cm<sup>2</sup> for 6 min). To strengthen the blotting of N68 to the membrane, the blotted membrane was fixed with 0.1% glutaraldehyde in Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) containing 0.05% Tween 20 for 30 min (6).

The blotted membranes were blocked with 1% bovine serum albumin in Tris-buffered saline and incubated with the specific MAbs diluted in Tris-buffered saline containing 0.1% bovine serum albumin. The subsequent washes, incubation with alkaline phosphatase-labeled anti-mouse IgG second antibodies (Santa Cruz Biotechnology), and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate reaction were carried out, as described previously (31).

Construction of pTracer bicistronic mammalian expression vectors. To transfectively express the TnT constructs in mammalian cells and investigate their cytotoxicity in the absence of myofilaemds, we subcloned the cDNA inserts in mammalian expression plasmid vector pTracer (Invitrogen) (Fig. 1D). In addition to expressing the cDNA insert under a SV40 promoter, pTracer plasmid contains a separate transcriptional unit encoding green fluorescence protein (GFP) under a cytomegalovirus promoter. Therefore, the TnT fragment-expressing cells can be traced under fluorescence microscope by the coexpression of GFP.

During the subcloning, the cDNA inserts encoding intact HcTnT, MsTnT<sub>T1-179</sub>, HcTnT-N68, MsTnT<sub>51met-154</sub> and MsTnT-T2 were isolated from the recombinant pAED4 plasmids as NdeI-EcoRI fragments. The pTracer vector DNA was cut by AflII-EcoRI. After ligation of the compatible EcoRI ends, Klenow polymerase fill-in and blunt-end ligation was followed to join the NdeI and AflII ends. The recombinant pTracer plasmid clones were screened by PCR, and the positive clones were verified by expression of

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<th>Table 1. Physical properties of the representative fragments of TnT</th>
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<td>TnT Fragments</td>
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<td>Human cardiac TnT NH&lt;sub&gt;2&lt;/sub&gt;-terminal region (HcTnT-N68)</td>
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<td>Mouse slow TnT middle region (MsTnT&lt;sub&gt;51met-154&lt;/sub&gt;)</td>
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<td>Mouse slow TnT COOH-terminal region (MsTnT-T2)</td>
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<td>Intact human cardiac TnT (HcTnT)</td>
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<td>COOH-terminal truncated mouse slow TnT (MsTnT&lt;sub&gt;T1-170&lt;/sub&gt;)</td>
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Number of amino acids, molecular weight, and isoelectric point of the troponin T (TnT) proteins were calculated from sequences (GenBank/EBI Data Bank accession numbers: human cardiac TnT, NM_001001430; mouse slow TnT, AF020946).

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GFP and TnT fragments in transfected human embryonic kidney (HEK)-293 cells.

**Cell culture and transient transfection.** HEK epithelial cell line HEK-293 (CRL-1573) (11) was used as a representative for non-muscle cells, and mouse adult myoblast line C2C12 (CRL-1772) (32) was used as representative for myocytes to test the cytotoxicity of TnT fragments. C2C12 and HEK-293 cells were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and 50 μg/ml streptomycin at 37°C in 5% CO2. Transient transfection of C2C12 and HEK-293 cells with purified pTracer plasmid DNA was carried out using two different protocols modified from the manufacturers’ suggestions. HEK-293 cells were transfected with a 1.5:1 ratio (μl/μg) of HEKfectin (Bio-Rad): DNA per well of 24-well plate was seeded with 1.5 × 105 cells the night before. HEKfectin and plasmid DNA were separately diluted in 25-μl serum-free DMEM and combined after incubation at room temperature for 5 min. The HEKfectin/DNA complexes were incubated at room temperature for an additional 20 min before being added to the monolayer cells in 0.25 ml of serum-free DMEM. The media was replaced with DMEM supplemented with 10% FBS 6 h after the state of transfection.

C2C12 cells were seeded at 0.5 × 104 cells per well in 24-well plates 12 h before transfection, which gave a density of 40–50% confluence. Cells were transfected with 2:1 ratio (μl/μg) mixture of LipofectAMINE 2000 (Invitrogen) and DNA. LipofectAMINE 2000 and DNA were diluted separately in 25 μl of serum-free DMEM and preincubated at room temperature for 30 min before being combined and incubated at room temperature for an additional 20 min. The mixture was then added to C2C12 cells in 0.45 μl of serum-free DMEM. The media was replaced with DMEM supplemented with 10% FBS 24 h after the state of transfection. Cultured for 30–32 h after transfection, the cells were examined for the expression of GFP under an epifluorescence microscope (Zeiss Axiovert 100). Each experimental group was tested in triplicate.

**MTT assay.** MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium; Sigma] assay (8) was applied to examine the cytotoxicity of the TnT fragments in transfected cells. The yellow MTT salt is reduced by NADH and NADPH in metabolically active cells, resulting in intracellular formazan that can be solubilized and quantified by spectrophotometry with a linear relationship to the number of living cells. When apoptosis or necrosis reduces metabolic rate in the cell membrane permissibility, Flow cytometry was employed to quantify the transfected GFP-positive cells, which are also PI positive, the cells were harvested rapidly by trypsinization, washed twice with PBS, and stained with 100 ng/ml PI in PBS at room temperature for 5 min. Flow cytometry analysis was carried out using a FACSCalibur flow cytometer (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA). GFP fluorescence was detected by excitation at 488 nm and emission at 515–545 nm with the FL1 channel. PI fluorescence was detected by excitation at 493–536 nm and emission at 564–606 nm with the FL3 channel. Background fluorescence of unstained non-transfected cells, transfected GFP-positive cells, or PI-stained non-transfected cells was used to set up gates and photomultiplier tube voltages and amperage gain for each channel. To exclude cell debris, cells were gated using standard forward and side scatter properties. Approximately 1 × 104 viable cell events were acquired for each sample, and the data were analyzed using the FlowJo 7.2.5 software (TreeStar).

**Cytoplasm-free nucleosome assay.** To detect apoptosis, cytoplasm-free nucleosome assay was employed using a Cell Death Detection ELISAPLUS kit from Roche Applied Science. This enzyme-linked immunosorbent assay detects the release of free nucleosomes from nucleus to cytoplasm before disintegration of the plasma membrane, which is an established hallmark of apoptosis (13).

The transfected cells were trypsinized and collected by centrifugation at 500 g for 15 min. The cells collected from each well of 24-well plates were lysed in 200 μl of lysis buffer at room temperature for 30 min. The cell lysate was centrifuged at 500 g for 15 min to remove nuclei. The lysate supernatant was measured for protein concentration using Bradford reagent (Bio-Rad) (4). An aliquot containing 200-μg proteins from each sample was added to a streptavidin-coated 96-well microplate. As described in the manufacturer’s instruction, one-step sandwich ELISA was performed by adding biotin-labeled anti-histone and peroxidase-conjugated anti-DNA MAb, and incubation occurred at room temperature for 2 h. After being washed three times, H2O2-ABTS [2,2’-azino-di(3-ethylbenzthiazoline-sulfonate)] substrate solution was added at 100 μl/well and incubated at room temperature for 20 min. Dual absorbance at 405 nm-to-490 nm ratio was measured using an automated microplate reader (Bio-Rad Benchmark). The 405-nm-to-490-nm ratio absorbance values were normalized by the transfection rate. The results from cells transfected with different TnT fragments were compared with that of control cells to determine the relative level of apoptotic events in the cell population.

**Statistical analysis.** Data are presented as means ± SE or SD, as noted in Fig. 3–6 legends. Statistically significant differences were established with two-tailed Student’s t-test, in which P < 0.05 denotes a significant difference.

**RESULTS**

**Engineering of representative fragments of TnT.** To investigate the cytotoxic effect of the established structure-function domains of TnT, we engineered intact TnT and four TnT fragments, representing the NH2-terminal variable region, the middle conserved region, the COOH-terminal conserved region, and a myopathic mutation containing the NH2-terminal and middle regions (Fig. 1).

The middle and COOH-terminal regions of TnT are highly conserved among isoforms and across species, so the use of MsTnT51met-154 and MsTnT-T2 fragments derived from mouse slow skeletal muscle TnT can serve as informative representatives. In contrast, the NH2-terminal region is hypervariable. We selected the NH2-terminal segment of HcTnT (HcTnT-N68) as a representative to test cytotoxicity, since it represents an NH2-terminal structure that is physiologically cleaved and released from the myofilaments under stress conditions (7, 33).

To examine the cytotoxic effect when the conserved and variable regions are resided in an intact TnT or large fragment, intact cardiac TnT (HcTnT) and COOH-terminal truncated slow TnT (MsTnT-T179) were also studied. Authenticity of the representative TnT fragments engineered was verified by expression in E. coli for SDS-PAGE and Western blotting examinations. Shown in Fig. 2, the intact TnT
four TnT fragments had anticipated gel mobility in SDS-PAGE, consistent with the predicted molecular weights (Table 1). Western blots using site-specific Mabs further confirmed that intact HcTnT and MsTnT1-179 were identified by MAb CT3, the NH2-terminal fragment HcTnT-N68 was identified by MAb 3G7, the middle fragment MsTnT51met-154 was identified by MAb 2C8, and the MsTnT-T2 fragment was identified by MAb 1G9.

Transient expression of TnT fragments resulted in cytotoxicity in myoblasts and nonmuscle cells. The efficiency of the transient transfections was first examined in culture plates using epifluorescence microscopy. The rates of transient transfection were further quantified using flow cytometry. The results in Fig. 3 showed that, despite the different expression constructs, an average of 54.6 ± 4.0% of the transfected HEK-293 cells and 18.91 ± 2.9% of the transfected C2C12 cells expressed GFP, reflecting the proportion of cells contained the recombinant pTracer plasmids that bicistronically also express the TnT fragment (Fig. 1D). C2C12 cells showed a lower efficiency in transient transfection than that of HEK-293 cells in these experiments. However, the transfection rates were reproducibly over 15%, providing a reliable experimental condition for our study of the cytotoxicity of TnT fragments. According to Poisson distribution, the <63.2% transfection rates in our experiments would not produce a significant occurrence of cells transfected with more than one expression vector. Assuming that the GFP-positive cells each contain only one copy of the expression plasmid DNA, the expression of GFP and TnT by the bicistronic vector allowed us to use GFP expression as a direct and quantitative indicator for TnT expression.

We first evaluated the overall viability of the cells transiently expressing each of the representative TnT fragments. By using MTT assay, we compared the proportion of viable cells at 30 h after the state of transfection with the different expression constructs. The results in Fig. 4 demonstrated that the
Fig. 3. Transfection of human embryonic kidney (HEK)-293 and C2C12 myoblast cell cultures. The rates of transient transfection of HEK-293 cells (A) and C2C12 myoblasts (B) in monolayer cultures were examined 30–32 h after the state of transfection and quantified by flow cytometry for cells emitting green fluorescence protein (GFP) fluorescence. The cell populations were gated for nontransfected cells or transfected GFP-positive cells using standard forward and side scatter properties, based on background fluorescence of unstained nontransfected cells and transfected GFP-positive cells (indicated with circles). The average rates of transfection with empty pTracer vector and the five TnT expression constructs were 54.6 ± 4.06% for HEK-293 epithelial cells and 18.91 ± 2.9% for C2C12 myoblasts (means ± SE). C: the presence or absence of TnT cDNA insert did not result in significant difference in the rate of transfection.
transfection and expression of GFP resulted in a low percentage of nonviable cells in the pTracer vector control groups (11.5 ± 3.0% for HEK-293 and 10.1 ± 0.02% for C2C12 cells). Expression of the NH2-terminal fragment of TnT (HcTnT-N68) did not significantly increase the percentage of nonviable cells compared with the GFP control (18.4 ± 8.1% in HEK-293 cells and 17.9 ± 2.3% in C2C12 cells) (Fig. 4).

MTT assay detected that the expression of intact HcTnT had a moderate but statistically significant cytotoxicity in HEK-293 cells (22.9 ± 2.6%), but not in C2C12 cells (12.5 ± 0.8%). The expression of MsTnT1-179 also showed intermediate cytotoxicities in both cell types (18.4 ± 8.1% in HEK-293 cells and 17.9 ± 2.3% in C2C12 cells) (Fig. 4).

Fig. 4. Expression of the middle and COOH-terminal fragments of TnT decreased cell viability. Calculated by subtracting the MTT-positive viable cells from the total number of cells, the results showed significantly higher percentage of nonviable cells in HEK-293 nonmuscle cells (A) and C2C12 myoblasts (B) transfectively expressing MsTnT1met-154 and MsTnT-T2 compared with that of cultures transfected with empty pTracer vector. In contrast, transfective expression of HcTnT-N68 did not result in a significant difference from the control. The results indicate that the expression of middle and COOH-terminal regions, but not the NH2-terminal region, of TnT caused cytotoxicity. MsTnT-ANM1-179 showed intermediate cytotoxicity in both HEK-293 and C2C12 cells. Intact HcTnT showed moderate cytotoxicity in HEK-293 but not C2C12 cells. Data are shown as means ± SD from 4 experiments. **P < 0.01 and ***P < 0.001 vs. pTracer vector control.

**DISCUSSION**

TnT is a muscle-specific protein and expresses in differentiated muscle cells as a major component of the myofilaments (27). Our laboratory previously observed that muscle cells have a high capacity of degrading nonmyofilament-incorporated TnT (31). Implied by the difficulty in establishing stable transfected cell lines expressing TnT or TnT fragments in the absence of myofilaments (unpublished results), a hypothesis was proposed that a significant level of nonmyofilament-associated TnT or TnT fragments may be harmful to the myocyte.

The present study investigated the potential cytotoxicity of TnT in mammalian cells. Using three independent assays (MTT analysis, PI staining, and detection of free nucleosomes) to examine cell viability, cell death, and apoptosis, the results clearly demonstrated apoptosis-based cytotoxicity of the mid-
dle and COOH-terminal fragments of TnT. The middle and COOH-terminal regions are highly conserved among the three muscle-type isoforms of TnT and across species (18). Therefore, their cytotoxicity demonstrated using constructs derived from slow skeletal muscle TnT represents a common feature of these regions of the TnT polypeptide. The middle and COOH-terminal regions of TnT contain binding sites for TnI, TnC, and tropomyosin, and it is plausible that they are normally integrated in the thin-filament protein complex, which would preventing the free structure-based cytotoxicity.

In contrast, the NH2-terminal variable region of TnT did not show any cytotoxicity when transfectively expressed in cells in

![Fig. 5](http://ajpheart.physiology.org/)

The expression of middle and COOH-terminal fragments of TnT increased late apoptotic and necrotic cells detected by propidium iodide (PI) staining. HEK-293 and C2C12 cell cultures transfected with the TnT expression constructs or pTracer vector control were stained with PI. GFP-PI double-positive cells were quantified using flow cytometry to identify the necrotic and late apoptotic cells. The cell populations were gated for unstained cells, transfected GFP-positive, and PI-stained nontransfected cells (indicated with the circles). The representative data plots (A and B) and summary (C and D, respectively) showed that the PI-positive cells were significantly higher in cultures transfectively expressing the middle (MsTnT51-154) or COOH-terminal fragment (MsTnT-T2) of TnT. In contrast, the expression of the NH2-terminal fragment HcTnT-N68 did not increase the rate of necrotic and late apoptotic cells. Transfective expression of intact HcTnT and MsTnT-ANM1-179 showed intermediate cytotoxicity in HEK-293 and C2C12 cells. Data are shown as means ± SD from 5 experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. pTracer vector control.
the absence of myofilament. The NH₂-terminal variable region of TnT does not bind any known thin-filament proteins. It can be cleaved off by restricted proteolysis as a physiological adaptation to stress conditions, leaving the conserved middle and COOH-terminal regions integrated in the myofilaments (7, 33). These structural and functional features are consistent with the fact that the NH₂-terminal fragment of TnT is nontoxic, as its acute release from the myofilament may occur in large quantities as a physiological adaptation to stress conditions (33), although subsequent decomposition of the myofilament in severe ischemia-reperfusion injuries may release the middle and COOH-terminal regions of TnT to cause cytotoxicity.

Intact cardiac TnT containing all three regions and a COOH-terminal truncated slow TnT found in a recessively inherited nemaline myopathy (15), which fails to incorporate into myofilaments due to much weakened binding to tropomyosin (31), exhibited intermediate cytotoxicities in cells lacking myofilaments. These results suggest that the combination with the NH₂-terminal segment may reduce the toxicity of the middle and COOH-terminal regions. A more native folding of intact TnT and larger fragments may also play a role in this reduced cytotoxicity.

A trend seen in the cytotoxicity experiments was that transfective expression of middle or COOH-terminal fragment of TnT had more predominant cytotoxicity in C₂C₁₂ myoblasts than that in HEK-293 nonmuscle cells (Figs. 4–6), although the transfection rates were lower for C₂C₁₂ cells than for HEK-293 cells (Fig. 3). A possible explanation is that the cellular environment in myoblasts has evolved for rapid synthesis of myofilament proteins, and, therefore, the transfective expression of TnT might be more effective in C₂C₁₂ cells than in HEK-293 epithelial cells.

The finding that nonmyofilament-associated TnT fragments induce apoptosis is of particular importance in cardiomyocytes. Adult cardiomyocytes of higher vertebrates are terminally differentiated cells that cannot regenerate. Therefore, acute or chronic death of myocytes in adult hearts would reduce the number of cardiac muscle cells and eventually cause heart failure. Apoptosis of cardiomyocytes is responsible for the progression of many cardiovascular disorders, including acute myocardial infarction and chronic ischemic cardiomyopathy (22, 28). These conditions involve significant myofilament decomposition or remodeling, in which TnT is released with a potential cytotoxic effect. On the other hand, TnT was found to cross-link in human apoptotic cardiomyocytes (10), which may prevent effective degradation and increase the likelihood to result in cytotoxicity. Therefore, the role of myofilament-released TnT or TnT fragments in triggering cell death in ischemic heart disease is worth further investigation.

Numerous studies have investigated the pathways that regulate cardiomyocyte apoptosis (29). Caspases are commonly occurring mediators of apoptosis and are induced and activated in cardiomyopathy (24). Caspase-3 was found to cleave cardiac TnT in the middle region between Asp96 and Asp97 in myofilaments to result in cytotoxicity. It would be interesting to investigate whether this fragmentation of cardiac TnT contributes to the apoptotic cascade downstream of the caspase cell signaling pathway.

There have been other suggestions for the involvement of nonmyofilament-associated TnT in inducing apoptotic cell death. Overexpression of muscleblind protein activates apoptotic cell death. Recent studies found that the splicing of TnT mRNA was affected by the muscleblind proteins in Drosophila (30) and in mammalian cells (12). Therefore, it is worth investigating whether the apoptosis signaling of muscleblind protein involves aberrantly spliced TnT polypeptides that are incapable of incorporating into myofilaments and result in cytotoxicity and cell death. There is also evidence from non-muscle cell studies to support the hypothesis that nonmyofilament-associated TnT may cause cell death. A microarray study found that the expression of slow skeletal muscle TnT in mouse brain was induced by kainate, a noncompetitive glutamate N-methyl-d-aspartic acid receptor antagonist that has

Fig. 6. The expression of middle and COOH-terminal fragments of TnT-induced apoptotic fragmentation of chromosomal DNA. Cytoplasm-free nucleosome assay was performed to detect apoptotic events after transfective expression of the representative TnT fragments in HEK-293 (A) and C₂C₁₂ (B) cells. The relative levels of cytoplasm-free nucleosomes are shown in arbitrary units normalized to transfection rate. The results demonstrated that the expression of MsTnT51-154, MsTnT-T2, intact HcTnT or MsTnT1-179, but not the NH₂-terminal fragment HcTnT-N68, induced apoptotic fragmentation of chromosomal DNA. Values are shown as means ± SD. Three repeated experiments were performed. *P < 0.01, **P < 0.005, ***P < 0.001 vs. pTracer vector control.
paradoxical effects on neuroprotection and neurotoxicity (20). Proteins immunologically cross-reactive with TnT have been observed in diverse types of nonmuscle cells from the animal, plant, and fungal kingdoms (19). Immunofluorescence microscopy and immunoblot analyses using a MAb generated against TnT found microtubule-associated and nonmicrotubule-associated TnT-like components in nonmuscle tissues, such as brain and cerebellar homogenates. Besides a possible role in Ca2+ or calmodulin-related cellular activities, the TnT-like proteins may have a function in regulating programmed cell death, which is worth further investigation.

In summary, we have demonstrated novel evidence that nonmyofilament-associated TnT and fragments containing the middle and COOH-terminal regions are able to induce apoptosis. For its abundant presence in the myofilaments, peak release of TnT and TnT fragments in myocardial injuries exceeding the capacity of proteolytic degradation would result in the death of myocytes. While the mechanism by which TnT fragments cause cytotoxicity remains to be established, the potential contribution of this mechanism to acute and chronic losses of adult cardiomyocytes and the development of heart failure merit further investigation.

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