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Cardiac troponin in ischemic cardiomyocytes: Intracellular decrease before onset of cell death

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ABSTRACT

Aim: Cardiac troponin I (cTnI) and T (cTnT) are the most important biomarkers in the diagnosis of acute myocardial infarction (AMI). Nevertheless, they can be elevated in the absence of AMI. It is unclear if such elevations represent irreversible cardiomyocyte-damage or leakage from viable cardiomyocytes. Our objective is to evaluate whether cTn is released from viable cardiomyocytes in response to ischemia and to identify differences in the release of cTn and its molecular forms.
Methods and results: HL-1 cardiomyocytes (mouse) were subjected to ischemia (modeled by anoxia with glucose deprivation). The total contents and molecular forms of cTn were determined in culture media and cell lysates. Cell viability was assessed from the release of lactate dehydrogenase (LDH). Before the release of LDH, the intracellular cTn content in ischemic cells decreased significantly compared to control (52% for cTnI; 23% for cTnT) and was not matched by a cTn increase in the medium. cTnI decreased more rapidly than cTnT, resulting in an intracellular cTnT/cTnI ratio of 25.5 after 24 h of ischemia. Western blots revealed changes in the relative amounts of fragmented cTnI and cTnT in ischemic cells.
Conclusions: HL-1 cardiomyocytes subjected to simulated ischemia released cTnI and cTnT only in combination with the release of LDH. We find no evidence of cTn release from viable cardiomyocytes, but did observe a significant decrease in cTn content, before the onset of cell death. Intracellular decrease of cTn in viable cardiomyocytes can have important consequences for the interpretation of cTn values in clinical practice.

Introduction

The cardiac troponin (cTn) complex is a heteromeric protein complex playing an important role in the regulation of cardiac muscle contraction and consists of three different subunits: cardiac troponin I (cTnI), T (cTnT) and C (cTnC). The cTn T–I–C-complex is predominantly structurally bound to the myofibrils with only a minor fraction of cTnT and cTnI (6–8%) present free and unbound in the cytoplasm as soluble intact protein (Bleier et al., 1998; Katus et al., 1991). Due to their cardiac specificity, cTnI and cTnT are accurate and sensitive markers of cardiac injury and the most important biochemical markers used in the diagnosis of acute myocardial infarction (AMI) (Morrow et al., 2007). Nevertheless, the cardiac troponins (cTns) have been reported to be elevated in the absence of AMI, in situations where irreversible cardiomyocyte damage is unlikely to play an important role (Hamm et al., 2002; Kelley et al., 2009), such as seen in subjects after strenuous exercise (Fortescue et al., 2007; Michielsen et al., 2008; Mingels et al., 2009).

It has been hypothesized that the elevated levels of cTn seen after exercise are the result of a transient increase in the cardiomyocyte membrane permeability, resulting in the release of cTn from the cytosolic cTn pool of cardiomyocytes (Neumayr et al., 2002, 2005; Remppis et al., 1995; Shave et al., 2007). In contrast, irreversible cellular damage, as observed after AMI or myocardial ischemia, will result in the release of both cytosolic and structurally bound cTn (and its complexes) from disintegrating myofibrils. AMI patients undergoing rapid reperfusion demonstrate a first cTnT peak concentration within 24 h after onset of symptoms, which has been attributed to the fast release of cytosolic cTnT (Bleier et al., 1998; Wu et al., 1998). This peak is followed by a second and persistent cTnT elevation which remains present for 7–14 days and is generally thought to represent the

Abbreviations: AMI, acute myocardial infarction; cTnI, cardiac troponin I; cTnT, cardiac troponin T; DMEM, Dulbecco's modified eagle medium; LDH, lactate dehydrogenase; mAb, monoclonal antibody; PBS, phosphate buffered saline.

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relatively slow dissociation of cTnT from the sarcomeres and release from the cells during necrosis. This biphasic release pattern has not been shown for cTnI, which release is characterized by an initial rise, directly followed by a gradual decrease to undetectable levels.

In addition to different release kinetics between cardiomyocytes with increased membrane permeability (reversible damage) and irreversible damage, there may also be a difference in released molecular forms of cTn. The cTns are thought to be targets for proteases such as caspase (Communal et al., 2002; Lancel et al., 2005) and calpain (Barta et al., 2005; Ke et al., 2008; Kositprapa et al., 2000), which are being activated and released during cell-death. Therefore, cTn is expected to be released partly fragmented from irreversibly damaged cells, whereas cytosolic cTn released after reversible damage will be predominantly intact. Moreover, the complexed (myofibril bound) forms of cTn might be more susceptible to proteolytic degradation than the free forms, as illustrated by Communal et al., who found that caspase-3 cleaves cTnT when it is in complex with cTnI and cTnC, but not as free cTnT (Communal et al., 2002).

Hessel et al. showed the release of intact cTnI from viable cardiomyocytes by stimulation of stretch-responsive integrins (Hessel et al., 2008a), a model in which irreversible damage is unlikely to occur. Conversely, irreversible cardiomyocyte damage, induced by metabolic inhibition with sodium azide, induced the simultaneous release of intact and fragmented forms of both cTnI and cTnT (Hessel et al., 2008a,b; Li et al., 2004). In a recent study, Cardinaels et al. clearly showed time-related degradation of cTnT after myocardial infarction (Cardinaels et al., 2013). Intact cTnT rapidly disappears from the serum after the ischemic event and progressively smaller cTnT fragments appear in time. Degradation and changes in the molecular forms of cTn may have consequences for the immunoreactivity of the antibodies used in the various clinical assays. Assays based on different antibodies may therefore generate different results when measuring cTn concentrations in the same serum sample, which complicates the clinical interpretation of those measurements.

In this *in vitro* study we investigated the release kinetics of cTn and its molecular fragments in cell culture using true conditions of ischemia. This was achieved by subjecting HL-1 cardiomyocytes to a complete anoxic environment with glucose deprivation. The HL-1 atrial cardiomyocyte cell line can contract in solution, maintains a differentiated adult cardiac phenotype up to at least 240 passages and can be recovered from frozen stocks (Claycomb et al., 1998). The aims of our study are, firstly, to investigate whether cTn can be released from ischemic, but viable, cardiomyocytes and secondly, to analyze the molecular forms of the cTns released from cells subjected to ischemia.

Methods

Cell culture of HL-1 atrial cardiomyocytes

HL-1 cardiomyocytes (derived from an AT-1 mouse atrial cardiomyocyte tumor lineage) were kindly provided by Dr. W. Claycomb (Louisiana State University, New Orleans, LA, USA), and cultured as described previously (Claycomb et al., 1998; Schwenk et al., 2010). Briefly, the cells were grown in Claycomb medium (Claycomb et al., 1998) supplemented with 10% Fetal Bovine Serum (FBS), 0.1 mM norepinephrine, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C and at an atmosphere of 5% CO_2 and 95% air. For routine passaging, the cells were maintained in T-75 flasks and split twice a week after reaching confluence. For each of our experimental conditions the cells were seeded onto 6-well culture plates at a density of 100,000 cells/cm^2 and grown for 24 h in supplemented Claycomb medium prior to the experimental treatment. The same batch of cells was used for all time points and for both the anoxia and control experiments. The medium used for the experimental treatment of the HL-1 cells consisted of Dulbecco's modified eagle medium (DMEM), supplemented with 2 mM L-glutamine, 100 μM non-essential amino-acids (NEAA), 0.5% albumin, 100 U/mL penicillin, 100 μg/mL streptomycin; with or without 4.5 g/L glucose.

Ischemia was modeled by anoxia and glucose deprivation. At the start of our experiment (t = 0), Claycomb medium was replaced with supplemented DMEM without glucose and the cells were immediately placed in an anoxic environment (MACS VA500 microaerophilic workstation, Don Whitley Scientific, Shipley, UK). The atmosphere in the chamber consisted of 5% CO_2, 5% H_2 and residual N_2. Anoxia treatment is performed for 1, 3, 5, 7, 9, 12 and 24 h.

As a control, non-treated HL-1 cells grown for 0, 1, 3, 5, 7, 9, 12 and 24 h in supplemented DMEM with glucose were grown alongside the ischemic cells. Fig. 1 depicts the workflow used in the cell culture experiments from routine cell passaging to the storage of individual medium and lysate samples.

Sample collection and cTn measurements

After each treatment period, the culture medium was collected and centrifuged for 5 min at 500 RCF and the supernatant was stored at −80 °C. Immediately after medium removal the cells were washed twice with cold (4 °C) phosphate buffered saline (PBS) and 600 μL cold (4 °C) RIPA lysis buffer, containing 150 mM NaCl, 50 mM Tris–HCl, 1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and 4% (v/v) complete protease inhibitor cocktail and 5% (v/v) PhosSTOP phosphatase inhibitor cocktail was added to each well. The cells were lysed for 2 h with mild agitation at 4 °C. Resulting cell lysates were stored at −80 °C until analysis.

Troponin concentrations were measured in DMEM and RIPA buffer aliquots. cTnT concentrations were measured using the 4th generation cTnT immunoassay on the Elecsys 2010 instrument (Roche Diagnostics, Mannheim, Germany), with a limit of detection (LOD) of <0.01 μg/L, a 10% CV of 0.03 μg/L and a linear measuring range of 0.010–25.00 μg/L, according to the product insert. cTnI concentrations were measured using the AxSYM® Troponin-I ADV assay (Abbott Diagnostics, Wiesbaden, Germany), with an LOD of 0.02 μg/L, a 10% CV of 0.16 μg/L and a linear measuring range of 0.02–22.78 μg/L.

Cell death

The release of lactate dehydrogenase (LDH) into the culture medium was used to quantify cell death. The cytosolic protein LDH is released from cells exhibiting a loss of membrane integrity as seen during primary and secondary necrosis and its release is commonly used to quantify cell-death (Hessel et al., 2008b; Seymour et al., 2003). LDH activity was determined with the LD-P lactate dehydrogenase assay on the Synchon LX20 pro clinical system (Beckman Coulter Inc., Palo Alto, CA, USA), with a linear measuring range of 20–2500 IU/L and a 5.3% CV of 22.5 IU/L. The release of LDH is expressed as the LDH activity in the culture medium as a percentage of the total LDH in the well:

$$LDH_{medium} / \left(LDH_{medium} + LDH_{lysates}\right) * 100\%.$$

In addition to the LDH release, the amount of viable cells was also estimated by measuring the total protein content in the collected cell lysates with the BCA (bicinchoninic acid) Protein Assay Reagent (Thermo Fisher Scientific, Inc., MA, USA), according to the manufacturer's instructions.

Validation of protein measurements

The clinical assays used to measure protein concentrations are optimized for the use in human serum. In order to exclude any matrix-effects from influencing our measurements, we validated their use in DMEM and RIPA buffer. Fig. 2 shows LDH (a), cTnI (b) and cTnT (c) concentration curves of untreated cell lysates serially diluted in either