Release characteristics of cardiac proteins after reversible or irreversible myocardial damage

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Chapter 2

Partial and delayed release of troponin-I compared with the release of lactate dehydrogenase from necrotic cardiomyocytes

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Abstract

Background: Although the troponins are the serum proteins most frequently used nowadays to diagnose myocardial infarction, controversy continues about whether troponins are released later from infarcted myocardium than the cytoplasmic enzymes used previously, like lactate dehydrogenase (LDH). The present study compared the release kinetics of troponin-I (TnI) and LDH from necrotic cardiomyocytes *in vitro*.
Methods: Cardiomyocytes prepared from neonatal rat ventricles were grown for 3 days. A total of 126 cultures were subjected to metabolic inhibition to induce cell necrosis. At various time intervals cells and media were collected for quantitative analysis of LDH activity and TnI concentration.
Results: Mean (±SD) LDH activity and TnI content of nine cultures at time t=0 were 2.07 ± 0.30 U and 1.52±0.30 µg per culture, respectively. Release of LDH from necrotic cardiomyocytes preceded release of TnI by about 60 min. The quantity of LDH released from the cultures after 210 min was 83.2±10.0%, whereas quantity of TnI released after 210 min was always less (33.8±22.2%). Cytochemical assessment of necrotic cardiomyocytes showed TnI-positive cells that were poor in LDH.
Conclusion: The delay of TnI release relative to LDH release may be explained by slow dissociation of TnI molecules from myofilaments and/or formation of TnI degradation products that are undetected by currently used ELISA assay.

Keywords

cardiomyocytes, necrosis, lactate dehydrogenase, troponin-I

Introduction

For about 50 years it is known that cytoplasmic proteins are released from cardiomyocytes upon necrosis. The proteins reported to become released upon necrosis were aspartate aminotransferase (ASAT)[1], lactate dehydrogenase (LDH)[2], isoenzymes of LDH, particularly LDH-1 and LDH-2[3], α-hydroxybutyrate dehydrogenase (αHBDH)[4], creatine kinase (CK)[5], the MB-isoenzyme of CK (CK-MB)[6] and, recently, the troponins, i.e. troponin-T (TnT)[7] and troponin-I (TnI)[8]. The impressive improvement of sensitivity and specificity of myocardial injury markers in the past 50 years has led to the consensus that TnT and TnI in plasma or serum should be measured to detect acute myocardial infarction (AMI) when anamnestic and electrocardiographic data are insufficient to diagnose AMI[9].

The troponins are bound, via tropomyosin, to the actin filaments of the sarcomeres. Only a small proportion (3-6%) of the troponins, but 100% of LDH, is found in the soluble cytoplasmic pool[10-13]. In patients with AMI, serum levels of both troponins remain elevated long after most other markers have normalized. This is probably not due to the slow clearance rate of troponins, but rather due to slow release kinetics, as the majority of troponin molecules must dissociate from the actin filaments[11]. The release kinetics of TnT in patients with AMI often result in a biphasic plasma concentration, particularly in patients with early reperfusion after thrombolytic therapy[10;12;14]. Despite the high sensitivity and specificity of cardiac troponins as markers of myocardial injury, the early increase of troponins in plasma is slower than the early increase of CK-MB, suggesting that the troponins are not excellent early markers for excluding AMI, particularly in patients presenting with non-ST-segment acute coronary syndromes[15-18].

We studied the release kinetics of cardiac troponin I (cTnI) from cultured cardiomyocytes and compared them with those of LDH as described earlier[19-22]. One of the advantages of this model system is the absence of protein clearance from this system. Our hypothesis was two-fold: that TnI is released from injured cardiomyocytes later than LDH, and that the relative quantity of cTnI release is lower than that of LDH. The model system consisted of neonatal rat ventricular cardiomyocytes in culture that were subjected to metabolic inhibition to induce cardiomyocyte necrosis. This preparation and incubation appeared very useful to unravel molecular mechanisms in metabolically

deprived cardiomyocytes[20;21]. An enzyme-linked immunosorbent assay (ELISA) was developed that captures TnI at aminoacids 40-50 and detects TnI at amino acids 189-195, thereby quantifying intact TnI molecules in the cells and in the medium. The severity of metabolic inhibition was varied by incubating the cardiomyocytes with varying concentrations of NaCN and 2-deoxyglucose (DOG), and in varying medium [Ca^{2+}]. As TnI may be a target for cellular proteases like calpain[23], the effects of calpain inhibitors on the release of intact TnI were also addressed. To this end, cardiomyocytes were preincubated with the protease inhibitors, calpain-I-inhibitor and leupeptin.

Materials and methods

Primary cultures of neonatal cardiomyocytes

Hearts were dissected aseptically from anaesthetized, 2-day-old Wistar male rats and transferred to a solution containing (in mmol/L) NaCl (137), KCl (5.4), Na_2HPO_4 (0.34), KH_2PO_4 (0.44), D-glucose (5.6), HEPES (20), 0.02% Phenol Red, pH 7.4. The ventricles were separated from the atria and minced into small fragments. These were further dissociated by incubating twice with an enzyme solution (containing 450 U/mL collagenase, type I CLS, Worthington) in a shaking water bath at 37°C for 20 min. The cell solution was put on ice for 15 min and centrifuged for 15 min (250g). The cell pellet was resuspended in growth medium containing Ham's F10 (Flow Laboratories), 10% fetal bovine serum (FBS, Invitrogen), 10% horse serum (HS, Invitrogen), 100 U/mL penicillin (Yamanouchi Pharma) and 100 µg/mL streptomycin (Radiumfarma-Fisiofarma). The cells were seeded in 6-cm diameter primaria-coated plastic culture dishes (Falcon, Becton Dickinson). After 45 min, non-myocytes (mainly fibroblasts) had attached to the culture dishes and non-adherent cells (representing the cardiomyocytes) were transferred to primaria-coated plastic culture dishes and incubated in a humidified incubator at 37°C and 5% CO_2. Culture medium was refreshed after 8 h by a 1:1 mixture of DMEM (Invitrogen) and Ham's F10 supplemented with 5% HS, penicillin (100 U/mL) and streptomycin (100 µg/mL) and 24 h later by the same growth medium. Three days of culture yielded a monolayer of spontaneously beating myocytes. The investigations had the approval of the Animal Experiments Committee of the LUMC, according to Dutch law.

Metabolic inhibition of cardiomyocytes

In each experiment, eight cultures of neonatal rat ventricular myocytes underwent metabolic inhibition by incubation in HEPES-buffered salt solution (in mmol/L): NaCl (125), KCl (5), $MgSO_4$ (1), KH_2PO_4 (1), $CaCl_2$ (2.5), $NaHCO_3$ (10), HEPES (20), pH 7.4 at 37°C to which NaCN and DOG were added at 2/4, 5/10 and 10/20 mmol/L (NaCN/DOG), for 0, 30, 60, 90, 120, 150, 180, and 210 min, at 37°C. In a separate series of experiments, the $CaCl_2$ concentration of HEPES-buffered salt solution containing 5 mmol/L NaCN and 10 mmol/L DOG was varied between 0 and 6 mmol/L. We varied the concentrations of metabolic inhibitors and extracellular Ca^{2+} to vary the severity of the insult, leading to early/late onset of irreversible cell damage and consequent protein release.

In two experiments cardiomyocytes were preincubated for 1 h in HEPES-buffered salt solution containing 10 µmol/L calpain-I-inhibitor (Roche) and 10 µmol/L leupeptin (Roche), and subsequently incubated in in HEPES-buffered salt solution containing 5 mmol/L NaCN, 10 mmol/L DOG and 2.5 mmol/L Ca^{2+}. At the end of a period of metabolic inhibition, medium (total volume 3 mL) was separated from the cells. Medium samples were kept on ice before assay of LDH activity on the same day, and then stored at -20°C until cTnI assay. To the cells, 1.5 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% ($^{v}/_{v}$) Triton X-100 and 10% ($^{v}/_{v}$) glycerol) was added per dish, the cells were scraped off, and transferred to small tubes kept on ice. The LDH activity was determined at the same day. Aliquots were stored at -20°C until cTnI assay. In 7 experimental series, 16 experiments have been done using a total of 126 cultures.

LDH activity assay

Loss of cardiomyocyte viability was quantified by measuring lactate dehydrogenase (LDH) activity released from the cells into the medium. At various time points of metabolic inhibition, LDH activities of medium and cell samples were determined using at 25°C in a spectrophotometer (Ultrospec 3000, Pharmacia Biotech)[2]. For each culture tested, the LDH activities in 3 mL medium and 1.5 mL cell extract were calculated. The percentage activity of LDH released from each culture was thus $[LDH_{medium}/(LDH_{medium} + LDH_{cells})]*100\%$. The detection limit of the LDH assay was 5 mU/mL, the intra-assay

variability was 3% (110 mU/mL) and 6% (48 mU/mL), and inter-assay variability was 12 % (110 mU/mL).

Troponin-I ELISA

Purified human cTnI (Calbiochem, 648480) was dissolved in urea/Tris buffer according to the manufacturer's instructions. This standard solution was kept in small tubes at -20°C. Monoclonal mouse anti-cTnI clone 19C7 (directed against amino acid sequence 40-50) and monoclonal mouse anti-cTnI clone 6F9 (directed against amino acid sequence 189-195) were purchased from HyTest.

The ELISA was based on the sandwich principle. The 96-well plates (Greiner Bio-one, 655092) were coated overnight at 4°C with the anti-cTnI-Ab clone 19C7 as capture antibody (concentration 2 µg/mL in 0.1 mol/L $NaHCO_3$, 0.5 mol/L NaCl, pH 9.0; 100 µL/well). Non-specific binding was blocked with chicken ovalbumin (10 mg/mL dilution buffer, 200 µL/well) to the wells for 1 h at room temperature. cTnI standard and samples of medium and cell extracts were added at different dilutions (100 µL/well) and incubated for 2 h at room temperature. The dilution buffer consisted of 50 mmol/L Tris-HCl, 100 mmol/L NaCl, and 0.1% ($^{v}/_{v}$) Tween-20, pH 7.4. Next, biotinylated monoclonal anti-cTnI-Ab clone 6F9 (concentration 2 µg/mL, 100 µL/well) was added and incubated for 2 h at room temperature. Finally, streptavidin conjugated to horseradish peroxidase (0.25 µg/mL, 100 µL/well, Pierce, 21126) was added, followed 1 h later by substrate solution, containing 0.4 mmol/L 3,3',5,5'-tetramethyl benzidine (TMB), 0.003% ($^{v}/_{v}$) H_2O_2, and 0.1 mol/L sodium-acetate, pH 5.5. Immediately thereafter, absorbances were read in a 96-well spectrophotometer (Spectra Thermo, Tecan) at 630 nm in the kinetic mode, providing data of initial rate of absorbance increase. The standards used ranged from 0 – 200 ng/mL. Between different incubation steps wells were washed with dilution buffer. Data were analysed and calculated in Excel (Microsoft). After correction for dilution the TnI contents of 3 mL medium and 1.5 mL cell extract were calculated. The percentage activity of TnI released from each culture was thus $[TnI_{medium}/(TnI_{medium} + TnI_{cells})]*100\%$.

The detection limit of the assay was 6 ng/mL. Inter-assay variability was 3% at 200 ng/mL and increased to 15% at 6.25 ng/mL. The intra-assay variability was 6% for 100 and 200 ng/mL TnI.

Cytochemistry

Cardiomyocytes were cultured on glass coverslips (diameter 25 mm) precoated with collagen type VII (Sigma) in plastic pertri dishes (35 x 10 mm, Falcon, Becton Dickinson) for 3 days. Cultures were subjected to metabolic inhibition by exposure to 5 mmol/L NaCN and 10 mmol/L DOG for 150 min, or incubation in HEPES-buffered salt solution for 150 min (control). After washing in phosphate-buffered saline (PBS) containing 1% FBS, the cultures were incubated in LDH staining solution, consisting of (in mmol/L) Tris-HCl (10), NaCl (150), and 0.05% ($^{v}/_{v}$) Tween-20, lactate (10), NAD^+ (5), 25 g/L nitroblue tetrazolium (Sigma), 10 mg/L 1-methoxyphenazine methosulfate (Serva), pH 8.0, for 2 h at 37°C. The NADH formed by LDH activity reduces nitroblue tetrazolium (yellow) to a blue-colored formazan. Next, the cultures were washed with PBS plus 1% FBS, and fixed in PBS plus 1% formalin for 1 h. After washing with PBS plus 1% FBS, the cultures were incubated in ice-cold PBS plus 1% FBS and rabbit polyclonal anti-cTnI antibody (clone 4T21/2, Hytest, 1:200) for 12 h at 4°C. After washing twice with PBS plus 1% FBS, the cultures were incubated with PBS plus 1% FBS and anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC, F9887, Sigma, 1:200) for 30 min at 4°C. Then cover-slips were washed twice with PBS plus 1% FBS, and mounted on a glass slide for microscopy using Vectashield (Vector Laboratories). Light microscopy of LDH-positive (blue) cells and fluorescence microscopy of TnI-positive cells (green) were performed at the same microscope (Eclipse E800, Nikon) with a 10x objective and a digital image-acquisition system. Shutter speeds and diaphragm openings were identical when imaging metabolically inhibited cells and control cells. No image enhancement techniques were employed.

Statistics

Where appropriate, means± SD are reported. Non-linear correlation analysis was performed with Prism (GraphPad software).

Results

LDH activity and TnI content of cardiomyocyte cultures

The mean LDH activity and TnI content at time t=0 were 2.07 ± 0.30 U and 1.52 ± 0.30 µg per culture, respectively (n= 9 cultures).

LDH release during metabolic inhibition

Release of LDH from cardiomyocytes incubated with 5 mmol/L NaCN and 10 mmol/L DOG was dependent upon the extracellular [Ca^{2+}] (Fig. 1a). The higher the latter, the earlier the onset of LDH release and the time to 50% LDH release.

At an extracellular [Ca^{2+}] of 2.5 mmol/L, LDH release was dependent upon the concentrations of metabolic inhibitors (Fig. 1b). The higher the concentrations of NaCN and DOG, the earlier the onset of LDH release and the time to 50% LDH release. Cardiomyocytes not incubated with metabolic inhibitors did not release significant amounts of LDH over 210 min.

LDH release from cardiomyocytes preincubated with calpain inhibitors and incubated with NaCN (5 mmol/L), DOG (10 mmol/L) and 2.5 mmol/L extracellular Ca^{2+} was similar to that of cardiomyocytes not preincubated with calpain inhibitors (Fig. 1c).

TnI release during metabolic inhibition

Release of TnI from cardiomyocytes incubated in the presence of 5 mmol/L NaCN and 10 mmol/L DOG was dependent upon the extracellular [Ca^{2+}] (Fig 2a). The onset of TnI release occurred roughly 60 min later than the onset of LDH release under identical incubation conditions.

At an extracellular [Ca^{2+}] of 2.5 mmol/L, TnI release was dependent upon the concentrations of metabolic inhibitors (Fig. 2b). Cardiomyocytes not incubated with metabolic inhibitors did not release significant amounts of TnI in 180 min.

TnI release from cardiomyocytes during metabolic inhibition following preincubation with calpain inhibitors was similar to that of cardiomyocytes not preincubated with calpain inhibitors (Fig. 2c).

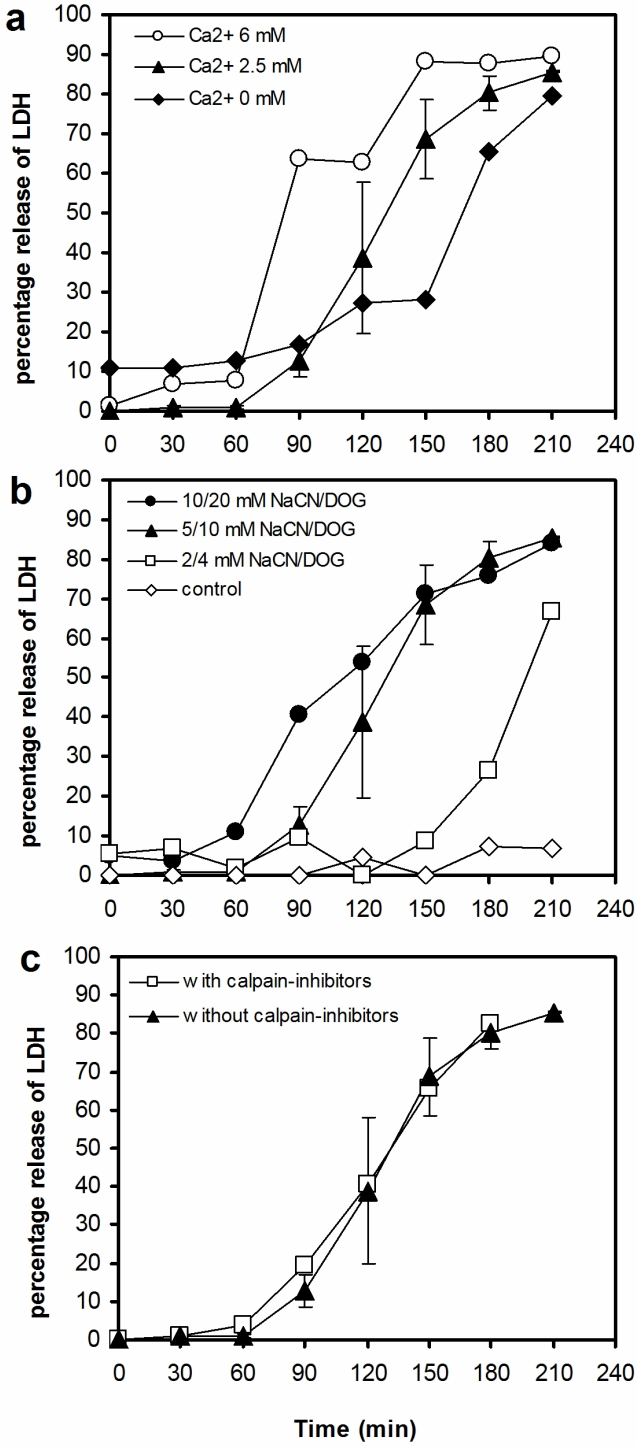

Figure 1. Time-dependent release of LDH from cardiomyocyte cultures incubated with 5 mM NaCN and 10 mM 2-deoxyglucose (DOG) with several extracellular Ca^{2+} concentrations (0, 2.5, and 6 mM) (n=2, 6 and 2, resp.) **(a)**, with NaCN and DOG at several concentrations (mM NaCN/mM DOG being 0/0, 2/4, 5/10, and 10/20) and an extracellular Ca^{2+} concentration of 2.5 mM (n=1, 1, 6 and 2, resp.) **(b)**, and with 5 mM NaCN, 10 mM DOG and 2.5 mM extracellular Ca^{2+} following 1 hour preincubation with 10 µM calpain-I-inhibitor and 10 µM leupeptin (n=2)**(c)**. The LDH activity of a culture at t=0 is 2.07 ± 0.30 U.

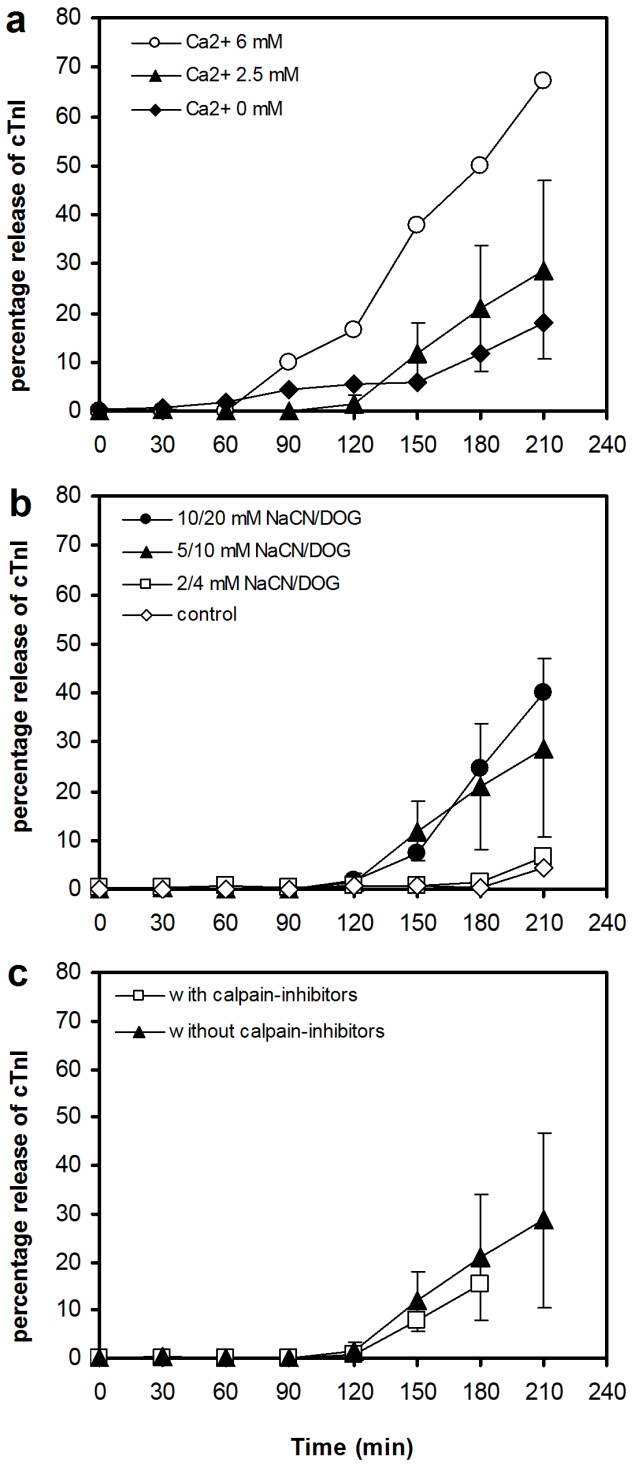

Figure 2. Time-dependent release of TnI from cardiomyocyte cultures incubated with 5 mM NaCN and 10 mM 2-deoxyglucose (DOG) with several extracellular Ca^{2+} concentrations (0, 2.5, and 6 mM) (n=2, 6 and 2, resp.) **(a)**, with NaCN and DOG at several concentrations (mM NaCN/mM DOG being 0/0, 2/4, 5/10, and 10/20) and an extracellular Ca^{2+} concentration of 2.5 mM mM (n=1, 1, 6 and 2, resp.) **(b)**, and with 5 mM NaCN, 10 mM DOG and 2.5 mM extracellular Ca^{2+} following 1 hour preincubation with 10 µM calpain-I-inhibitor and 10 µM leupeptin (n=2) **(c)**. The TnI content of a culture at t=0 is 1.52 ± 0.30 µg.

Relationship between release of TnI and release of LDH

Figure 3 shows the percentage release of TnI released as a function of the percentage of LDH release for all cultures preincubated without or with calpain inhibitors and incubated with various concentrations of NaCN, DOG and extracellular Ca^{2+}. Non-linear correlation analysis showed a a highly significant relationship ($r=0.946$: $p<0.0001$, $n=55$). It is evident that the first 60% of LDH release is associated with hardly any TnI release. TnI release thus appears to be delayed compared with LDH release. Once substantial amounts of LDH had been released (about 60% or higher), TnI release followed, suggesting that a considerable part of TnI release occured from cardiomyocytes that have been dead for some time already. Pooling the data from all experiments with metabolic inhibition, the quantity of LDH released from the cultures after 210 min was 83.2 ± 10.0%, whereas the quantity of TnI released after 210 min was always less (33.8 ± 22.2%).

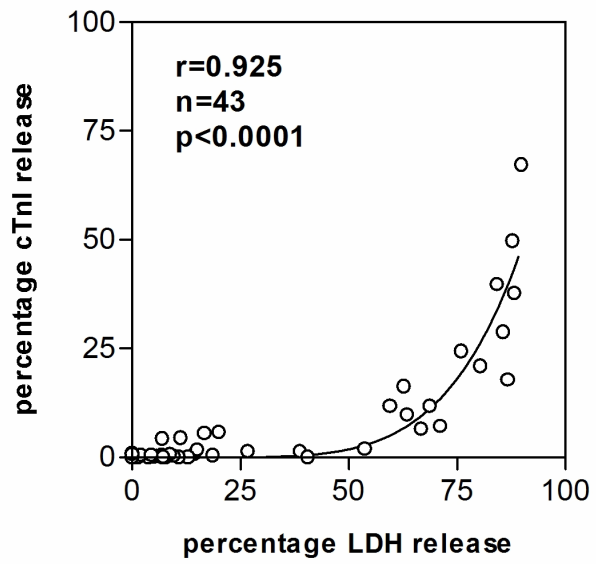

Figure 3. Relation between TnI release and LDH release from several series of preincubation without or with calpain inhibitors and incubations with metabolic inhibitors and extracellular Ca^{2+} concentrations at various time points. Non-linear correlation analysis demonstrated a highly significant correlation.

Cytochemical analysis

Healthy cardiomyocytes in culture stained positively for TnI (by fluorescence microscopy) and for LDH (by light microscopy) (Fig. 4, left four panels). Cardiomyocytes that had been incubated with metabolic inhibitors (5 mmol/L NaCN and 10 mmol/L DOG with 2.5 mmol/L extracellular Ca^{2+}) for 150 min still stained positively for TnI, but poorly for LDH (Fig. 4, right four panels).

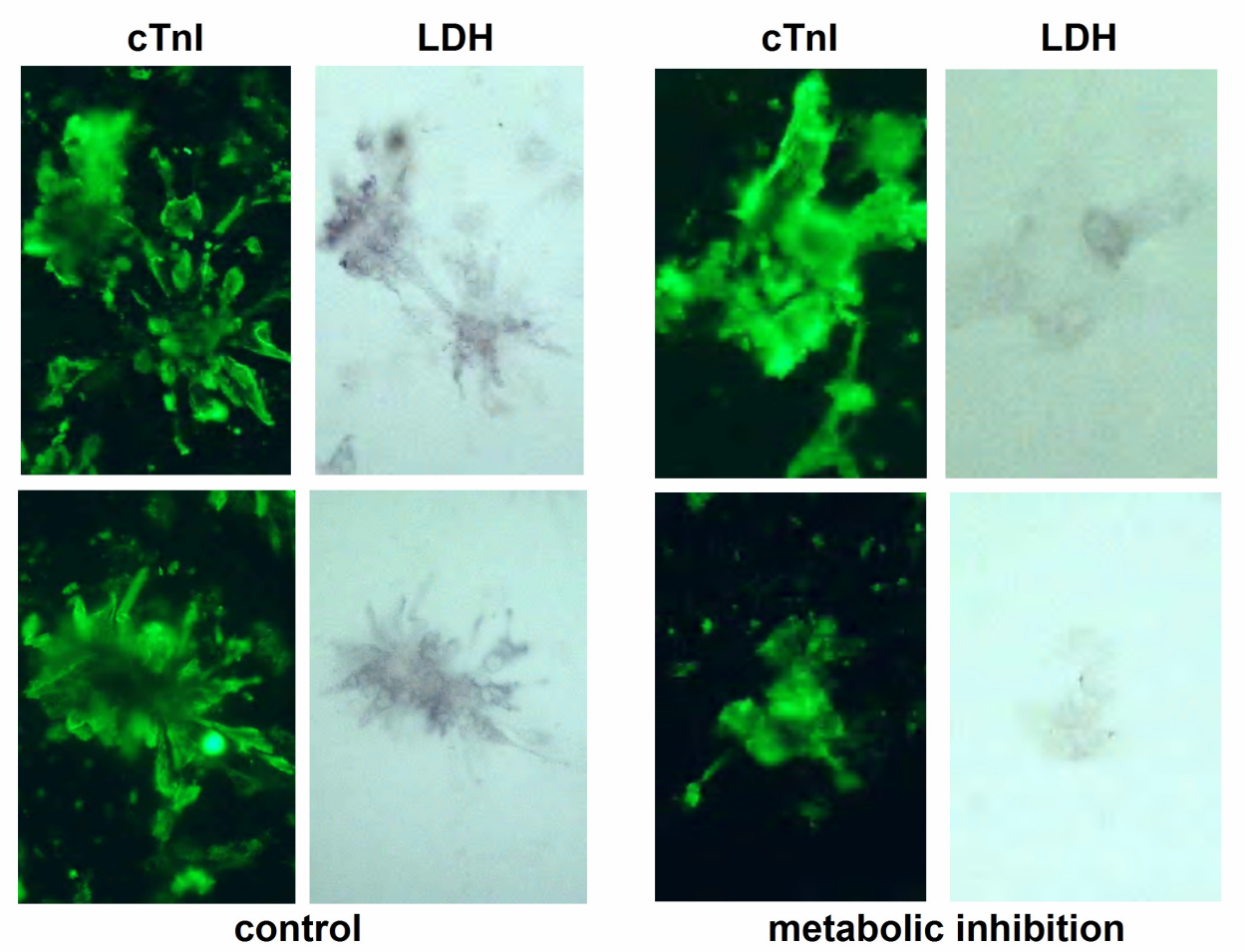

Figure 4. Fluorescence microscopy images (TnI) and light microscopic images (LDH) of cardiomyocyte cultures that were incubated without metabolic inhibitors for 150 min (control, LEFT), and with metabolic inhibitors (5 mM NaCN and 10 mM DOG with 2.5 mM extracellular Ca^{2+} concentration) for 150 min (RIGHT). The control cells are positive for TnI (green) and LDH (blue), whereas the metabolicly inhibited cells are positive for TnI but stain faintly for LDH. (*For color figure see back of book*)

Discussion

The main findings of the present study are first, that the release of LDH from necrotic cardiomyocytes precedes that; second, that the quantity of LDH released from the cultures is 80-90% of their content, whereas the highest value of TnI release found was roughly 50%; third that the calpain inhibitors, calpain-I-inhibitor and leupeptin, have no effect on the release of TnI or LDH, and finally, cytochemical assessment of necrotic cardiomyocytes revealed TnI-positive cells that were poor in LDH, demonstrating that certain factors are responsible for delayed release of TnI compared to LDH release.

Theoretically, there are two explanations for the delaye of TnI release compared with LDH release. First, time is required for TnI to dissociate from its binding to the tropomyosin-actin complex. Only 6±1% of total myocardial TnT content is present in free, *i.e.* unbound, form[10] that could be expected to be released as early as LDH, and earlier than bound TnT. Kragten *et al.*[11] calculated that, after 72 h, for each gram-equivalent of myocardium infarcted based on αHBDH release there is only 39 mg-equivalent of myocardium infarcted based on TnT release, increasing to 69 mg-equivalent of myocardium infarcted based on TnT release in the first 168 h. In other words, from the infarcted area only 6.9% of TnT is recovered in the circulation in the first 168 h, compared with 100% of αHBDH in the first 72 h. Although the release of TnI release in the present study exceeded the 6.9% seen for cTnT[11], the explanation of time-dependent dissociation of TnI from myofilaments remains plausible. Second, TnI may become fragmented intracellularly by proteases like calpain[20;23], mekratin[24] and matrix metalloproteinases[25] or extracellularly, e.g. in the circulation[26;27]. Any TnI fragment that does not contain the two antigenic sites of cTnI that are used in the ELISA (aa 40-50 and aa 189-195) will not be recovered, as the TnI ELISA used detects the full molecule only. There is ample evidence that under certain conditions some fragmentation of TnI occurs, intracellularly as well as extracellularly[23;25-33]. If in the present study fragmentation of TnI had occurred, release of TnI fragments would not be detected. On the other hand, metabolic inhibition-induced TnI release was not influenced by preincubation of the cardiomyocytes with calpain inhibitors, suggesting that fragmentation of released TnI either does not occur, or is not caused by calpain.

The finding that released TnI complexed to other troponins like TnT and TnC[31;32] may add to the problem of detection of full-length TnI. A C-terminal portion of 17 amino acids, containing part of the antigenic site of the detection antibody (clone 6F9) we used, is split off before forming covalent complexes between TnI_{1-193} and $TnT_{191-298}$ and between TnI_{1-193} and TnC_{1-94}[32]. If these complexes would be formed in necrotic cardiomyocytes *in vitro*, they will not be detected in our ELISA.

A typical biphasic release pattern of TnI, as observed with TnT in patients with AMI who had early reperfusion[10;12;14], has not been observed in necrotic cardiomyocytes *in vitro*. Probably the action of macrophages is responsible for the second peak of serum TnT.

The cardiomyocyte cultures used in the present study were free of monocytes and macrophages, but contained fibroblasts. Although the protocol for preparing cardiomyocyte cultures included specific steps for remove fibroblasts, the remaining fibroblasts proliferate due to serum stimulation. These cells contain LDH but no TnI, and are less sensitive to metabolic inhibition than cardiomyocytes. The presence of a low fraction of fibroblasts in the cardiomyocyte cultures may account for maximally attained LDH release of 80-90%, as observed earlier[20].

The cardiomyocyte cultures we used had an average TnI content of 1.52 µg/culture and an average LDH activity of 2.07 U/culture. An average value of 0.75 µg TnI/U LDH in these cultures compares favourably with TnT/LDH values in human myocardium, being 1.3 µg TnT/U LDH (0.234 mg TnT/g of tissue and 183 U LDH/g of tissue)[11].

Conclusion

The release of full-length TnI molecules from necrotic cardiomyocytes *in vitro* is delayed compared to the release of LDH. Secondly, the relative quantity of TnI released (30-40%) is less than that of LDH (80-90%). Thirdly, the release of intact TnI is not dependent on cellular calpain activity. Further studies should reveal whether intracellular and extracellular TnI degradation and complex formation between TnI and other troponins mask early release of TnI that is not detected by the ELISA assay used in the present study.

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