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Fragmentation of human cardiac troponin I from serum samples of AMI patients

Katrukha I.[1], Vylegzhanina A.[1], Kogan A.[2], Bereznikova A.[2], Koshkina E.[3], Mizov A.[4], Moiseev V.[4], Katrukha A.[1]

1 - HyTest Ltd, Turku, Finland; 2 - School of Biology, Moscow State University, Moscow, Russia; 3 - 67th City Hospital, Moscow, Russia; 4 - 64th City Hospital, Moscow, Russia

Introduction

IMMUNODETECTION OF CARDIAC TROPONIN I (cTNI) in the blood sample is one of the most reliable methods of diagnostics of acute myocardial infarction (AMI). In blood of AMI patients cTnI is present by the intact molecule and a repertoire of its proteolytic fragments. Degradation may have a significant negative influence on the immunodetection of cTnI and determination of its real concentration. In the current work we aimed to border the cTnI fragments, present in blood of AMI patients and study the time-course of cTnI degradation.

Methods

SERIAL SERUM SAMPLES were from 9 patients (all with STEMI followed by coronary stenting) and were collected 4-6, 9-10, 13-19 and 25-36 hours after the onset of chest pain.

MYOCARDIAL EXTRACT was prepared by homogenization of 1 g of postmortem human heart tissue in 3 ml of 20 mM Tris-HCl buffer, 0.15 M KCl, 5 mM $CaCl_2$, 7.5% w/v BSA, pH 7.5, and incubation for 3 h at 37°C under shaking. Then cell debris was removed by centrifugation. This preparation stands for a time point "0 h" and was subsequently incubated at 37°C for up to 48 h. After incubation aliquots were frozen at -70°C until use.

IN VITRO **DEGRADATION OF cTNI** was performed by incubation of AMI serum samples or cTnI (in a form of a ternary ITC complex) spiked into the NHS sample at 37°C for up to 48 h. After the incubation, samples were stored at -70°C until use.

IMMUNOPRECIPITATION of cTnI and its fragments was performed on the Sepharose CL-4B (GE Healthcare) conjugated with mAbs, specific to the different epitopes of cTnI molecule (HyTest).

SEPARATION OF THE PROTEINS was performed by means of 10-20% Tris-Gly SDS PAGE with subsequent electrotransfer onto the nitrocellulose membranes (GE).

IMMUNODETECTION of cTnI and its fragments was performed by means of ECL with 15 HRP-conjugated mAbs, specific to the different epitopes of the cTnI molecule (see Fig. 1).

IMAGE QUANTIFICATION was performed using ImageLab 5.2.1 software (BioRad). To calculate the ratio of the individual band cTnI was stained by mAb 560, specific to the central part of TnI molecule (83-93 aar) and capable of staining all visible fragments of cTnI. The intensity of the band was divided by the total intensity of the stained bands and expressed as percentages.

Results and discussion

STUDY OF cTNI FRAGMENTS FROM THE SERUM SAMPLES OF AMI PATIENTS revealed the presence of the full-sized cTnI and 11 proteolytic fragments. The samples of all patients comprised the same set of proteolytic fragments.

The staining with mAbs specific to the different epitopes of cTnI revealed that the shortest fragment detected by WB was stained by mAb 4C2 from the N-terminus and mAb M46 from the C-terminus and thus bordered by approximately 23-148 aar (Fig. 1).

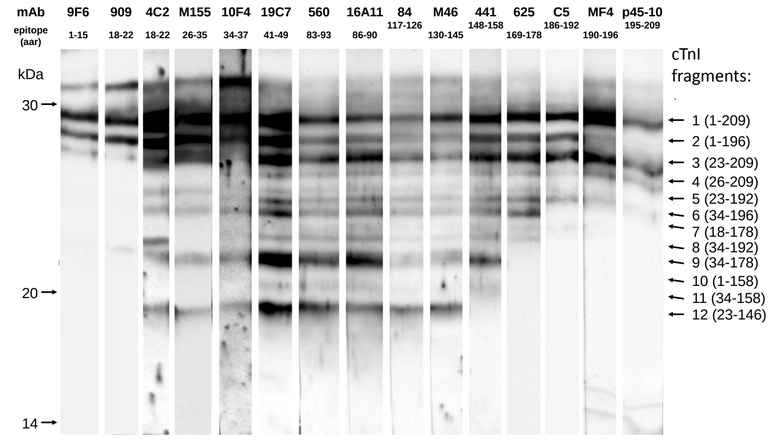

FIGURE 1. **A representative ECL of cTnI and it's fragments immunoprecipitated from serum of an AMI patient and stained by anti-cTnI mAbs, specific to different epitopes.** The approximate borders of each fragment are indicated in brackets on the right. Band 1 stands for the full-sized cTnI.

The analysis of the relative abundance of cTnI and its fragments from the serum samples, collected ~6 hours after the onset of the chest pain and stained by mAb 560, specific to the central part of the molecule revealed that mAbs which epitopes fall within the range 23-196 aar are capable of staining more than 80% of all cTnI from the samples of AMI patients (see Table 1).

TABLE 1. **Relative abundance of cTnI and its fragments in the serum samples of AMI patients (n=9).**

Serum samples collected 4-6 hours after the onset of chest pain. cTnI and its fragments were immunodetected by mAb TnI560, specific to the central part of cTnI molecule (83-93 aar) and capable of staining all visible cTnI fragments.

mAb	Epitope (aar)	Bands that comprise the epitope	Amount of stained cTnI (%)
9F6	1-15	1, 2, 10	49.3±10.4
909	18-22	1, 2, 7, 10	51.5±9.7
4C2	23-29	1, 2, 3, 5, 8, 10, 12	88.3±3.7
M155	26-35	1-4, 6, 8, 10, 12	92.1±3.3
10F4	34-37	1-4, 6, 8, 9, 10, 12	96.6±1.2
19C7, 560, 16A11, 84, M46	37-148	1-12	100
441	148-158	1-11	96.6±1.2
625	169-178	1-9	82.3±5.2
C5	186-192	1-6, 8	80.4±5.7
MF4	190-196	1-5, 7	80,1±5.7
P45-10	195-209	1, 3, 4, 5, 7	72.2±6.3

TIME CHANGE OF cTNI FRAGMENTS' COMPOSITION IN BLOOD OF AMI PATIENTS was studied by WB. The analysis of the abundance of cTnI and its fragments showed no significant changes in the ratio of full-sized cTnI or any of its fragments in the range of 3-36 hours (Fig. 2).

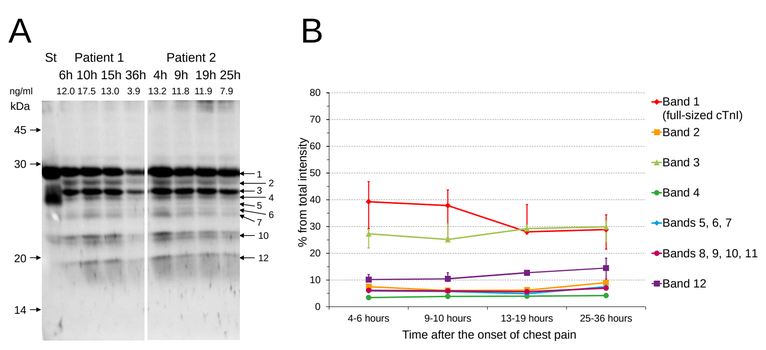

FIGURE 2. **Time change of cTnI fragments' ratio in serum samples of AMI patients.**

A – a representative ECL demonstrating abundance of cTnI and its fragments in serial blood samples. Staining with mAb 560.
B – median values of the ratio of different cTnI fragments from serum samples of 9 AMI patients. Whiskers are shown for the ratio of the three most abundant bands (Band 1, Band 3 and Band 12) and represent 25-th and 75-th percentiles.

IN VITRO **DEGRADATION OF cTNI** was performed by incubation of myocardial extract and serum samples of AMI patients at 37°C for up to 48 hours. Incubation of myocardial extract showed the substantial decrease of the ratio of full-sized cTnI and its 25-29 kDa fragments, while the ratio of a low-molecular weight fragments increased in time (Fig. 3A and B). In contrast, incubation of the serum samples of AMI patients (containing endogenous cTnI) resulted in no changing of the ratio of different cTnI fragments (Fig. 4D and E). The same results were obtained for a recombinant TnI used as a control (Fig. 4C). These results suggest that most likely, no degradation of cTnI takes place in serum, thus necrotic myocardium is the only place where cTnI is proteolytically cleaved.

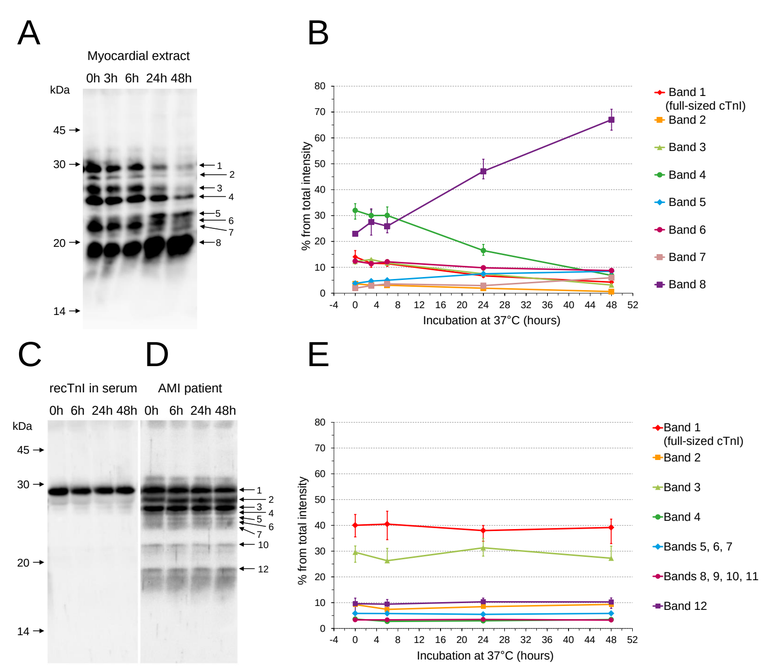

FIGURE 3. ***In vitro*** **degradation of cTnI in myocardial extract and serum samples.** Incubation at 37°C. Staining with mAb 560.

A – cTnI and its fragments from myocardial extract.
B – Median ratio values of different cTnI fragments obtained during *in vitro* incubation of myocardial extract. Whiskers represent 25th and 75th percentiles for 3 most abundant bands.
C – recTnI (50 ng/ml) incubated in normal human serum.
D – A representative ECL of an AMI patient serum sample incubated *in vitro*.
E - Median ratio values of different fragments detected after *in vitro* incubation of serum samples of 3 AMI patients. Whiskers represent 25th and 75th percentiles for 3 most abundant bands.

Conclusions

1. In AMI blood cTnI is presented by the full-sized molecule and 11 major proteolytic fragments. The set of cTnI fragments is the same in the blood of all studied patients.

2. The ratio of cTnI fragments remained almost unchanged in time (6-36 hours after the onset of the chest pain).

3. More than 80% (by abundance) of studied cTnI fragments include amino acid residues 23-196, so antibodies, specific to this region, could be utilized in diagnostic systems.

4. Degradation of cTnI occurs mainly in the necrotic cardiac tissue, but not in blood.

HyTest Ltd | Intelligate 1, 6th floor, Joukahaisenkatu 6, FI-20520 Turku, FINLAND | Tel. +358 2 512 0900, Fax +358 2 512 0909, e-mail: hytest@hytest.fi | **www.hytest.fi**