Cardiac Troponin I Is Modified in the Myocardium of Bypass Patients

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Background—Selective proteolysis of cardiac troponin I (cTnI) is a proposed mechanism of contractile dysfunction in stunned myocardium, and the presence of cTnI degradation products in serum may reflect the functional state of the remaining viable myocardium. However, recent swine and canine studies have not demonstrated stunning-dependent cTnI degradation.

Methods and Results—To address the universality of cTnI modification, myocardial biopsy samples were obtained from coronary artery bypass patients (n = 37) before and 10 minutes after removal of cross-clamp. Analysis of biopsy samples for cTnI by Western blotting revealed a spectrum of modified cTnI products in myocardium both before and after cross-clamp, including degradation products (7 products resulting from differential N- and C-terminal processing) and covalent complexes (3 products). In particular, a 22-kDa cTnI degradation product with C-terminal proteolysis was identified, which may represent an initial ischemia-dependent cTnI modification, similar to cTnI1–193 observed in stunned rat myocardium. Although no systematic change in amount of modified cTnI was observed, subgroups of patients displayed an increase (n = 10, 85±5% of cTnI remaining intact before cross-clamp versus 75±5% after) or a decrease (n = 12, 67±5% before versus 78±5% after). Electron microscopy demonstrated normal ultrastructure in biopsy samples, which suggests no necrosis was present. In addition, cTnI modification products were observed in serum through a modified SDS-PAGE methodology.

Conclusions—cTnI modification, in particular proteolysis, occurs in myocardium of bypass patients and may play a key role in stunning in some bypass patients. (Circulation. 2001;103:58-64.)

Key Words: ischemia • proteins • cardiopulmonary bypass

Myocardial ischemic injury produces a spectrum of damage, ranging from myocardial stunning (reversible contractile dysfunction in the absence of cell death) to infarction (irreversible dysfunction accompanied by cell death). The specific and selective proteolysis of cardiac troponin I (cTnI) has recently been proposed to play a key role in the contractile dysfunction observed in myocardial ischemia/reperfusion injury, including myocardial stunning.1–6

In the isolated perfused rat heart, cTnI is progressively and selectively posttranslationally modified through proteolysis, the formation of covalent cross-links, and phosphorylation, with increases in the severity of the ischemic insult1–3 (see Reference 4 for review). In particular, myocardial stunning (15 minutes of ischemia followed by 45 minutes of reperfusion), in which no myocardial protein release occurs, results in the proteolysis of cTnI from the C-terminus to produce a degradation product comprising amino acid residues 1 to 193 (rat isoform).1–3 More severe ischemia/reperfusion (60 minutes of ischemia followed by 45 minutes of reperfusion) produces further cTnI degradation from the N-terminus to cTnI residues 63 to 193 and 73 to 193.1

The importance of cTnI proteolysis over other protein modifications is suggested by its crucial role in the control of calcium-dependent muscle contraction.7 This theory is supported by a recently developed line of transgenic mice that express cTnI1–193, the cTnI degradation product found in stunned rat myocardium.8 These mice exhibited ventricular dilation and decreased myofilament contractility, demonstrating that cTnI proteolysis alone is sufficient to recapitulate the cellular phenotype of stunning.8 However, in vivo swine and canine models of stunning have failed to demonstrate stunning-dependent cTnI degradation.9,10 Therefore, the universality of cTnI degradation as a mechanism of contractile dysfunction is in question.

However, if these various disease-induced cTnI modification products are present in human cardiac diseases, they may be necrotically released into the blood. A profile of modified...
cTnI products in serum may be observed that reflects the progression of the disease, as well as the functional state of the remaining viable myocardium. This information could affect treatment of patients, enabling more precise and individualized long-term prognosis and management.

To address this issue of universality of cTnI modification in stunning, we have previously demonstrated cTnI proteolysis in myocardial biopsy samples taken from 2 coronary artery bypass patients. Here, we present analysis of biopsy samples from 37 bypass patients, providing further evidence that cTnI undergoes extensive modification in the myocardium of bypass patients into 7 degradation products and 3 covalent complexes, all of which have a C-terminal proteolytic cleavage. In particular, an initial, or first, 22-kDa degradation product was observed with a similar C-terminal cleavage to TnI (n = 19). Although no systematic change in cTnI modification was observed with cross-clamp, subgroups of patients experienced an increase or decrease in the amount of modified cTnI, and modified cTnI products were observed in postoperative serum samples when a modified SDS-PAGE protocol was used. Electron microscopy (EM) on a subset of myocardial biopsy samples demonstrated normal cellular ultrastructure within biopsy samples, which suggests that cTnI degradation can occur in the absence of necrosis. Therefore, TnI is specifically and selectively modified in the myocardium of bypass patients.

Methods

Surgical Technique

Thirty-seven patients undergoing elective coronary bypass were recruited at Kingston General Hospital (KGH) between June 1998 and February 1999. The Human Research Ethics Board of Queen’s University approved this study, and all patients gave informed consent. Exclusion criteria were age under 21 or over 75 years and congenital heart defects or valvular heart disease. Patients underwent multiple coronary artery bypass grafts (n = 3 to 7) with internal mammary artery and saphenous vein grafts. Briefly, a single cross-clamp technique was performed on full cardiopulmonary bypass (flow rate 5 to 6 L/min, perfusion pressure 50 to 70 mm Hg) using blood cardioplegia at a 4:1 crystalloid-to-blood ratio (High K; Plasma-Lyte 56 with 5% dextrose, 50 mmol/L NaHCO3, 50 mmol/L KCl, and 200 mg of lidocaine) infused at 37°C (n = 13) or 4°C (n = 24) with an initial bolus of 600 or 1000 mL (High K) followed by intermittent boluses of 400 or 250 mL after each proximal vein anastomosis. On completion of the mammary anastomosis, a hotshot of 400 mL (Low K; Plasma-Lyte 56 with 5% dextrose, 50 mmol/L NaHCO3, 6 mmol/L KCl, and 50 mg of lidocaine) at 37°C was infused before cross-clamp removal. No statistical differences existed between patients with the 2 cardioplegic methodologies (data not shown). Systemic normothermia was maintained, and standard anesthetic techniques with a neuromuscular blocking agent were used. Cardiopulmonary bypass was maintained with moderate hemodilution (hematocrit 20 to 25) with a centrifugal pump and membrane oxygenator via single arterial and venous (2-stage) cannulae. Preoperative and postoperative serial ECGs were assessed, and the diagnosis of myocardial infarction was made based on new Q waves or loss of R wave.

Tissue and Blood Sampling

Epicardial biopsy samples (50 to 100 μg) were obtained from both the right (anterior or inferior) and left (anterolateral) ventricles in areas remote to the visually underperfused muscle, with no visible scar or epicardial fat, after initiation of bypass before clamping (RVpre and LVpre) and 10 minutes after cross-clamp removal (RVpost and LVpost); these samples were immediately frozen in liquid nitrogen and stored at −70°C. No clinical complications resulted from the procurement of biopsy samples. An additional subset of patients (n = 3) were recruited, and biopsy samples were analyzed by EM. For EM, biopsy samples were fixed immediately after initiation of bypass before clamping (time = 0) and at 10 and 30 minutes and 3, 24, and 72 hours after cross-clamp removal. Aliquots of serum were retained and frozen at −70°C for further analysis.

Results

cTnI Is Specifically and Selectively Modified in Human Myocardium

To determine whether cTnI is modified in bypass patients in a similar manner to isolated rat heart, epicardial biopsy samples from bypass patients (n = 37, Table 1) were analyzed for cTnI by Western blot (MAB 8I-7, Figure 1A). Biopsy samples were obtained from the inferior or anterior right ventricle (RV) and anterolateral left ventricle (LV) before application of cross-clamp (LVpre, RVpre) and 10 minutes after...
cross-clamp removal (LV<sub>post</sub>, RV<sub>post</sub>) from regions of the heart remote to the coronary occlusions.

cTnI was extensively modified into both degradation products and covalent complexes (Figure 1A) in the myocardium of 70% of bypass patients (Table 2). In fact, many patients had modified cTnI products in the myocardium even before application of the cross-clamp. A maximum of 7 degradation products (molecular weight [MW] 22, 18, 16, 15, 12, 10, and 9 kDa; Deg 1 through Deg 7, respectively) and 3 covalent complexes (MW 60, 50, and 40 kDa; Cov 1 through Cov 3, respectively) (TnI MW 26 kDa by SDS-PAGE; Figure 1A) were present. Competitive Western blotting with recombinant cTnI confirmed that all immunoreactive bands were indeed cTnI products (data not shown). Although the number and quantity of cTnI modification products varied between patients, the products (when present) were the same across all patients, which suggests that the modification process is specific and selective. The particular products Deg 1, Deg 2, and Deg 4, corresponding to MW 22, 18, and 15 kDa, were the most abundant in individual patients (Table 3) and were also seen most frequently across patients (Table 2), and so they were considered the best candidates for further analysis.

![Figure 1](http://circ.ahajournals.org/)

**Figure 1.** TnI is extensively modified in myocardium of bypass patients. A, Representative Western blots of myocardial biopsy samples from LV and RV of bypass patients (n = 37), obtained before (LV<sub>pre</sub>, RV<sub>pre</sub>) and 10 minutes after (LV<sub>post</sub>, RV<sub>post</sub>) removal of cross-clamp and analyzed for cTnI modification by Western blotting with a monoclonal anti-cTnI antibody (8I-7). All immunoreactive bands are indeed cTnI products, as determined by competitive Western blotting with recombinant cTnI (data not shown). A maximum of 7 degradation products (Deg 1 through Deg 7) and 3 covalent complexes are present. B, Representative Western blots performed on LV biopsy sample, either native or dephosphorylated with alkaline phosphatase (LV-dephos), using anti-cTnI antibodies of different epitopes on cTnI.

### TABLE 1. Patient Demographics

<table>
<thead>
<tr>
<th>Surgical variables</th>
<th>LV&lt;sub&gt;pre&lt;/sub&gt;</th>
<th>LV&lt;sub&gt;post&lt;/sub&gt;</th>
<th>RV&lt;sub&gt;pre&lt;/sub&gt;</th>
<th>RV&lt;sub&gt;post&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>Bypass time, min</td>
<td>98±19</td>
<td>72±17</td>
<td>22±5</td>
<td>6±1</td>
</tr>
<tr>
<td>Cross-clamp time, min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reperfusion time, min</td>
<td></td>
<td></td>
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<tr>
<td>Median number of grafts (range)</td>
<td></td>
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<tr>
<td>Pacing used, n (%)</td>
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<tr>
<td>Inotropes used, n (%)</td>
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<tr>
<td>Preoperative systolic pressure, mm Hg</td>
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<tr>
<td>Postoperative systolic pressure, mm Hg</td>
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<tr>
<td>Hospital stay, d</td>
<td></td>
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</table>

### TABLE 2. Most Bypass Patients Experience cTnI Modification

<table>
<thead>
<tr>
<th>LV&lt;sub&gt;pre&lt;/sub&gt;</th>
<th>LV&lt;sub&gt;post&lt;/sub&gt;</th>
<th>RV&lt;sub&gt;pre&lt;/sub&gt;</th>
<th>RV&lt;sub&gt;post&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients with cTnI modification, n (%)</td>
<td>25 (68)</td>
<td>26 (70)</td>
<td>29 (78)</td>
</tr>
<tr>
<td>Number of patients with specific cTnI products</td>
<td>Deg 1</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Deg 2</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Deg 4</td>
<td>15</td>
<td>13</td>
</tr>
</tbody>
</table>

*Percentage of total patients (ie, of total n = 37).
Characterization of cTnI Modification Products

The most prominent degradation products (Deg 1, Deg 2, and Deg 4) were characterized in part through Western blotting with a variety of anti-cTnI antibodies, with different epitopes on cTnI. An LVpre biopsy sample (LV, Figure 1B), both native and dephosphorylated by treatment with alkaline phosphatase (LV-dephos, Figure 1B), was analyzed with the anti-cTnI antibodies 8I-7, 3E3, 3I-35, and MP. 3E3 binds to the extreme N-terminus of cTnI, where protein kinase A (PKA)–dependent and protein kinase C (PKC)–dependent phosphorylations occur (serine residues 23 and 24 and serine residues 42 and 44, respectively), whereas 3I-35 and MP bind to the extreme C-terminus, where cTnI is specifically proteolyzed in stunned rat heart (amino acid residue 193 in rat, 192 in human).

Deg 1, Deg 2, and Deg 4 did not interact with MP or 3I-35, and therefore these products likely have a C-terminal truncation in the region of cTnI amino acid residue 192. Because all but 1 patient with cTnI modification had Deg 1 (Table 2), Deg 1 likely represents the first, or initial, degradation product with a C-terminal proteolytic cleavage.

The smaller cTnI degradation products were modified at both N-termini and C-termini, but each product was modified to different degrees. Dephosphorylation significantly increased the immunoreactivity of Deg 1, Deg 2, and Deg 4 to the anti-TnI antibody 3E3, which suggests phosphorylation at the PKA and/or PKC (amino acid residues 22 and 23 and residues 42 and 44, respectively) sites. Further characterization of the individual modification products is ongoing and will require extensive biochemical analysis.

cTnI Modification Is a Progressive Process

To determine whether cTnI degradation in bypass patients is ischemia/reperfusion dependent, the quantity of modified cTnI in the myocardium was compared before versus after the cross-clamp episode. Each modification product was quantified as a percentage of the total amount of cTnI, in the same manner as previous reports in isolated rat hearts.1 The amount of intact cTnI remaining was therefore a direct (inverse) measure of the total amount of modified cTnI. For example, if intact cTnI represents 85% of total cTnI, then 15% of total cTnI is posttranslationally modified. Therefore, increases in the proportion of intact cTnI from 75% to 85% do not indicate increases in the absolute amount (ie, micrograms) of intact cTnI but rather show that the proportion of cTnI remaining intact has increased relative to total cTnI. Interestingly, we found on average that there was no difference between values before and after cross-clamp for any of the cTnI modification products (including intact cTnI, data not shown). However, on an individual level, most patients (60%) did experience a change in cTnI modification during cross-clamp, but the changes varied extensively (Figure 2), comprising 3 subgroups of patients: those who experienced an increase in degradation after surgery (Figure 2 A and D; n = 10), those

### TABLE 3. cTnI Modification Changes in an Ischemia-Dependent Manner

<table>
<thead>
<tr>
<th>Patients in Whom TnI Products Appearance Changed</th>
<th>Did Not Change (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVpre</td>
<td>LVpost</td>
</tr>
<tr>
<td><strong>cTnI product</strong></td>
<td></td>
</tr>
<tr>
<td>Intact cTnI</td>
<td>$85\pm5%^{\dagger\ddagger}$</td>
</tr>
<tr>
<td>Deg 1</td>
<td>$9\pm3%^{\dagger\ddagger}$</td>
</tr>
</tbody>
</table>

*Proportion of cTnI (% of total cTnI ± SE) present as either intact cTnI or Deg 1 in LV myocardium before and after cross-clamp (LVpre, LVpost). Significant differences (P<0.05) are indicated as follows: †between before and after surgery (LVpre vs LVpost); ‡between patients in whom products appeared and those in whom products disappeared; §between patients in whom products disappeared and those with no change; and ¶between patients in whom products appeared and those with no change.

Figure 2. Bypass patients experienced heterogeneous changes in cTnI modification. A, B, and C show anti-cTnI antibody (8I-7) Western blots of representative patients who demonstrate 3 different observed changes in TnI modification: increase in cTnI modification (A, n = 10), decrease in cTnI modification (B, n = 12), and no change in modification (C, n = 10), respectively. D and E graphically demonstrate proportion of cTnI remaining intact (a measure of total cTnI modification) for all patients who experienced a change between before and after cross-clamp. Note that increase in proportion of cTnI remaining intact does not indicate increase in absolute amount of cTnI but rather increase in relative proportion of intact cTnI to total of all cTnI products.
who experienced a decrease in degradation after surgery (Figure 2 B and E; n=12), and those who experienced no change after surgery (change <2%, Figure 2C; n=10).

Before cross-clamp, patients who experienced an increase in cTnI modification with surgery were equivalent to those who did not experience a change (Table 3), but patients who experienced a decrease in modification with surgery initially demonstrated extensive modification (P<0.05, Table 3).

During cross-clamp, increases or decreases in cTnI modification, when they occurred, were significant (P<0.05, Table 3). After cross-clamp removal, differences between subgroups did not reach significance (Table 3).

To determine whether the loss of degradation products in a subset of patients (Figure 3, B and E; Table 3) results from necrotic release, serum cTnI was observed by 2 methods: the diagnostic kit used by KGH laboratories (ACCESS, Sanofi Diagnostics, Pasteur SA) and a newly developed SDS-PAGE methodology for the electrophoretic separation and immunoblot visualization of serum proteins. Under normal SDS-PAGE conditions, serum albumin overwhelms all other proteins on the blot (owing to its high concentration) and interferes with the proper separation of lower-abundance proteins such as cTnI and CK-MB. Through the use of a combination of detergents, the effect of albumin on the separation of lower-abundance and lower-MW proteins is minimized. Figure 3 shows serum cTnI (and CK-MB) release profiles as detected by diagnostic kit (Figure 3A) and by an anti-cTnI Western blot using our new approach (2 µL of serum per lane), with the diagnostic kit cTnI values given below the blot (Figure 3B) (note: 0 minutes indicates measurement taken before cross-clamp). Although both methods clearly show a postoperative rise in total serum cTnI, our new methodology demonstrates that cTnI modification products are present in addition to intact cTnI, including a degradation product similar to Deg 1. Surprisingly, cTnI was detected in a presurgery serum sample (0 minutes) at levels below the detection limit of the diagnostic kit.

Serum cTnI and CK-MB release, however, cannot be directly attributed to specific myocardial regions and may not reflect the local environment of the biopsy samples. Therefore, 3 additional patients were recruited, and the biopsy samples were analyzed by EM (Figure 4). Cardiac muscle fibers in both longitudinal and cross-sectional orientations were examined. In general, all ventricular samples examined showed normal ultrastructure, and there were no detectable differences before versus after cross-clamp. Many muscle fibers showed a serrated sarcolemma (Figure 4A). Bundles of myofibrils were arranged along the length of each muscle

![Figure 3. cTnI modification products are necrotically released into serum. A, Average (±SD) serum TnI and CK-MB levels from before (0 hours) to 72 hours after cross-clamp removal. B, Representative Western blot of serum samples from single bypass patient (2 µL per lane), separated by modified SDS-PAGE protocol (see Methods), with values determined by diagnostic kit given below.](http://circ.ahajournals.org/issue/Jan_29/2001/62/Circulation_January_29_2001_62.pdf)

![Figure 4. Biopsy samples showed normal ultrastructure both before and after cross-clamp. A, B, and C show EM images of uranyl acetate and lead citrate–stained sections of biopsy samples obtained from subset (n=3) of bypass patients, demonstrating normal sarcomeres (A), intercalated disks (ICD; A), gap junctions (GJ; C), and mitochondria (B), both before and after cross-clamp.](http://circ.ahajournals.org/issue/Jan_29/2001/62/Circulation_January_29_2001_62.pdf)
fibre and interspersed with numerous mitochondria (Figure 4B). Within each myofibril, thin and thick myofilaments were organized into typical sarcomeres; however, the 1 bands of nearly all muscle fibers examined were in a contracted stage, rendering them invisible unless the fibers were cut in cross section. Intercalated disks were found adjoining adjacent muscle fibers (Figure 4A), and gap junctions were located at the longitudinal portion of the sarcolemma of adjacent myocytes (Figure 4C). Overall, this suggests that at least 1 subpopulation of patients does not experience necrosis either before or after cross-clamp. However, measurements of regional function in the area of biopsy and biochemical analysis on EM-analyzed samples were not performed, which prevents any conclusive determinations regarding cTnI degradation in myocardial stunning.

Discussion

The specific and selective posttranslational modification of cTnI has been proposed to play an important role in the contractile dysfunction observed in stunned and ischemia/reperfused myocardium.1-6 However, in vivo swine and canine models of stunning have failed to demonstrate the ischemia-dependent cTnI degradation observed in isolated rat heart models.9,10 Thus, demonstrating selective and progressive ischemia/reperfusion-dependent modification of cTnI in human disease is of fundamental importance.

Previously, we demonstrated that cTnI is specifically modified in the myocardium of 2 bypass patients, without modification to other myofilament proteins (actin and the regulatory myosin light chain).8 In the present study, analysis of myocardial biopsy samples from 37 bypass patients demonstrated that cTnI is modified in a selective and progressive manner, both before and after cross-clamp. Importantly, myocardial cTnI is degraded in approximately 2 thirds of patients before cross-clamp; when there is little or no cell necrosis at the site of the biopsy. This is the first study to demonstrate ischemia-dependent TnI modification in human myocardium.

The first, or initial, degradation product in bypass patients (Deg 1) is similar in MW (22 kDa) and immunogenicity to the C-terminal degradation product found in stunned rat myocardium. In the isolated rat heart, this product has been identified as cTnI1-193, resulting from proteolytic cleavage of 17 C-terminal amino acids.1 This suggests that ischemia/reperfusion activates similar processes (ie, the activation of Ca2+-dependent proteases) in both rats and bypass patients.

The presence of modified cTnI in the myocardium of so many patients before application of cross-clamp, which suggests myocardial injury (possibly stunning) in "normal" perfused myocardium, is both intriguing and unexpected. This basal level of modified cTnI may result from 2 processes: (1) undetected prior or ongoing disease, not necessarily directly resulting from the repaired coronary occlusions; and (2) pre–cross-clamp myocardial damage resulting from surgical preparation (including anesthetic influences). The relative influence of these 2 processes cannot be established at this time; however, it is known that anesthetics such as halothanes can modulate myocardial preconditioning in animal models.13,14 On the other hand, all patients had preexist-
32-49126.96, Dr Atar), as well as a Heart and Stroke Foundation of Canada research scholarship (Dr Van Eyk) and research traineeship (J.L. McDonough). The authors would like to thank Spectral Diagnostics (Toronto, Canada) for anti-cTnI monoclonal antibodies; Dr Anne Murphy, Pediatric Cardiology, The Johns Hopkins University, for advice; and John DaCosta for his assistance in processing and sectioning cardiac tissue samples.

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