REVIEW ARTICLE

CARDIAC TROPONIN-I: A BIOCHEMICAL MARKER FOR CARDIAC CELL NECROSIS

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Ischaemic heart disease represents the most common of the serious health problems in contemporary society and acute myocardial infarction (AMI) is the major cause of cardiovascular morbidity and death. The accurate determination of infarct size and the volume of myocardium at risk at the time of initial insult is important in the choice of treatment; however, it is probable that it is the ultimate infarct area which determines the longer term outcome. Myocardial ischaemia first appears in the sub-endocardial region. Initially, the ischaemic cells are reversibly injured and if these initial changes are not promptly reversed, it will result in the death of the myocytes. Thereafter, irreversible myocyte necrosis travels transmurally towards the epicardium in the form of a wave-front (Alpert, 1989).

Progress in the field of thrombolytic therapy has significantly affected the management of patients with AMI (Verstraete, 1992). A timely and successful reperfusion during an evolving infarction often prevents immediate mortality, reduces the risk of infarct and thus preserves the left ventricular function.

The familiar triad, clinical history, ECG and serum enzyme analysis, is still of utmost significance in the diagnosis of myocardial infarction (Willerson, 1989). However, none of these criteria reveal quantitative and precise information regarding the extent and location of the infarct. Efforts to relate infarct size to serum level changes of the marker substances released in blood from necrosing myocytes, have met with little success. In addition, a number of specialist techniques such as planar radioisotope imaging, single photon emission computed tomography (SPECT), positron emission tomography (PET), echocardiography, ventriculography and NMR imaging have been employed to substantiate diagnosis in patients showing ambiguous symptoms and ECG findings. However, most of these procedures are not available to patients due to their very high costs.

In recent years, major advances have been made in the methods based on immunological techniques to improve the detection and estimation of infarcts. These

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methods are exclusively based on the production and availability of specific antibodies against intracellular, cardiac-specific substances. The membranes of irreversibly injured and necrosing myocardial cells lose their property of semi-permeability and hence a number of intracellular cytoplasmic components become released into the circulation. The rise in the serum concentration, as well as the activities of these substances, form the basis of many of the presently available biochemical tests for MI. The results of the studies by Serrano *et al.* (1990) and Vannen *et al.* (1990) revealed that although non-specific, serum myoglobin (Mb) levels rise very early in the event of MI. The simultaneous measurement of serum Mb and serum carbonic anhydrase-III (CA-III), a skeletal muscle-specific protein, could be used to assess myocardial damage. Similarly, there is considerable evidence in the literature that there is leakage of mitochondrial and lysosomal enzymes from necrotic myocytes following myocardial infarction (Sylvén *et al.*, 1987; Puleo *et al.*, 1988; Pappas, 1989; Doran *et al.*, 1990).

There is an ever growing list of enzymes which have been suggested as being suitable for the detection of myocardial injury. However, only a very small number of enzymes, such as creatine kinase (CK) (Lee *et al.*, 1986 & 1987; Apple, 1989; Puleo, 1990 & Prager *et al.*, 1992) and lactate dehydrogenase (LD) (Loughlin *et al.*, 1988; Wolf 1989, Smith *et al.*, 1987, Jensen *et al.*, 1990 & Galbraith *et al.*, 1990) have been singled out as being of particular significance. A serious limitation associated with most of these markers is the lack of cardiac specificity — this often results in a high percentage of false positive results. For this reason, research in the past decade has been focussed on structural as well as functional proteins of the cardiac muscle, that show a very high degree of specificity for the myocardium.

Khaw *et al.*, 1976, undertook a study to visualise damaged myocardium using a radiolabelled polyclonal anti-myosin antibody. Encouraged by the results obtained various groups of research workers then reported producing monoclonal antibodies (McAb) against the light and heavy chains of cardiac myosin (Haber *et al.*, 1981; Khaw *et al.*, 1984; Leger *et al.*, 1985 & 1991; Hoberg *et al.*, 1988 & Jin *et al.*, 1990). Recent reports from Bhattacharya *et al.* (1991), Johnson *et al.* (1992) and Nakata *et al.* (1992) showed that AMA planar and tomographic imaging can detect myocyte necrosis in both Q-wave and non-Q-wave infarctions, with a high degree of sensitivity regardless of infarct age, size and location. The system of cardiac myosin-antimyosin was chosen for the *in vivo* visualisation of myocardial infarction because cardiac myosin is present at a relatively high intracellular concentration. It is also easy to isolate and purify, shows strong immunogenicity and its exposure to the extracellular environment only occurs after cellular damage under pathological conditions (Khaw, *et al.*, 1989). However, there are many problems and questions, associated with anti-myosin monoclonal antibody (AMA) imaging, which still remain unanswered. An important example is the non-specific uptake of AMA Fab in the area of the thorax due to cross reactivity with skeletal myosin (Khaw *et al.*, 1983); more evidence of the non-specific uptake in AMA is provided by the reports of Hoefnagel *et al.*, 1987, Cox *et al.*, 1988 & Planting *et al.*, 1990. Moreover, the very complex situation regarding the specificity of cardiac myosin for myocardial tissue, together with its isoform switching at various developmental stages and under a number of pathological conditions has jeopardised its position as an ideal cardiac-specific marker (Gorza *et al.*, 1984).

The use of cardiac-specific troponin-T (cTn-T) is an interesting new development that promises better diagnostic efficacy and a higher degree of cardiospecificity than the existing markers. Like many other major contractile and regulatory proteins, cTn-T is biochemically and immunologically different from its counterparts in skeletal muscle (Gusev *et al.*, 1983). Its release profile has been reported to be similar to cTn-I and tropomyosin (Tm) after AMI (Cummins *et al.*, 1981 & 1987); however, recent evidence indicates that cTn-I has a significantly different pharmacokinetic profile with a subsequent earlier release of the molecule. Nevertheless cTn-T shows a biphasic release pattern and provides a long diagnostic window of 10.5–140h after the onset of pain (Katus *et al.*, 1991 & 1992; Mair *et al.*, 1991, Gerhardt *et al.*, 1991). The determination of cTn-T in the serum offers several distinct advantages for the diagnosis of AMI such as very low normal serum levels, cardiospecificity, and as a subcellularly compartmentalised protein, its release into serum lasts longer than many other marker substances.

CARDIAC TROPONIN-I

Troponin plays a key role in the thin filament regulation of both skeletal and cardiac muscle contraction. Depending upon the state of bound Ca^{2+} , it positions tropomyosin along F-actin to either allow or prevent the interaction of myosin with actin (Potter *et al.*, 1982). In other words, it serves as a Ca^{2+} sensitive switch on the thin filament which is involved in regulating muscle contraction.

Troponin (Tn) is a complex of troponin-I (Tn-I), troponin-T (Tn-T) and troponin-C (Tn-C) (Ebashi *et al.*, 1972; Tsukui *et al.*, 1973). The Tn-C component (18.5KD) is a Ca^{2+} binding component which, by an unknown mechanism, conveys a calcium associated signal through Tn-T (38KD), the Tm binding component and Tn-I (23.9KD), the inhibitory component of actomyosin ATPase activity (Greaser *et al.*, 1972). Each of these molecules has been reported to exist in multiple isoforms and is expressed in a tissue-specific and developmentally regulated manner. The troponin complex proteins are single polypeptides and show a relatively simple tissue distribution, compared to myosin or tropomyosin.

In the case of Tn-I, three principle isoforms have been reported — these are associated with cardiac muscle, slow skeletal muscle and fast skeletal muscle, in humans (Cummins *et al.*, 1978). These isoforms may contribute to the functional differences of the various muscle types and separate genes have been found to encode for the three isoforms (Vallins *et al.*, 1990; Hunkler *et al.*, 1991).

The Tn-I isoforms have been found to be tissue specific but species non-specific (Dhoot *et al.*, 1978). They are located in different types of cells in the myofibrils and the cells of the fibres of the type-1 contain only the slow isoform of Tn-I whereas the fast isoform is present in type-2 cells. However, in some developmental and pathological conditions, cells can have both isoforms concomitantly. Studies in a variety of different mammals, including rabbit, cow, baboon, monkey and man have indicated that the structural relationship of cTn-I is strongly conserved in all mammalian species. Its distribution pattern is uniform throughout the atrial and ventricular chambers possibly due to the presence of only one cell type in the heart tissue; moreover, it is uniquely located in the myocardium (Humphreys *et al.*, 1984). A comparative study of cTn-I from different species by Berson *et al.* (1978) showed

that all cTn-I appeared similar with respect to mol.wt but with different overall charges. These different cTn-I molecules have the same inhibitory effect on skeletal actomyosin ATPase activity and the same ability to be phosphorylated. However, a marked variation on the phosphate content was observed between one species and another. This was possibly related to the conditions in which heart was removed as the adrenaline surge before the death of an animal was reported to be capable of phosphorylating cTn-I (Solaro *et al.*, 1976).

cTn-I is a single polypeptide chain of 211 amino acids which exhibits no evidence of heterogeneity in its amino acid sequence. A comparative study of the sequence of cTn-I with that of sTn-I showed that the inhibitory region is almost identical in the two proteins, the former having an additional 26 residues at the N-terminal region not present in skeletal muscle specific troponin-I (sTn-I). cTn-I should, therefore, prove to be a heart-specific biochemical marker which can be detected in blood plasma of a patient with AMI and also can be targetted, *in vivo*, using radiolabelled cTn-I specific McAbs for localisation and visualisation of the infarct site. Apart from this, there is strong overall homology between the two proteins especially at the C-terminal end, which is the most conserved part of the molecule (Grand *et al.*, 1976). This sequence contains two adjacent serine residues which become phosphorylated in response to β -adrenergic stimulation of the heart; this is an important feature of cTn-I but it should be noted that the amino acid sequence of this phosphorylation site is conserved between all species. cTn-I, isolated from bovine heart contains two adjacent phosphoserines (Swiderek *et al.*, 1988); absence of the N-terminal serine residues confers reduced responsiveness of the heart to β -adrenergic stimulation (Mittamann *et al.*, 1990).

The amino acid sequence from 104–115 is the peptide imparting ATPase inhibitory activity to the Tn-I molecule. This region has also been found to be important for Tn-I interaction with Tn-C and Tm and any alteration in this sequence results in loss or reduction in the inhibitory activity of Tn-I (Eyck *et al.*, 1988). Laure *et al.* (1992) have recently carried out epitope analysis of cTn-I with synthetic peptides using 40 apparently cTn-I specific McAbs. The study revealed the presence of six different epitopes on the cTn-I molecule several of which were highly specific for cTn-I and not found in sTn-I.

Developmental changes in the functional properties of cardiac muscle, particularly Ca^{2+} and pH sensitivity of the contractile system, have been related to Tn-I isoform switching. An embryonic to adult Tn-T switching has been clearly demonstrated in mammalian heart but the results concerning Tn-I are conflicting. Saggin *et al.* (1989), using a set of McAbs, investigated cTn-I changes in the developing heart. The data showed the presence of two antigenically and electrophoretically distinct isoforms during cardiac development. However, studies on the developmental expression of cTn-I in human heart by Bhavsar *et al.* (1991) and Hunkler *et al.* (1991) demonstrated that cTn-I in human fetal heart corresponded to the slow skeletal muscle isoform which lacks the N-terminal serine residues. During development between 20 weeks post-gestation and the 9 month post-natal period, the transition from slow sTn-I to adult cTn-I isoform occurs. However, the molecular mechanism of this transition is unknown although the developmental changes in the hormonal levels, particularly thyroid hormones, may be directly involved in the process (Diekman *et al.*, 1990). The developmental regulation of Tn-I has been reported to

be at the mRNA level in rat hearts. With maturation, the quantity of slow skeletal mRNA decreases and the quantity of cTn-I mRNA increases (Murphy *et al.*, 1991). Recently, production of some McAbs have been reported which can apparently differentiate between the phosphorylated and dephosphorylated cTn-I isoforms (Cummins *et al.*, 1991).

ANTIBODIES TO cTn-I

In myocardial infarction, the pattern of degradation of Tn subunits together with preservation of Tn-T and tropomyosin was reported to be similar to the skeletal muscle in dystrophy. The anaerobic activation of proteolytic enzymes like cathepsin, reduction in tissue ATP, accumulation of lactate, elevation of pH and changes in the intracellular distribution of electrolytes, were all suggested as being responsible for this degradation (Katagiri *et al.*, 1981). These alterations in the intracardiac environment might directly affect the stability of troponin or act by indirectly activating proteolytic enzymes. Although the exact mechanism of this is unclear, the degradation in the early stages of myocardial ischaemic necrosis, together with reduction in myosin and α -actinin, is well documented in the literature. Cummins *et al.* (1979) proposed that antibodies specific to cTn-I could be used to assess cardiac damage; based on this assumption, Cummins *et al.* (1983) reported the development of a RIA for cTn-I to diagnose MI. The results of the study showed that the cTn-I release profile resembled that of CK-MB earlier on in the event of MI. The lower limit of assay sensitivity was 10ng/ml which was the upper limit of cTn-I serum level of normal patients. A later study of MI patient blood samples showed cTn-I to exhibit a biphasic release profile with serum levels elevations within 4–6h, reaching a mean peak levels in 112ng/ml (range from 20–550ng/ml) at 18h and remaining above normal for up to 6–8d post-infarction (Cummins *et al.*, 1987). Moreover, cTn-I levels in patients with chest pain were found to be unchanged or slightly elevated while they were always normal in patients with chest pain of a non-cardiac origin.

The relatively early release of cTn-I could be the result of rapid myofibrillar breakdown but may also reflect the presence of free cTn-I within the sarcoplasm that has not yet been incorporated into the myofibrillar texture; evidence for a free cytoplasmic pool of myosin LC is in existence (Wikman-Coffelt *et al.*, 1973). cTn-I shows a prolonged biphasic release pattern which is probably the result of the loss of the cytoplasmic pool in the first instance. This given an early peak at around 18h post-infarction while a second increase in the serum level is observed at 48h which continues for 8–10d; the later possibly derived from myofibrillar degradation. Hence, rapid loss of cTn-I from the cytoplasmic pool superimposed on the prolonged myofibrillar degradation suggests that cTn-I can be useful for both immediate (6–8h) as well as long term (2–8d) diagnosis of MI when the other clinical diagnostic parameters have returned to normal. Similar prolonged release and biphasic patterns have also been reported for tropomyosin and myosin LC (Nagai *et al.*, 1979).

In order to investigate the possible use of cTn-I as a diagnostic agent, in more detail, Cummins *et al.* (1987) established an experimental canine model of MI and reported an ELISA for cTn-I with enhanced specificity and sensitivity which used a polyclonal antiserum. A prominent feature to emerge from the study was the prolonged release profile of cTn-I of up to 200h post infarction. The cTn-I serum

levels rose to detectable levels within 4–6h and peaked between 10–16h post ligation. However, the infarct size was found to show a lower correlation with cTn-I values as compared with CK-MB serum concentrations, possibly due to the complex nature of the release pattern from the necrotic tissue.

Hunt *et al.* (1991) investigated the incidence of myocardial cell damage following angioplasty by monitoring CK-MB and cTn-I serum level changes as cardiac specific markers for cell injury. The results supported the idea of no cell damage after successful coronary angioplasty as was evident from results showing normal serum cTn-I levels. However, some discrepancy was observed between the results when CK-MB was analysed by immunoassay and an immunoinhibition enzyme assay, due to the false positive results produced by the latter technique; up to 20% of the patient samples were shown to give small but significant elevations of CK-MB. The results supported the idea that cTn-I is the most reliable and specific biochemical marker for myocardial cell necrosis, available at present.

The superiority of cTn-I as a cardiac specific marker was also shown by the results of a comparative study involving measurement of cTn-I, CK and its isoenzymes, Tm, Mb and C-reactive protein in serum samples from marathon runners. While no rise in cTn-I and C-reactive protein was observed in any serum sample, the rest of the markers did register serum level elevations suggestive of cardiac muscle trauma after severe exercise. However, studies using myocardial 'hot spot' scintigraphy and Tl²⁰¹ perfusion imaging suggest that serum CK-MB increases do not necessarily reflect cardiac injury but more likely derive from skeletal muscle damage (Cummins *et al.*, 1987).

Most of the work published regarding the use of cTn-I as a marker for myocyte damage has been carried out *in vitro*, using cTn-I specific polyclonal antisera. Recently, some reports have been published indicating the production of cTn-I specific McAbs. Russell *et al.* (1989) characterised anti-cTn-I McAbs raised against primate cTn-I, and some of the cell lines were reported to show species-cross reactivity as well as tissue non-specificity. Ladenson *et al.* (1990) raised a group of McAbs against human cTn-I and developed a two-site sandwich ELISA for cTn-I measurement in cases of suspected myocardial infarction; this had a minimum detection limit of 1.9 µg cTn-I/l (Bodor *et al.*, 1990 & 1992). The results of their studies strongly emphasise the significance of cTn-I as a valuable cardiac-specific diagnostic marker for the assessment of myocardial infarction.

Loss of membrane integrity should allow the entry of radiolabelled antibodies into cells to bind to any remaining myofibrillar cTn-I; Cummins *et al.* (1989) took this point into account and reported the potential use of I¹³¹-labelled Fab fragments of cardiac specific IgG for *in vivo* imaging, in a cardiac infarct model. Images of heart muscle slices showed areas of Fab uptake corresponding with areas of histochemically defined necrosis. Images using In¹¹¹-labelled Fab showed a seven-fold improvement over previous results (Cummins *et al.*, 1990).

A more detailed account of radioiodinated, affinity purified cTn-I antibody uptake in MI was published by Cummins *et al.* (1990). The study showed an inverse relationship between blood flow and antibody uptake in the necrotised myocardium. The study clearly showed that the injected radiolabelled antibodies to cTn-I did localise in the necrotic tissue and that the targeting was mainly due to direct antigen binding. An uptake of up to 0.003% of the injected dose per gram of the infarcted

tissue could be achieved; more important was the uptake ratio of the antibody in infarcted and normal myocardium which ranged from 1.399–3.94. The antibody uptake was reported to be 24 times greater towards the centre of the infarct compared to the peripheral tissue. It has been suggested that the use of Fab fragments in place of the whole antibody molecule and specific high affinity McAbs, in place of polyclonal antibodies, could also improve the uptake significantly. The unique degree of cardiac specificity and both short and long term availability suggest that cTn-I may be a valuable parameter both for *in vitro* serum detection and *in vivo* scintigraphic studies of MI.

In a recently concluded study involving a brain dead model in pigs, the authors have successfully employed radiolabelled anti-cTn-I McAbs to assess the damage to the cardiac tissue resulting from brain death. The results of the study are significant in relation to cardiac transplantation from brain dead donors. The McAbs used in this study were produced in our laboratory against bovine cTn-I and showed a high degree of specificity for the cardiac isoform of Tn-I. However, the antibodies did not show any species specificity; thus allowing their use in this study (Haider & Stimson, *in press*).

In summary, cTn-I possesses cardiac specificity, a simple tissue distribution pattern in atrial and ventricular myocardium, and a unique location in the myofibrillar organisation; this makes the protein more accessible for antibody targeting. The fact that cTn-I is only exposed to the extracellular environment after myocardial cell necrosis, should ensure that it is a reliable cardiac specific biochemical marker for both *in vitro* diagnosis as well as *in vivo* imaging for myocyte necrosis. However, as a number of biochemical markers already exists, it will take extensive experimentation in a clinical setting before cTn-I is finally recognised as the valuable marker molecule we believe it to be.

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