Clinical Research

Time Course of Degradation of Cardiac Troponin I in Patients With Acute ST-Elevation Myocardial Infarction

The ASSENT-2 Troponin Substudy


Abstract—Although measurement of troponin is widely used for diagnosing acute myocardial infarction (AMI), its diagnostic potential may be increased by a more complete characterization of its molecular appearance and degradation in the blood. The aim of this study was to define the time course of cardiac troponin I (cTnI) degradation in patients with acute ST-elevation myocardial infarction (STEMI). In the ASSENT-2 substudy, 26 males hospitalized with STEMI were randomized to 2 different thrombolytic drugs within 6 hours after onset of symptoms. Blood samples were obtained just before initiation of thrombolysis and at 30 minutes intervals (7 samples per patient). Western blot analysis was performed using anti-cTnI antibodies and compared with serum concentrations of cTnI. All patients exceeded the cTnI cutoff for AMI during the sampling period; at initiation of therapy, 23 had elevated cTnI values. All patients demonstrated 2 bands on immunoblot: intact cTnI and a single degradation product as early as 90 minutes after onset of symptoms. On subsequent samples, 15 of 26 patients showed multiple degradation products with up to 7 degradation bands. The appearance of fragments was correlated with higher levels of cTnI (P<0.001) and time to initiation of treatment (P=0.058). This study defines for the first time the initial time course of cTnI degradation in STEMI. Intact cTnI and a single degradation product were detectable on immunoblot as early as 90 minutes after onset of symptoms with further degradation after 165 minutes. Infarct size and time to initiation of treatment was the major determinant for degradation. (Circ Res. 2006;99:1141-1147.)

Key Words: myocardial infarction ■ troponin ■ troponin degradation ■ diagnostics

The criteria for diagnosing acute coronary syndrome and myocardial infarction (AMI) changed in the year 2000 with the endorsement of the American College of Cardiology/European Society of Cardiology guidelines, which designated cardiac troponin (cTn) as the biochemical marker of choice.1 Troponin is a regulatory protein of the thin filament of striated muscle and consists of 3 tightly interacting subunits: T (37 kDa), I (24 kDa), and C (18 kDa). In addition, there are generally thought to be free cytoplasmic components2,3: for cardiac troponin T (cTnT), amounting to approximately 6% to 8% of the total pool; and for cardiac troponin I (cTnI), 3% to 4%. Troponin is released into the bloodstream 4 to 6 hours after AMI, peaks after approximately 18 to 24 hours, and can stay elevated for up to 14 days. Assessment of cTnI or cTnT by automated assay is today the most sensitive and specific method for diagnosing AMI. However, cTn is not released only in response to ischemic insults but by any condition that is associated with and/or causes cardiac injury, eg, decompensated heart failure, pulmonary embolism, end-stage renal disease, and stroke.4–11 In addition, there is significant variability in the sensitivity, selectivity, and specificity among various diagnostic cTnI immunoassays.12 Some of these assay differences have been speculated to be attributable to the presence of multiple forms of cTn such as posttranslational modifications, in particular specific degradation. As there is limited knowledge of the degradation of cTn and the exact forms of cTn in the blood of patients after myocardial injury, the aim of this study was to systematically define the initial time course of cTnI degradation in patients with acute ST-elevation myocardial infarction (STEMI) in the ASSENT-2 study by use of immunoblotting with antibodies that recognize different epitopes on the cTnI molecule.

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Materials and Methods

Patient Samples

Blood samples were collected from 26 patients (all males; age 33 to 72 years) hospitalized with STEMI in the ASSENT-2 study. Patients had to have onset of symptoms of AMI within 6 hours of randomization and have ST-segment elevations of 0.1 mV or more in 2 or more limb leads or 0.2 mV or more in 2 or more contiguous precordial leads or have left bundle branch block. After informed consent, patients were randomly assigned to a bodyweight-adjusted bolus of tenecteplase plus bolus and infusion of placebo or a bolus and infusion of alteplase and a bolus of placebo. Blood samples were obtained immediately before initiation of therapy (baseline, time 0) and at 30 minutes intervals up to 3 hours (a total of 7 samples per patient). We did not receive any information regarding randomized treatment, concomitant medicine, or reperfusion status after thrombolysis was administered to the patient. The ASSENT-2 study showed the 2 treatments to be equivalent in terms of both 30-day and 1-year mortality.

Biochemical Testing

Platelet-poor plasma was collected from all patients at intervals stated above and assayed immediately for routine biochemistry tests including creatine kinase (CK) and its MB isoenzyme (CK-MB). The diagnosis of AMI was confirmed by a typical time profile observed with at least a doubling of baseline values for at least 1 of the assays, as defined in the ASSENT-2 study. Regarding further analysis processing and handling of blood samples were standardized and serum aliquoted and frozen at −80°C until analysis. Samples were thawed only once before immunoblotting. Cardiac TnI was assayed on Access AccuTnI (Beckman Coulter) with the minimal detectable concentration reported by the manufacturer of 0.01 ng/mL, a 99th percentile upper reference limit of 0.04 ng/mL, and a recommended cutoff for AMI of 0.1 ng/mL. Values above 100 ng/mL were labeled “>100” without further attempts to define the exact values. The coefficient of variation of the assay is reported to be 20% at 0.03 ng/mL, 14% at 0.04 ng/mL, and 10% at 0.06 ng/mL. Also according to the manufacturer, the antibodies selected for the Access AccuTnI assay bind to epitopes located in the stable region of the molecule (amino acids [aa] 30 to 110) and has been proposed to measure both complexed and intact free cTnI but may or may not bind degradation products.

Electrophoresis and Western Blot–Direct Serum Assay

Detection of serum cTnI by Western blot–direct serum assay (WB-DSA) was performed under denaturing and reducing conditions. Serum was diluted 20-fold in sample buffer containing 16.5 mg/mL sodium dodecyl sulfate, 16.5 mg/mL 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 1.65% Nonidet P-40, 0.5 mol/L dithiothreitol, 1 mol/L urea, 0.25 mol/L Tris-HCl (pH 6.8), 0.01% wt/vol bromophenol blue, and 50% SDS-PAGE (40% acrylamide/bis solution, 37.5:1 [C 2.6%]; Mini SDS-PAGE, 1.65% Nonidet P-40, 0.5 mol/L dithiothreitol, 0.25 mol/L Tris-HCl (pH 6.8), 0.01% wt/vol bromophenol blue, and 50% mL of glycerol. One microliter of neat serum was resolved by 12% SDS-PAGE and film (X-Omat Blue XB-1, 18x24 cm, Kodak) and were within the linear range. Sera were analyzed 2 times, and the corresponding results were consistent.

Purification Methods

IDM (Interaction Discovery Mapping) Affinity Beads (Ciphergen Biosystems Inc, Fremont, Calif) were used for pull-down purification and enrichment of cTnI. The protocol of coupling an antibody to IDM Affinity Beads and subsequent capture and elution of antigen, as described by the manufacturer, was used. The beads (10 μL) were washed with water and added 200 μL of coupling buffer (50 mmol/L sodium bicarbonate, pH 9.2) and 5 μL of 8-17 antibody. After incubation overnight at 4°C, the beads were washed with coupling buffer and then by a Tris/Triton X buffer (0.5 mol/L Tris HCl, pH 9.0, 0.1% Triton X-100). To reduce unspecific reactions, the beads were blocked with 1 mg/mL BSA in Tris/Triton X buffer for 1 hour at room temperature. After blocking, the beads were washed with PBS, added to 50 μL of serum and 150 μL of PBS, and incubated for 1 hour at room temperature. The beads were subsequently washed 4 times in 1 mol/L urea, 50 mmol/L Tris HCl (pH 7.2), 0.1 mol/L NaCl, 0.1% CHAPS, and finally in PBS and water before they were added 20 μL of SDS-PAGE sample buffer, to elute bound protein from the beads.

Image Quantification

The ImageQuant TL Software (Amersham Biosciences) was used to visualize the release pattern and to estimate the size of the individual degradation products. As a standard, we used purified human cTnI from HyTest Ltd (Turku, Finland) with a molecular mass of 23,876 kDa reported by the manufacturer, which allowed for an internal consistency check regarding the localization of cTnI after gel migration during electrophoresis. To express the release pattern semiquantitatively, we adjusted the intensity of the signal from each band to the signal of the intact purified human cTnI, which was loaded in equal concentrations on each immunoblot.

Statistical Analysis

All data with a normal distribution are presented as mean±SD with range in parentheses. Differences between groups were examined with Student’s t tests. A value of P<0.05 was considered statistically significant. All statistical analyses were performed using SPSS 11.5 software.

Results

Of the 26 patients, 2 had previous diagnoses of diabetes mellitus, 4 had known hypertension, 2 experienced a previous AMI, 1 previously underwent coronary angiogram, 1 previously underwent a percutaneous coronary intervention with stenting, and 1 had previously undergone coronary bypass surgery. One patient died (day 11), and 17 were alive and 8 were lost to 1-year follow-up.

At baseline, ie, immediately before initiation of thrombolytic treatment, 23 of 26 patients (88%) had cTnI levels above the minimal detectable limit with thirteen patients (50%) above the diagnostic cutoff for AMI (≥0.1 ng/mL). Although there can be a higher rate of false-positive results near the lower detection limit, cTnI could be detected by WB-DSA in all patients at baseline. Time from onset of symptoms to initiation of treatment was 206±68 minutes (mean±SD; range, 105 to 420 minutes) with time from baseline to initiation of treatment 9±5 minutes (mean±SD;
Increasing levels of cTnI occurred over the course of the sampling and all patients eventually exceeded the cTnI cutoff for AMI. The anti-cTnI antibody 3-E3 detected in all patients a minimum of 2 bands on immunoblot, namely intact cTnI and a single degradation product (molecular mass, 23.5 and 20 kDa, respectively) as early as in their baseline samples (from 90 minutes after onset of symptoms). Eleven patients showed only increases in the intensity of the signals from the 2 fragments, but no further degradation. Of these patients, 5 reached maximum CK and CK-MB levels of 2 to 5 times the upper reference limit (URL); the remaining 5 times the URL. Progressive degradation, on the other hand, was found in 15 of the 26 patients, all of which reached maximum CK and CK-MB levels >5 times the URL. Immunoblot showed multiple degradation products with a molecular mass less than intact cTnI with up to 7 degradation bands, all molecular mass >12 kDa, appearing at a mean of 261 ± 64 minutes after onset of chest pain (range 165 to 355 minutes). In these patients, time from initiation of treatment to the appearance of more than 1 degradation product was 68 ± 53 minutes (0 to 145 minutes). The appearance of more than 1 detectable degradation fragment was dependent on the cTnI level. Only 1 degradation band appeared at low concentrations, followed by an increasing number of degradation products with increasing measured concentrations (P<0.001).

The finding of intact cTnI and up to 7 smaller fragments in some patient samples was consistent for both primary antibodies, but anti-cTnI antibody 3-E3 was able to demonstrate all bands, whereas anti-cTnI antibody 8-I7 was unable to detect the presence of the 2 smallest fragments. Also, unlike anti-cTnI antibody 3-E3, the anti-cTnI antibody 8-I7 was only able to visualize intact cTnI, but not the initial degradation fragment in the baseline sample of 6 patients, which was independent of measured concentrations of cTnI. The third primary antibody, G-131-C, was used only to confirm the bands as being products of troponin degradation when verification was needed (data not shown). All samples were tested with secondary antibody alone and showed no cross-reactivity.

Figure 1 depicts representative blots showing progressive cTnI degradation from time 0 through 180 minutes in 2 patients with ultimately high concentrations of cTnI using 2 different primary anti-cTnI antibodies. In contrast to these findings, Figure 2 shows representative blots of cTnI degradation from time 0 through 180 minutes in 2 patients with relatively low concentrations of cTnI using the same antibodies. In both blots, human purified intact cTnI was used as a positive control. Timing began immediately before initiation of thrombolytic treatment (t=0), and subsequent time intervals are listed for each Western blot.

The concentration of cTnI in serum is at least 100 000 times less than that of the most abundant serum proteins. Results from Western blotting with anti-cTnI antibodies using crude serum should therefore be interpreted with care because of the possibility of unspecific binding to more abundant proteins. To verify the cTnI identity of the Western blot bands, we performed pull-down procedures, ie, immunoprecipitation with an anti-cTnI antibody. This procedure could not be expected to give pure cTnI, but a strong reduction of contaminating proteins, and hence an enrichment of cTnI could be possibly expected. The pull-down products were subsequently analyzed by SDS-PAGE and Western blotting. We found that bands corresponding to bands from crude serum presumably marking intact cTnI and degradation products indeed were enriched, whereas dense bands from crude serum (probably corresponding to albumin and immunoglobulin) were strongly reduced after the pull-down procedure (Figure 3). This reduces the risk of our results being false positives.

The release and clearance pattern for intact cTnI (band 1) and the primary degradation band (band 2) are shown in Figure 4, including SDs. The remaining degradation products are not shown in this format, but Figure 5 summarizes the release pattern for bands 1 to 8. The figure shows bands 1 and 2 being present at an earlier stage than the remaining bands following the same release pattern, with a less steep increase in concentration at the end of the sampling period. The other degradation fragments show a more rapid increase in concentration as measured by their corrected intensity.
The molecular mass estimate based on mobility in the SDS-PAGE gel for intact cTnI and the smaller degradation product confirmed that there was consistency in the appearance of the different fragments when using blots with both anti-cTnI antibody 3-E3 and 8-I7, except for the difference in sensitivity to show the presence of the 2 bands. For intact cTnI, we found an approximate molecular mass of 23.5 kDa (band 1), with subsequently decreasing size of the following degradation fragments of 20.0 (band 2), 18.5 (band 3), 17.0 (band 4), 16.5 (band 5), 15.5 (band 6), 14.0 (band 7), and 12.0 kDa (band 8) (data from immunoblots visualized by anti-cTnI antibody 3-E3). The particular products “intact cTnI” and “band 2” corresponding to molecular mass 23.5 and 20.0 kDa were present at baseline in all patients as previously described, whereas bands 3 and 5 were the most abundant of the subsequent degradation products. In patients who demonstrated several degradation products, there was a trend toward earlier initiation of treatment after onset of symptoms (184 ± 41 minutes) as compared with patients with only 2 bands on immunoblot (235 ± 87 minutes; P = 0.058). We could not detect any significant relation between infarct location and fragment patterns, just as we did not find any relationship between the relative increase in intensity of bands and time from onset of symptoms to initiation of treatment in patients who demonstrated several degradation products as a possible indicator of differences in degree of reperfusion.

**Discussion**

The cardiovascular community has embraced the troponins as the new state-of-the-art biochemical markers for diagnosing myocardial damage and AMI. Clarifying the degradation of cardiac troponins is a further step in achieving and evaluating the full diagnostic potential of these biochemical markers. Perhaps the most imminent issue is whether the presence of specific fragments of troponin or a combination of fragments would be characteristic of a specific time or severity of an AMI. The present study began to evaluate this critical question by rigorously defining the time course of degradation of cTnI in patients with unequivocally diagnosed STEMI. We showed intact cTnI and the primary degradation fragment of 20 kDa detectable on immunoblot as early as 90 minutes after onset of symptoms, with further degradation after 165 minutes. It appears that anti-cTnI antibody 8-17 is more strongly connecting to intact cTnI as opposed to...
anti-cTnI antibody 3-E3, which seems more sensitive to the initial degradation product of 20 kDa.

Previous studies have shown a correlation between concentrations of cTnT and cTnI and infarct size, suggesting levels 72 hours after AMI the most specific for evaluating infarct size using commercial tests that may or may not detect the degradation products. Ohlmann et al were able to demonstrate a significant correlation between infarct size and cTnI levels as early as 3 hours after primary percutaneous coronary intervention. In our study, infarct size, as estimated by the concentration of cTnI also 3 hours after thrombolysis, was the major determinant for the presence of several degradation products. Up to 7 degradation fragments from both the N and C termini were seen in addition to intact cTnI, and these results are in agreement with previous studies conducted by Labugger et al. The fact that antibody 8-I7 is not able to detect the 2 smallest fragments suggests that the cleavage is within its epitope (aa 137 to 148) and therefore splits the molecule essentially in half.

As suggested by earlier studies, the pattern of release of the different cTnI forms after AMI may vary over time, with intact ternary complexes predominantly early, followed by binary complexes, free forms, and immunoreactive fragments mostly later in the course. In our study, we were not able to visualize complexes of cTnI. Intact cTnI was the primary product detected and it was detected early after the onset of symptoms. Subsequently, we observed degradation products with a smaller molecular mass. This may represent the fact that early release comes from the free form of cTnI present in the cytosol but does not exclude the presence of larger complexes being the primary form in the blood after a myocardial infarction. Also we do not know whether thrombolysis or preparation of blood samples for immunoblotting is of importance in degradation of cTn.

As Figures 4 and 5 show, the increase in concentration diminishes after the initial release with the smaller fragments dominating the picture in peripheral blood by the end of the sampling period. This is suggestive of differences in clear-

Figure 4. Corrected intensity of intact cTnI (band 1) and initial degradation fragment (band 2).
The release pattern of troponin may be dependent on the time from onset of the coronary occlusion to recanalization, if achieved, and the abruptness of the reperfusion. In addition, comparisons are problematic because of a different amount of troponin being released into the blood depending on the degree of revascularization. Our data show that patients with only 2 bands on immunoblot had low concentrations of cTnI in the very early phase of AMI (ie, the sampling period; 0 to 300 minutes) and generally lower values and/or later peak concentrations of CK/CK-MB during hospitalization. Combining these facts with the concept suggested previously that most of the troponin is degraded in the myocardium and not in peripheral blood, one could speculate whether the patients with the relatively early release of multiple degradation products represent the patients who benefited the most from thrombolytic treatment, whereas the remaining patients have not been sufficiently reperfused and will show a relatively larger, but slower increase in cTnI concentration at a later time point. Some patients, however, have low levels of CK-MB, but peak at the same time as patients with high levels, and could simply represent a cohort with smaller infarctions. The range of cTnI concentration in which there is no further degradation beyond intact cTnI and band 2 is 0.02 to 30.50 ng/mL. We see further degradation in a range of 0.07 to >100 ng/mL. This does not exclude, but diminishes, the possibility of not visualizing degradation products because the severity of the injury is not enough to release cTnI in a concentration sufficient to visualize degradation products on the gels.

### Strength and Limitations

The study is a single-center study with a randomized design within the framework of a major STEMI trial and with uniform time frequency of blood drawing. Limitations were the small number of patients and the lack of further clinical information, including follow-up. Unfortunately, we also do not have any information regarding the degree of achieved reperfusion by the administered fibrinolytic therapy, just as we do not know the time course of cTnI concentrations past the initial 180 minutes after treatment initiation (ie, our sampling period).

### Conclusion

Taken together, this study defines, for the first time, the initial time course of cTnI degradation in STEMI. Intact cTnI and a single degradation product was detectable on immunoblot as early as 90 minutes after onset of symptoms, with further degradation after 165 minutes. Infarct size and time to initiation of treatment was the major determinant for degradation.

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### Disclosures

None.

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