Time-Dependent Degradation Pattern of Cardiac Troponin T Following Myocardial Infarction

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BACKGROUND: Cardiac troponin T (cTnT) is widely used for the diagnosis of acute myocardial infarction (AMI). However, it is still unclear whether degraded cTnT forms circulate in the patient’s blood. We therefore aimed to elucidate which cTnT forms are detected by the clinical assay.

METHODS: Separation of cTnT forms by gel filtration chromatography (GFC) was performed in sera from 13 AMI patients to examine cTnT degradation. The GFC eluates were subjected to Western blot analysis with the original antibodies from the Roche immunoassay used to mimic the clinical cTnT assay. To investigate the degradation pattern with time, standardized serum samples of 18 AMI patients collected 0–72 h postadmission were analyzed by Western blot analysis.

RESULTS: GFC analysis of AMI patients' sera revealed 2 cTnT peaks with retention volumes of 5 and 11 mL. Western blot analysis identified these peaks as cTnT fragments of 29 and 14–18 kDa, respectively. Furthermore, the performance of direct Western blots on standardized serum samples demonstrated a time-dependent degradation pattern of cTnT, with fragments ranging between 14 and 40 kDa. Intact cTnT (40 kDa) was present in only 3 patients within the first 8 h after hospital admission.

CONCLUSIONS: These results demonstrate that the Roche cTnT immunoassay detects intact as well as degraded cTnT forms in AMI patients' sera during the period of diagnostic testing. Moreover, following AMI, cTnT is degraded in a time-dependent pattern.

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ments on Western blots of human serum from renal failure and post-AMI patients (14, 15). Other investigations have not confirmed these findings and have revealed only intact cTnT on gel filtration chromatography (GFC) of serum from such patients (16, 17). To date it is still not clear which cTnT forms circulate in the serum of AMI patients and if they are detected by the Roche cTnT assay.

In this study, we aimed to elucidate whether degraded cTnT forms are present in the circulation following ischemic myocardial damage. We examined the sera of patients with AMI using a novel approach. First, the patients’ sera were fractionated by GFC, as in previous studies (16, 17). Second, these GFC fractions were subjected to a unique Western blotting technique, in which the capture and detector antibodies of the commercial cTnT assay were incorporated to mimic the working principle of this assay. In a second AMI patient population, we aimed to characterize the changes in the molecular forms of cTnT with time. Serum samples collected for each patient at standardized time points after symptom onset were subjected directly to this Western blotting technique.

Materials and Methods

STUDY POPULATIONS
GFC followed by Western blotting was performed on residual serum samples from 13 patients with AMI followed by rapid revascularization. Blood samples were obtained from the routine laboratory at 2 time points, mean (SD) 3 (1) as well as 15 (2) h after admission to the emergency department of Maastricht University Medical Center, and frozen at −80°C. One serum sample was missing from each of 2 patients with AMI, 1 at 3 h and 1 at 15 h postadmission. As a result, 12 AMI serum samples were analyzed for both time points.

The molecular changes of cTnT with time and their subsequent effect on infarct size estimation were examined in a second well-characterized AMI population. Serum samples of 18 patients with ST-segment elevation myocardial infarction (STEMI) were collected at hospital admission and at standardized time points, 4, 8, 12, 24, 36, 48, 60, and 72 h after hospital admission, and immediately stored at −80°C. These patients were enrolled in the control group of a clinical trial investigating the effects of ventricular pacing during revascularization and thus were receiving standard treatment (http://clinicaltrials.gov/ct2/show/NCT00409604). Inclusion criteria for the study were as follows: age >18 years, presentation with first myocardial infarction, admitted to hospital 3 (1) h from symptom onset, treated with percutaneous coronary intervention (PCI) and exhibiting a typical release pattern of cTnT, creatine kinase (CK), and lactate dehydrogenase (LD).

The study was conducted according to the principles of the Declaration of Helsinki and approved by the local ethics committees. Informed consent was obtained from the patients.

BIOCHEMICAL TESTING

cTnT was measured on the Elecsys 2010 (Roche) using the fourth generation cTnT assay with a limit of detection <0.01 μg/L and 10% CV cutoff at 0.03 μg/L. N-terminal fragment of the prohormone brain-type natriuretic peptide (NT-proBNP) was also measured on the Elecsys 2010 (Roche) with a limit of detection of 5.08 ng/L (0.6 pmol/L) and interassay CV 6.8% of 74.4 ng/L (8.78 pmol/L). Albumin was measured on the Synchro LX20 (Beckman Coulter) using the microalbumin assay with measuring range of 2.0–970 mg/L. CK and LD were measured on the Synchro LX20 (Beckman) as well. cTnI was measured using the Axsym troponin I-Adv assay (Abbott Laboratories) with the 10% CV cutoff at 0.16 μg/L. All assay characteristics were given by the manufacturer.

FRACTIONATION BY GFC

GFC was performed on an HP 1100 system (Agilent Technologies) equipped with a 1.6 × 60–cm Sephacryl-S100 column (GE Healthcare) and a diode array detector. The column was equilibrated with 0.26 mol/L NaCl, 2.5 mmol/L CaCl₂, 10 mmol/L Tris, 6 mmol/L Na₂S, and 1 g/L BSA buffer, pH 7.4, and operated at 0.5 mL/min. The void volume (V₀) determination and calibration was conducted using the Gel Filtration Calibration kit (GE Healthcare) containing Dextran Blue [molecular weight (MW), 2000 kDa], conalbumin (MW, 75.0 kDa), ovalbumin (MW, 43.0 kDa), carbonic anhydrase (MW, 29.0 kDa) and ribonuclease (MW, 13.7 kDa), supplemented with myoglobin (MW, 16.9 kDa; Sigma). Dextran Blue (1 g/L) and globular protein standards (3–4 g/L) were dissolved in running buffer, 0.5 mL was loaded on the column, and absorbance was studied at 280 nm, all in duplo. V₀ was determined to be 37 mL.

In addition, purified human cardiac troponin T-I-C complex (MW, 77 kDa; Hytest) and purified free cTnT, cTnI, and TnC (MW 37, 24, and 18 kDa, respectively; all Advanced ImmunoChemical) were dissolved as prescribed by the manufacturer and were further diluted in running buffer or spiked in serum pool of healthy volunteers (cTnT <0.01 μg/L and cTnI <0.03 μg/L).

To obtain the standard calibration curve, the gel-phase distribution coefficient (Kav = VR/(Vc – V₀)) was plotted against the logarithm of the MW of globular proteins and purified cardiac troponin standards. VR signifies the retention volume adjusted to V₀, and
\( V_c \) indicates column volume and corresponds to 120 mL.

For serum samples, 1.0 mL was loaded on the column. Serum albumin (67 kDa) and serum NT-proBNP were used as internal standards in the patients’ sera.

For each sample loaded, fractions of 1.25 mL were collected, kept on ice until immunoassay measurements (≤1 h), and subsequently stored at −80°C.

**PURIFICATION AND WESTERN BLOT DETECTION OF cTnT**

Purification and characterization of cTnT were performed with the antibodies from the Roche cTnT assay (as kindly provided by Roche Diagnostics, www.roche.com). The capture cTnT antibody (M11.7, epitope a.a.r. 136–147) was used for immunoprecipitation of cTnT. To each milliliter of magnetic streptavidin-coated dynabeads (Invitrogen), 10 μg biotinylated M11.7 was added. Additionally, beads were crosslinked 3 times with dimethyl pimelimidate. Subsequently, 200 μL of serum was precipitated with 50 μL of M11.7-coated beads for 1 h at 4°C. Because of low cTnT concentrations in the GFC fractions, more sample was added to the beads, ranging from 200 to 5000 μL. In some cases, up to 4 fractions were merged to generate larger volumes. The mean cTnT yield after immunoprecipitation was >90% (data not shown). After being washed with PBS containing 0.1% Tween-20 (PBST), the immunoprecipitates were eluted with 1 mol/L glycine (18 μL), pH 3, for 15 min at 56°C (see Fig. 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vo59/issue7).

As negative controls for immunoblotting of GFC samples and AMI patients’ sera, running buffer or the serum pool of healthy volunteers was used, respectively. As positive controls, the cardiac troponin T-I-C complex was spiked in running buffer and cTnT negative pooled serum, respectively. Only GFC fractions containing sufficiently high cTnT concentrations could be visualized on the immunoblot, corresponding to 9 of 12 serum samples collected at ±15 h postadmission.

Immunoprecipitates were diluted with XT sample buffer (6 μL) (Bio-Rad Laboratories), heated for 7 min at 56°C, and separated on a 12% Criterion XT SDS-PAGE gel (Bio-Rad) together with the Precision Plus protein standard (Bio-Rad). After stacking for 15 min at 100 V the proteins were resolved for 45 min at 200 V in XT MOPS electrophoresis buffer (Bio-Rad) and were transferred to the nitrocellulose membrane (Bio-Rad, 0.45 μm) at 100 V for 1 h at 4°C. The membranes were treated with the vacuum SNAP i.d. protein detection system (Millipore) according to manufacturer instructions. Nonspecific binding was blocked with 0.5% nonfat dry milk Blocking-Grade blocker (Bio-Rad) in PBST. Primary and secondary antibodies were Roche M7 anti-cTnT antibody (epitope a.a.r. 125–131; 10 μg/L) and goat anti-mouse peroxidase (0.4 μg/mL; Dako) in PBST, respectively. Subsequently, membranes were incubated for 5 min in Super Signal West Femto substrate (Thermo Scientific) and bands were detected using the ChemiDoc XRS scanner (Bio-Rad) and Quantity One Software (Bio-Rad, version 4.6.5).

**STATISTICAL ANALYSIS**

Results are presented as mean (SD) or when not normally distributed as median [interquartile range (IQR)]. The Wilcoxon signed-rank test was applied to investigate differences in peak-to-peak ratios of the GFC elution profiles obtained 3 and 15 h postadmission. All statistical analyses were performed using SPSS (Statistical Package for Social Sciences; version 18). A P value <0.05 was regarded as statistically significant.

**Results**

**VALIDATION OF cTnT DEGRADATION BY GFC FOLLOWED BY WESTERN BLOTTING**

Elution profiles of globular protein standards (MW varying from 16.9 to 75.0 kDa) and purified cardiac troponin standards (cardiac troponin T-I-C complex, free cTnT, cTnI, and TnC) were determined. A schematic overview of the obtained retention volumes adjusted to \( V_0 \) (\( V_R \)) is given in Fig. 1A. With spiking of the intact cTnT standard in cTnT-negative serum (cTnT, 19.9 μg/L), cTnT elutes with \( V_R \) of 5 mL before the elution of the internal standard serum albumin (\( V_R = 11 \) mL) (see online Supplemental Fig. 2A). Western blotting of these GFC fractions confirmed that this peak corresponded to intact cTnT [estimated MW (MW_adj) of 40 kDa] and for a small part to a 29-kDa cTnT fragment, as shown in online Supplemental Fig. 2B. Identical elution profile and immunoblot analysis results were obtained when the purified cTnT standard was spiked in running buffer (data not shown). For the other purified cardiac troponin standards, \( V_R \) was determined to be 3 mL, 16 mL, and 20 mL for the cardiac troponin T-I-C complex, intact cTnI, and TnC, respectively. (Fig. 1A) Moreover, the correlation between \( V_R \) and MW of the globular proteins can be represented by a linear calibration curve following the equation: \( y = -0.44x + 0.93 \), as depicted by Fig. 1B. In contrast, all cardiac troponin proteins (cardiac troponin T-I-C complex, free cTnT, cTnI, and TnC) deviated clearly from the calibration curve.

Median (IQR) cTnT concentrations measured in serum of AMI patients, subjected to GFC, were 2.17 (5.07) μg/L and 6.94 (6.46) μg/L for 3 and 15 h postadmission, respectively. After GFC separation, overall median (IQR) recovery of loaded cTnT (%) was 79% (27%) (see online Supplemental Table 1). The GFC
elution profiles of each patient showed 2 cTnT peaks at an adjusted \( V_R \) of 5 and 22 mL, eluting before and after the internal standard serum albumin (\( V_R = 11 \) mL). As shown in Fig. 2, A and B, this was observed at 3 as well as 15 h postadmission, respectively. Western blot analysis using the antibodies of the clinical immunoassay identified the first cTnT peak at \( V_R = 5 \) mL as a degradation product of cTnT with an \( M_{\text{West}} \) of 29 kDa (Fig. 2, C and D). The second peak (\( V_R = 22 \) mL) was allocated to smaller cTnT degradation products of \( M_{\text{West}} \) between 14 and 18 kDa. Moreover, there was a significant relative increase in the second peak at 15 h postadmission as compared with 3 h postadmission (median ratio of cTnT peak at 22 mL/5 mL of 0.54 and 1.89 for 3 and 15 h postadmission, respectively; \( P = 0.001 \)), suggesting a different cTnT degradation pattern that changed with time.

**TIME COURSE OF cTnT DEGRADATION AT STANDARDIZED TIME POINTS**

The release kinetics of cTnT, CK, and LD determined in 18 patients with STEMI [66% males with mean(SD) age 60 (9) years] (see online Supplemental Table 2) are shown in Fig. 3A and online Supplemental Figs. 3 and 4, respectively. On average, peak cTnT concentrations were reached 8 h after admission, with a median (IQR) concentration of 6.68 (4.54) \( \mu \)g/L. Furthermore, cTnT concentrations were still increased 72 h postadmission, with a median (IQR) concentration of 2.65 (1.83) \( \mu \)g/L.
Fig. 3B depicts a representative blot of an AMI patient and shows that the antibodies of the clinical assay detected mainly cTnT degradation products in the circulation. As many as 5 different degradation products were reproducibly identified (MW of 14, 16, 18, 27, and 29 kDa). Moreover, a breakdown pattern of cTnT was observed over time. Western blot analysis of all patients showed that intact cTnT (MW of 40 kDa) was present in only 3 patients during the first 8 h after admission (Fig. 3C). Instead, the 29-kDa fragment of cTnT was detected in highest abundance up to 24 h postadmission. Afterward, the larger fragments (MW of 27 and 29 kDa) disappeared, and primarily low MW fragments between 14 and 18 kDa were identified. When blotting was performed with an antibody directed against the C terminus of cTnT, the same degradation pattern for cTnT was visualized (see online Supplemental Fig. 5). On the contrary, when we applied an antibody directed against the N terminus of cTnT, none of the cTnT fragments were detected.

Discussion

The present study shows for the first time that circulating cTnT is highly degraded in cases of AMI followed by rapid revascularization, as demonstrated by fractionation of serum from AMI patients with GFC and Western blotting and the use of the antibodies of the clinical cTnT assay.

GFC enables detailed characterization of noncovalent complexes present in the patient’s serum, but also requires extensive and careful validation. Herein, we report similar elution profiles as previously found by Bates and colleagues (16), although they assigned their main peak to intact cTnT. As determined from the calibration curve obtained with globular protein stan-
standards, the expected adjusted $V_R$ for intact cTnT would be 20 mL. However, in this study we clearly demonstrated that purified cTnT, and also other cardiac troponin molecules, eluted much earlier and therefore deviated from the calibration curve. For this reason, MW assignment of cardiac troponin elution profiles should not be based on the MWs of globular proteins. This is especially important for structures with elongated shapes like cTnT (18, 19). In the present study, we observed that the most abundant form of circulating cTnT detected by the cTnT immunoassay is actually a mixture of cTnT fragments. Previous findings have also led to the postulation that ternary structures of the cardiac troponin complex are the most frequent form present in the patient’s circulation (7, 16, 17, 20). Using the same gel filtration technique (16, 17), we observed that this type of GFC column was not suitable for adequate separation of the ternary-bound cardiac troponin T-I-C complex ($V_R$ = 3 mL) and intact cTnT ($V_R$ = 5 mL) molecules. Therefore, the presence of ternary-bound cardiac troponin molecules in the patient’s circulation requires further study.

Furthermore, sera from patients with AMI, drawn at standardized time points after admission to the emergency department and analyzed directly with Western blot analysis, revealed that cTnT is degraded in a time-dependent pattern. Intact cTnT was the largest cTnT form that could be detected and it was present only during the early hours (4 – 8 h) after admission in 3 of the 18 patients. Primarily 29-kDa cTnT fragments were visible up to 24 h after AMI, corresponding to the peak concentrations of the cTnT release curve and the period of diagnostic testing. Later on, 29-kDa fragments seemed to diminish, and degradation products with a smaller molecular mass (MW low of 14 and 16 kDa) dominated in peripheral blood. The immunoreactivity on the Western blot, however, seemed to differ between intact and low MW cTnT molecules, thereby limiting quantification of Western blot bands.

Because Roche Diagnostics is the only manufacturer of clinical cTnT assays, cTnT degradation does not affect harmonization between different assays, as previously reported for the cTnI assays (11). To ensure quality of the cTnT measurement in blood samples, Roche Diagnostics recommends the use of recombinant human cTnT spiked in a human serum matrix for monitoring the calibration and precision of the assay. However, as shown in the present study, this recombinant intact cTnT is not representative for AMI patient serum, which contains mainly cTnT fragments. Furthermore, although studies have indicated the magnitude of cTnT measurements to be of prognostic value (21–23) and strongly correlated with infarct size (24, 25), whether the detection of different cTnT forms will affect the clinical utility of cTnT measurements has to be examined.

A limitation to this study is that the fourth-generation cTnT assay was used. However, the epitopes of the capture and detection antibodies are the same for the current high-sensitivity cTnT assay (26). Subsequently, the same cTnT forms will be detected by both assays. Because cTnT fragments were observed using 2
different techniques and cTnT degradation exhibited a time-dependent pattern, these fragments are unlikely to have been introduced by our method, and the time-dependent pattern suggests they are the products of an enzymatic process. Moreover, when the purified cardiac troponin T-I-C standard (NIST-SRM 2921) was spiked into serum or heparin plasma, cTnT was shown not to be susceptible to degradation when stored for 72 h at 4 °C or for 24 h at 37 °C (27, 28). It should be noted that the NIST-SRM 2921 standard already contained the 29-kDa fragment of cTnT before incubation. These findings further suggest that the cTnT degradation observed in this study is induced in vivo. In addition, several in vitro studies have demonstrated that the degradation of cTnT occurred upon ischemia, due to the activity of calpain-I (29–31) and caspase-3 (32) in the intracellular environment of cultured cardiomyocytes, possibly leading to the cleavage of the N terminus of cTnT. This all leads to the intriguing question of what modification patterns of cardiac troponin are present when myocardial necrosis is thought to be absent, for instance after physical activity (33, 34), and the subsequent question of whether the diagnostic potential of cTnT can be further improved. Of note, the present study describes cTnT degradation in serum of AMI patients; the presence of cTnT fragments in heparin plasma samples has yet to be investigated.

In conclusion, this study demonstrates that circulating cTnT undergoes degradation in serum following AMI and the degradation pattern is dependent on time after symptom onset. Importantly, this is the first study to reveal that the cTnT immunoassay widely applied in the clinic binds both intact cTnT and degradation products of cTnT during the time frame of diagnostic testing.

### References


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