Extensive Troponin I and T Modification Detected in Serum From Patients With Acute Myocardial Infarction

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Background—Cardiac troponin I and T (cTnI and cTnT) are specific biochemical serum markers for acute myocardial infarction (AMI). However, cTnI diagnostic assays are plagued by difficulties, resulting in ≥20-fold differences in measured values. These discrepancies may result from the release of the numerous cTnI modification products that are present in ischemic myocardium. The resolution of these discrepancies requires an investigation of the exact forms of cTnI present in the bloodstream of patients after myocardial injury.

Methods and Results—A western blot–direct serum analysis protocol was developed that allowed us to detect intact cTnI and a spectrum of up to 11 modified products in the serum from patients with AMI. For the first time, we document both a cTnI degradation pattern and the existence of phosphorylated cTnI in serum. The number and extent of these modifications reflect patterns similar to the time profiles of the routine clinical serum markers of total creatine kinase, creatine kinase-MB, and cTnI (determined by ELISA). Data from in vitro experiments, which were undertaken to study the degradation of human recombinant cTnI and cTnT when spiked in serum, indicate that some modification products present in patient serum existed in the myocardium and that recombinant cTnI alteration dramatically reduces the detectability of cTnI by the Immuno1 assay over time (our assay was unaffected).

Conclusions—This pilot study defines, for the first time, what forms of cTnI and cTnT appear in the bloodstream of AMI patients, and it clarifies the lack of standardization between different cTnI diagnostic assays. (Circulation. 2000;102:1221-1226.)

Key Words: troponin ■ myocardial infarction ■ biological markers ■ diagnosis ■ blotting, western
This was accomplished by the development of a western blot–direct serum analysis (WB-DSA) procedure. The serum from 12 patients diagnosed with AMI was analyzed to demonstrate the capability of this method in a clinical setting.

**Methods**

**Patient Samples**

Blood samples from 12 patients admitted to the Kingston General Hospital Emergency Department with chest pain and unequivocal signs of AMI (on the basis of ECG findings) were approved for use by the Human Research Ethics Board of Queen’s University. Serum sampling was performed according to routine care protocols and not by a defined study time course. This led to different intervals between successive blood samples, which gave us the opportunity to study the occurrence of biochemical markers in a “real-life” scenario. Because this was essentially a pilot study to investigate the effectiveness of our new method on human samples, no further clinical data were obtained for these patients.

**Routine Biochemical Testing**

Blood was collected in serum separator tubes, centrifuged, and assayed immediately for routine biochemistry tests. Samples were then frozen until WB-DSA. Routine testing included total creatine kinase (CK; measured by CX7, Beckman Coulter), its MB isoenzyme (CKMB), and cTnI (both measured by Technicon Immuno1, Bayer Corporation). A diagnosis of AMI was confirmed if a typical time profile was observed for CK, with at least a doubling from baseline values. Confirmatory testing by either CKMB or cTnI was also required on at least one sample. CKMB was considered positive if the absolute value was >8 μg/L and the relative index (CKMB×100/CK) was >3%. cTnI was considered positive when >0.9 μg/L.

**Stability Studies for cTnI and cTnT**

Electrophoresis and Western Blot Analysis

To determine the proteolytic susceptibility of cTnI and cTnT in serum, full-length human recombinant cTnI (209 amino acids), human recombinant cTnT (amino acid fragment 1 to 192), and human recombinant cTnI (rcTnI, rcTnI[1-192], and rcTnT, respectively) were added to 3 separate serum pools at a final concentration of 100 mg/mL and incubated at 37°C for up to 48 hours. The serum was obtained from a 28-year-old healthy male volunteer (hereafter referred to as normal serum).

Polyacrylamide gel electrophoresis was performed under denaturing and reducing conditions using a sample buffer containing 0.33% SDS, 0.33% CHAPS, 0.33% NP-40, 0.1 mol/L DTT, 4 mol/L urea, and 50 mmol/L Tris-Cl (pH 6.8) in 50% glycerol. Serum was diluted 12.5× in sample buffer to prevent precipitation of serum proteins during boiling. Diluted samples were then boiled for 10 minutes to assure separation of the troponins from serum proteins and to break up binary and ternary complexes. Twenty-five microliters of serum was added to 3 separate serum pools at a final concentration of 100 mg/mL, able to detect all isoforms of cTnT.

Primary antibodies were detected with horseradish peroxidase–conjugated goat anti-mouse IgG or rabbit anti-goat IgG (both from Jackson Laboratories), and signals were visualized using chemiluminescence substrate (Boehringer Mannheim) and X-Omat Scientific Imaging Film (Eastman Kodak Company). All antibodies were diluted in 1% blocking reagent and incubated for 1 hour at room temperature.

**Dephosphorylation of Serum**

On the basis of previously published protocols, dephosphorylation of serum was performed as follows: 100 U of calf intestinal alkaline phosphatase (New England Biolabs) and 1.6 μL of 10× dephosphorylation buffer (50 mmol/L Tris, 100 mmol/L NaCl, 10 mmol/L MgCl₂, and 1 mmol/L DTT; pH 7.9) were added to 4 μL of serum (~100 mg/mL serum proteins) and incubated for 30 minutes at 30°C (1 U of alkaline phosphatase hydrolyzes/1 nmol of p-nitrophenyl phosphate per minute at 30°C; pH 8.5). Reactions were terminated by adding 4 μL of 5× sample buffer and boiling for 5 minutes. The activity of alkaline phosphatase in serum was confirmed by its ability to dephosphorylate ³²P-labeled myelin basic protein when added to normal serum (data not shown).

**Data Analysis**

Because this was a pilot study designed to address qualitative rather than quantitative alterations of the troponin molecules, no formal statistical analysis of these results was performed.

**Results**

In Figure 1, we present the western blots for 5 representative AMI patients. In addition to intact cTnI, as many as 8 truncated degradation products and 3 products of higher molecular weight were observed. The number and extent of cTnI modifications in each patient changed over time after the infarction. This profile of the visually detectable cTnI modification products (and their intensity, as indicated by WB-DSA) corresponded with the time profiles of serum CK, CKMB, and cTnI, as determined by CX7 and Immuno1.

Western blot analysis of serum samples from patient 1, using an anti-cTnT polyclonal antibody, showed massive degradation of intact cTnT to a single truncated product with a molecular weight of ~26 kDa (Figure 1a). In addition, 2 further products appeared in the final sample. Like cTnI, the amount of cTnT detectable in patients’ serum changed over time after an AMI. This profile also corresponded to the time profiles of serum CK, CKMB, and cTnI. It is interesting to note that for this particular patient, cTnI was detected before cTnT.

Dephosphorylation of serum verified that some of the cTnI (intact and modified products) found in patients’ serum was phosphorylated. Although some antibodies (Figures 2b through 2d) seemed to change their immunoreactivity toward cTnI due to the dephosphorylation of serum, others (Figure 2a) were not affected. Phosphorylated cTnI has not been shown previously in the serum of patients with AMI. Serum from an AMI patient incubated in dephosphorylation buffer in the absence of alkaline phosphatase served as a negative control for the dephosphorylation experiment and showed no difference in the cTnI pattern when compared with native conditions (data not shown).

The cTnI fragments all arose from C-terminal truncations, as shown by the lack of immunoreactivity to the C-terminal anti-cTnI antibody 10F2 (Figure 2d). In addition, it was clear that degradation products below a molecular weight of 22
kDa resulted from both N- and C-terminal truncations, as evidenced by the lack of interaction with the N-terminal anti-cTnI antibody P1 (Figure 2a).

The determination of whether the protein modifications detected in the serum occurred before or after release from the myocardium was addressed by adding human rcTnI, rcTnI1–192, or rcTnT to normal serum, followed by incubation at 37°C for up to 48 hours (Figure 3). The Immuno1 results demonstrated a dramatic decline in detectable cTnI for both the intact form and the 1 to 192 fragment (Figure 3). When using WB-DSA, rcTnI underwent degradation within 30 minutes in serum, forming a fragment that migrated in a gel to the same position as rcTnI 1–192 (Figure 3). No additional cleavage products were detected, and we observed no further substantial degradation and reduction of total rcTnI after 2 hours that could explain the dramatic decline in detectable cTnI by Immuno1. These data are supported by control experiments in which a 5-fold excess of spiked rcTnI, relative to the amount resolved in Figure 3, showed the same discrete proteolysis (data not shown).
Degradation of rcTnI1–192 (Figure 3) occurred to a lesser extent than that observed for rcTnI and, again, no reduction of total protein was detected over a period of 48 hours. In contrast, human rcTnT did not degrade in normal serum (data not shown). We noted that freeze/thawing of both normal serum containing rcTnI and rcTnT and patients’ serum did not change the pattern of protein degradation detectable by WB-DSA.

All western blots used for this study were repeated, and samples of the serum time courses from patients 1 and 5 (see Figure 1) were frequently used as positive controls. We have not yet determined any change in the cTnI patterns of these samples.

Discussion

The full diagnostic potential of cTnI is currently impeded by discrepancies between commercially available detection kits.8,22,23 We report a WB-DSA procedure that, for the first time, directly detects the cTnI and cTnT in the serum from patients with diagnosed AMI. Our procedure has overcome the main problems limiting the application of SDS-PAGE to serum. The large quantities of albumin and IgG hamper migration within the gel and overwhelm the small amount of cTnI and cTnT that are commonly present in serum after AMI, which limits the sample volume that can be applied to a polyacrylamide gel. By modifying our sample preparation, we could optimize the method so that only 2 μL of serum was required for the reliable detection of cTnI and cTnT. Our small volume requirements also circumvent another problem. Manipulating the serum to concentrate or to isolate the cardiac troponins11,13 to enable further analysis increases the inherent risk of missing some forms of the proteins; this problem is not encountered with our protocol.

The continuum of changing cTnI profiles observed in AMI patients contains specific modification products that are not identified by an investigation of rcTnI proteolytic susceptibility in normal human serum. Obvious discrepancies include the absence of any higher molecular weight products and degradation products <22 kDa. This finding is in contrast to those of Morjana,13 who showed extensive degradation of spiked rcTnI; this discrepancy is most likely due to differences in methodology, including the 30-fold higher concentration of spiked rcTnI used by Morjana13 (3.2 mg/L compared with 0.1 mg/L in our protocol).

Although rcTnT shows no proteolytic susceptibility in normal serum, AMI patients possess only a small amount of intact cTnT, but 1 major and 2 minor degradation products of cTnT. Therefore, it is likely that these forms of cTnI and cTnT, which are found only in AMI patients, are generated in the diseased myocardium itself and then subsequently released into serum. Indeed, some modifications of cTnI observed here are reminiscent of those seen in the myocardium of animal models and bypass patients.15,16,18 In a recent study, it was reported that in the myocardium of bypass patients, the presence of specific cTnI degradation products correlated with the extent of myocardial injury.18 Similarly, the patterns of cTnI degradation products present in our patients’ serum seem to correspond with the severity of the AMI, as assessed by routine biochemical markers (ie, CK, CKMB, and cTnI; Figure 1).

Although rcTnI remains relatively stable in serum, as demonstrated by WB-DSA, it was interesting to note that an analysis by Immuno1 measured a dramatic decrease of rcTnI over a period of 48 hours (Figure 3). The onset of rcTnI degradation within 30 minutes may mitigate detection by Immuno1, yet the continuous decline in serum cTnI concentration, as determined by the Immuno1 assay, is not attributable to further general proteolysis of rcTnI. We hypothesize that some as-yet-unidentified modification (other than degradation) is responsible for altering the immunogenicity of rcTnI in the serum, which influences its detectability by the Immuno1 assay. Thus, the accuracy of this diagnostic assay decreases with time after the onset of the ischemic event. This increases the risk of false-negative diagnosis in patients with minor myocardial damage and late admittance to the emergency department.

The reason for this change in accuracy may lie in the inability of the antibodies used in the diagnostic assay to detect the present forms of modified troponin, rather than to the actual serum concentration of cTnI. If so, we should lower the detection level, which would increase the sensitivity of the diagnostic assays. This change should be achievable by...
optimizing the antibody combination to detect all of the various forms of cTnI. This concept is supported by data from the WB-DSA performed on the serum of patients undergoing bypass surgery, in which cTnI was found in samples diagnosed as negative by Immuno1 (unpublished data).

By comparing the data from the WB-DSAs of patients’ time courses with results from patient serum probed with antibodies raised against different epitopes of cTnI (compare Figures 1 and 2), we confirmed previous findings that the cTnI found in patients’ serum after an AMI is indeed both C- and N-terminal clipped. The C-terminal cleavage occurs as the first of numerous proteolytic steps, leading to up to 8 degraded products found in serum after AMI. Therefore, we support the suggestion by Katrukha et al. to avoid the use of antibodies against extreme C- or N-terminal epitopes of cTnI for the diagnosis of AMI. This leads to the conclusion that the total amount of detectable troponin is related to the antibody used and underlines the importance of antibody selection for a diagnostic assay. Herein lies the difficulty with the standardization of cTnI assays. Whether the same holds true for cTnT cannot be verified, because only one manufacturer currently offers commercially available assays. Because this manufacturer has already released modified versions of these assays to overcome problems with false-positives, it is likely that, with other manufacturers marketing cTnT assays using different antibodies, variations in the performance of these tests (similar to those seen with cTnI) will occur.

This pilot study was designed to address qualitative rather than quantitative alterations of the troponin molecules and to demonstrate the capability of our new procedure to detect even these alterations in a clinical setting. Indeed, a thorough inspection of Figure 1 reveals unequivocal changes in the visually detectable modifications of the troponins during the time course after AMI. In fact, these changes correspond with the rising and declining patterns in the time profiles of serum CK, CKMB, and cTnI. Obviously, because of different methods of signal detection, a direct comparison of the intensity of bands appearing on western blots with the numeric concentration values obtained by ELISA would be inappropriate and misleading; this was not a part of the scientific design.

In conclusion, this pilot study using our WB-DSA procedure defines, for the first time, the exact forms of cTnI and cTnT that appear in the blood stream of patients after acute myocardial injury. We demonstrated that the cTnI and cTnT found in patients’ serum after an AMI shows modifications that reflect primary damage occurring intramyocardially, as well as changes arising after the release of troponin into the bloodstream. The number and extent of cTnI and cTnT modifications in each patient change throughout the time course after the infarct, and the continuous change of their visually detectable amounts corresponds with the time profiles of serum CK, CKMB, and cTnI (as determined by CX7 and Immuno1). Some modification of cTnI (other than degradation), occurring after its release from the myocardium, alters its detectability by Immuno1.

The following question arises: Does the appearance of a certain troponin modification product or a distinct pattern of products over time correlate with a distinct cardiovascular condition, a specific time point after the onset of an AMI or, possibly, the severity of an infarct or even reinfarction? These issues must be addressed in larger clinical studies.

Our findings should help direct the future design of new immunological diagnostic tools using the variety of forms of troponin in a patient’s blood to detect myocardial damage and to provide more information about the condition of the diseased myocardium, which would reflect its viability. Ultimately, this tool may have therapeutic implications and lead to a more differentiated risk stratification of patients with acute coronary syndromes.

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References


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