Radioimmunoassay of human cardiac tropomyosin in acute myocardial infarction

P. CUMMINS, B. McGURK AND W. A. LITTLER
British Heart Foundation Department of Cardiovascular Medicine, Clinical Research Block, University of Birmingham, Queen Elizabeth Hospital, Birmingham, U.K.

(Received 26 February 1980; accepted 22 October 1980)

Summary

1. Tropomyosin was prepared from fresh human myocardium and antisera raised in rabbits. A sensitive radioimmunoassay was developed for the detection of human cardiac tropomyosin in human sera down to levels of 1 ng/ml.

2. Values for human cardiac tropomyosin in normal patients ranged from less than 1 to 3 ng/ml. In 18 patients with acute myocardial infarction all had elevated tropomyosin levels ranging from 41 to above 200 ng/ml with a mean peak level of 101 ng/ml. In this study there were no false positive or false negative results.

3. In the initial stages of infarction the time course of appearance and peak levels of cardiac tropomyosin, total creatine kinase and creatine kinase MB isoenzyme were similar. Although total creatine kinase and creatine kinase MB isoenzyme levels were normal after 72 h in patients with single, uncomplicated infarction, cardiac tropomyosin levels were still significantly elevated above normal after this time, being 30–60% of peak values.

4. Radioimmunoassay of human cardiac tropomyosin may prove useful in the diagnosis and in the management of patients with acute myocardial infarction, particularly in the long-term postinfarction period.

Key words: cardiac tropomyosin, myocardial infarction.

Introduction

The detection of different cytoplasmic components that appear in the serum has been used extensively in the clinical assessment of tissue damage, particularly in the diagnosis of acute myocardial infarction. These include the commonly measured enzymes, creatine kinase (EC 2.7.3.2), glutamic-oxaloacetic transaminase (EC 2.6.1.1) and lactate dehydrogenase (EC 1.1.1.27), in addition to substances such as myoglobin (for review see Apps & Tinker, 1978). Although most of these substances appear in the blood stream within a few hours of onset of myocardial infarction, there are various problems associated with their measurement, including loss of enzymic activity, lack of specificity and insensitivity of assay leading to false positive and false negative results. Nevertheless although these problems may sometimes preclude a more quantitative estimate of the extent of tissue damage, in particular in estimating the size of a myocardial infarct (Sobel, Markham & Roberts, 1977), they do provide a useful diagnostic tool.

However, the detection of these components in the immediate and long-term postinfarction period where an estimate of cell damage might be of benefit in patient management is limited owing to a number of factors. In the majority of patients who suffer uncomplicated acute myocardial infarctions the cytosolic nature of these components leads to rapid cell release and return to normal levels. For this reason a search has been centred on cell components that may have a different time course of release.

Owing to their structural role in the myocardial cell the contractile and regulatory proteins of the myocardium (for review see Katz,
P. Cummins, B. McGurk and W. A. Littler (1970) could provide a useful diagnostic tool in the long term assessment of tissue damage. Although the major contractile protein, myosin, has been assessed in the serum of patients with acute myocardial infarction (Trahern, Gere, Krauth & Bigham, 1978), its time course of release has been found to be similar to that of creatine kinase. However, no assay has been developed for the detection of the regulatory proteins, tropomyosin or troponin, and it is not known whether these are present in the serum of normal subjects and become elevated after acute myocardial infarction.

In the results presented here we describe the development of a sensitive radioimmunoassay for the detection of human cardiac tropomyosin in serum and present our preliminary findings on its measurements in patients with acute myocardial infarction.

Some aspects of this work have been briefly reported elsewhere (Cummins, Littler & McGurk, 1979a; Cummins, McGurk & Littler, 1979b).

Methods

Preparation of human cardiac tropomyosin

Human cardiac tissue was obtained immediately after death from renal transplant donors from the Renal Unit, Queen Elizabeth Hospital, University of Birmingham with the informed consent of the relatives. If not used immediately, tissue was flash-frozen in liquid nitrogen and stored at -30°C.

Tropomyosin was prepared by the method of Cummins & Perry (1973) with modifications as described by Phillips, Lattman, Cummins, Lee & Cohen (1979). In some cases tropomyosin was further purified by gel filtration on a Sephadex G-100 column (100 cm x 2.5 cm) equilibrated with urea (8.0 mol/l)/Tris/HCl (20 mmol/l) dithiothreitol (1 mmol/l) (pH 7.4). After renaturation and dialysis of the fractionated human cardiac tropomyosin in 2-mercaptoethanol (15 mmol/l), the protein was freeze-dried. No difference was observed between preparations that had been further purified in this manner and pure preparations that had not been subject to gel filtration in denaturing conditions. Both types of preparation gave similar standard curves on subsequent radioimmunoassay. Purity of preparations was assessed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the presence of Tris/borate buffer as described by Cummins & Perry (1973) (Fig. 1).

Preparation of antisera

Antisera to human cardiac tropomyosin were raised in rabbits. The purified protein at a concentration of 1–5 mg/ml was dissolved in sodium chloride (0.145 mol/l)/sodium phosphate (10 mmol/l) buffer, pH 7.2, and diluted with an equal volume of Freund’s complete adjuvant. Samples (5 mg) were injected intramuscularly at weekly intervals for 3 weeks and then at monthly intervals with 100 μg/dose until a satisfactory response had been obtained on subsequent testing of the antiserum for its ability to bind 125I-labelled human cardiac tropomyosin. This usually required up to 3 months. Preliminary testing for the presence of antibody was carried out by Ouchterlony immunodiffusion analysis with agar plates (Cummins & Perry, 1974).

Preparation of 125I-labelled human cardiac tropomyosin

The iodination method was based on that of Greenwood, Hunter & Glover (1963). A 5 μg portion of tropomyosin solution (1 mg/ml) in sodium phosphate buffer (50 mmol/l), pH 7.5, was added to 50 μl of sodium phosphate (0.5 mol/l), pH 7.5, and 1 mCi of Na125I. After the addition of 20 μl of chloramine-T (17-75 mmol/l), the reaction was allowed to proceed for 1 min.
Radioimmunoassay of tropomyosin in infarction

and then terminated by addition of 750 μl of sodium metabisulphite (12.6 mmol/l). Labelled tropomyosin was immediately separated from unreacted Na\(^{125}\)I by gel filtration on a Sephadex G-25 column (15.7 cm x 1 cm) equilibrated in sodium phosphate buffer (50 mmol/l), pH 7.5. Losses of labelled tropomyosin by adsorption were minimized by prior priming of the column with 1 ml of 0.5% potassium iodide, 0.5% ovalbumin in sodium phosphate (50 mmol/l), pH 7.5, and eluting with 30 ml of the same buffer. The fractionated \(^{125}\)I-labelled tropomyosin was stored at 4°C in 2.5% ovalbumin/0.05% sodium azide/sodium phosphate (50 mmol/l) (pH 7.5).

Assay procedure

The radioimmunoassay method used was a typical postprecipitation double-antibody technique (for reviews see Midgeley, Rebar & Niswender, 1969; Kirkham & Hunter, 1971). Assays were carried out in sodium chloride (0.15 mol/l)/EDTA (0.01 mol/l)/0.1% sodium azide/0.1% ovalbumin in sodium phosphate buffer (40 mmol/l), pH 7.0, with disposable glass tubes (7.5 cm x 0.9 cm). A 50 μl portion of 0.5% carrier non-immune rabbit serum was used in a total assay volume of 300 μl. Anti-(human cardiac tropomyosin) serum at a concentration known to give a 10–30% binding of total added labelled tropomyosin was used in a volume of 50 μl. Some tubes in each assay contained no unlabelled tropomyosin to give an estimate of maximum binding, whereas in others the antiserum was replaced by buffer to act as a check on the level of non-specific binding of radioactivity. Freshly purified \(^{125}\)I-labelled tropomyosin containing 8–12 000 c.p.m. in 50 μl of assay buffer was finally added and tubes were mixed and left for 48 h at 4°C. After the addition of donkey anti-(rabbit) IgG (Wellcome Reagents Ltd, Beckenham, Kent, U.K.) tubes were incubated for a further 24 h at 4°C and then centrifuged at 150 g for 30 min. The activity in the precipitate was determined in a gamma-counter (Gamma Set 500, ICN Instruments Division, Surrey, U.K.). All assays were carried out in triplicate and tubes counted for a minimum of 3 min.

The amount of \(^{125}\)I-labelled tropomyosin added per assay on the basis of a specific radioactivity of 100 μCi/μg (see the Results section) and 10 000 counts/min per assay was 0.1 ng.

Creatine kinase and creatine kinase isoenzyme assay

Creatine kinase MB isoenzyme levels were determined at 25°C by using both an ion-exchange method as modified by Mercer (1974) and an immunological method based on the presence of creatine kinase M-subunit antibodies. Ion-exchange test-combination kits were obtained from Boehringer Mannheim, West Germany and antibody Merck-1-test kits from E. Merck, Darmstadt, West Germany. Total creatine kinase levels were determined at 25°C by an N-acetylcysteine-activated system obtained from Boehringer Mannheim, West Germany. Precipath E Control Sera (Boehringer Mannheim) with known levels of creatine kinase MB isoenzyme and total creatine kinase activities were used to standardize the different methods. Both methods for determining creatine kinase MB isoenzyme levels gave comparable results.

Serum collection

Serum samples (5 ml) were stored at −30°C in the presence of 10 μl of 2-mercaptoethanol (150 mmol/l) to protect creatine kinase thiol groups. All serum samples were assayed for total creatine kinase and creatine kinase MB isoenzyme within 3 days of collection. Cardiac tropomyosin radioimmunoassay was carried out within 14 days of collection, although serum could be stored at −30°C for at least 1 year without any detectable change in tropomyosin levels.

Control subjects

Fifteen healthy subjects (eight men and seven women) who ranged in age from 20 to 54 years were used as controls. They had no history of heart disease.

Acute myocardial infarction patients

Eighteen patients (13 men and five women) aged between 48 and 70 years who had a typical history of myocardial infarction of less than 6 h duration gave informed consent for extra blood samples to be withdrawn. Acute myocardial infarction was subsequently confirmed from ECG criteria, i.e. appearance of pathological Q waves accompanied by elevation of the ST segment and subsequent inversion of the T wave, together with a significant rise in serum glutamic-oxaloacetic transaminase and creatine kinase levels.

Venous blood samples were collected from a forearm Venflon catheter inserted on admission at 2 h intervals during the daytime and at 4 h intervals overnight for a period of 72 h.
Results

Iodination

The iodination procedure used was very reproducible and gave preparations of high specific radioactivities. The percentage incorporation of $^{125}$I achieved varied from 47 to 72%, yielding labelled tropomyosin with specific radioactivities ranging from 94 to 144 $\mu$Ci/µg. Iodinations (17) were carried out over a period of 13 months with a mean specific radioactivity of 119.1 $\mu$Ci/µg (SD 14.9).

The labelled tropomyosin deteriorated somewhat during storage owing to dissociation of $^{125}$I from the protein. After 1 week of storage up to 20–25% of the radioactivity was lost. This free $^{125}$I could be separated from the labelled tropomyosin by a further passage through Sephadex G-25 and this was therefore routinely carried out if the labelled material was more than 1 day old. This allowed a constant amount of antiserum to be used in different assays, although some loss of binding occurred with time after iodination.

The iodinated tropomyosin was immunologically homogeneous as demonstrated by the fact that the leading edge, main peak and trailing edge of the protein fraction all behaved in the same way in the immunoassay. The labelled tropomyosin also yielded dilution curves parallel to the normal standard curve, demonstrating that the immunoreactivity of the labelled material was indistinguishable from the unlabelled material. This correlates well with the extremely stable structure of tropomyosin, which contains almost 100% $\alpha$-helical content (Holtzer, Clark & Lowey, 1965). Furthermore the identical behaviour in the immunoassay of tropomyosin that had previously been denatured and then renatured (see the Methods section) was further indication of its high stability.

Antiserum potency

Different batches of antisera were tested by assaying serial dilutions against a constant amount of labelled tropomyosin. The antiserum dilution curve of the most potent antiserum obtained is shown in Fig. 2. This was capable of a 21% binding of total added label at an antiserum dilution of 1/228 571 (log reciprocal dilution 5.36) and was the antiserum selected for use in this study. When the same antiserum dilution curve was carried out in the presence of a constant amount of 20 ng of unlabelled human cardiac tropomyosin/ml in each assay the binding was lowered at the highest antiserum dilutions to 11.8% of the total (Fig. 2).

Sensitivity of assay and operating range of dose–response curve

Unlabelled tropomyosin in added concentrations ranging from 0.012 to 97.7 ng/50 µl was used to construct the dose–response curve. Assays carried out in the absence of antisera were used to obtain an estimate of non-specific binding and these values were subtracted before estimating the dose–response curve. The data from 14 triplicate standard curves obtained over a period of 13 months by using the same batch of antiserum at a dilution of 1/228 571 are shown in Fig. 3. The minimum concentration of tropomyosin that was consistently distinguishable from zero was used to define the lower limit of assay sensitivity. The coefficient of variation of counts in the zero standard was 3–4% so that detection limit was selected as that tropomyosin concentration which gave 8% inhibition of binding. This concentration was about 0.8–1.0 ng/ml in undiluted serum. The maximum working limit was 200 ng/ml, giving an effective operating range for the assay of 1–200 ng/ml in undiluted serum. Fifty per cent of the maximum binding of labelled tropomyosin in the absence of unlabelled protein was obtained at 20 ng of tropomyosin/ml in agreement with the results above.
Radioimmunoassay of tropomyosin in infarction

**Fig. 3.** Radioimmunoassay standard curve of human cardiac tropomyosin in the presence and absence of normal serum. Radioimmunoassay was carried out as described in the Methods section at an antiserum dilution of 1/228,571 in the presence and absence of 50 µl of normal serum. Data in the absence of serum represent the means of 14 standard curves ± SD. ●, Standard curve; ○, + 50 µl of normal serum.

Recovery of added tropomyosin

This was determined by addition of known amounts of human cardiac tropomyosin to a series of sera to give increments in tropomyosin concentration of 2, 50 and 150 ng/ml. The results are shown in Table 1, indicating that recovery of added tropomyosin was complete.

Assay reproducibility

The within-assay reproducibility was calculated at three different tropomyosin concentrations and is shown in Table 2. The between-assay reproducibility at three different tropomyosin concentrations is also shown in Table 2.

**Assay procedure**

Although the standard procedure for the assay required a total of 4 days and the results presented here were obtained with this time period, attempts were made to shorten the assay. The standard procedure included a 2 day incubation at 4°C before the addition of second
antibody followed by a 1 day incubation at 4°C before centrifugation and counting. It was found that the initial 48 h incubation could be cut to 15 h (i.e. overnight) while keeping the assay at 4°C without any decrease in the percentage of the total counts precipitated or precision of the dose–response curves.

Increasing the temperature up to 22°C or even incubation at 37°C for only 1 h decreased the maximum binding considerably and could not be routinely used. However, once the second antibody had been added, incubation of the assay at 37°C for either 30 or 60 min before centrifugation gave the same percentage precipitation of counts and dose–response curve as incubation at 4°C for 24 h. It is therefore possible to set the assay up on the first day and add second antibody, incubate and centrifuge the next morning. The total assay with this modified procedure can therefore be conveniently carried out inside 24 h.

Specificity

The specificity of several different batches of rabbit anti-(human cardiac tropomyosin) sera was tested by Ouchterlony immunodiffusion tests (Cummins & Perry, 1974) and radioimmunoassay under the standard conditions. Antisera failed to react with any of the myofibrillar contractile and regulatory proteins which would be the most likely contaminants of cardiac tropomyosin preparations. These were all purified from human myocardium and included actin, myosin, troponin-I, T and C (Cummins & Perry, 1978). None of these proteins interfered in the radioimmunoassay when added at concentrations up to 500 ng/tube.

Cross-reactivity of human cardiac tropomyosin antisera with purified preparations of tropomyosin from human skeletal muscle was also examined by both Ouchterlony immunodiffusion tests and radioimmunoassay. Skeletal tropomyosin showed slight reactivity with antisera depending on the skeletal muscle from which the tropomyosin was isolated. This almost certainly results from the varying proportions of α- and β-subunits that exist in different skeletal muscles depending on the speed of muscle contraction (Cummins & Perry, 1974). Although skeletal muscles contain significant amounts of both α- and β-subunits, human cardiac muscle contains very little β-subunit (Fig. 1). Although it is not known which subunit the antisera used in this study is primarily directed against some slight cross-reactivity might be expected if the β-subunit was involved.

Similar cross-reactivity was also detected in the radioimmunoassay depending on the source of the skeletal muscle tropomyosin, but this did not appear to interfere with patient measurements (see the Discussion section).

Normal serum

Serum was collected from 15 healthy subjects who had no history of heart disease. Between 50 and 200 µl of serum was assayed for the presence of human cardiac tropomyosin. In the presence of 50 µl of serum, the volume routinely used when assaying serum from patients after myocardial infarction, no change was observed in the radioimmunoassay standard curve in any of the control subjects (Fig. 3). Likewise, no difference was seen in the immunoassay curve in any of the 15 subjects when 100 and 150 µl of serum was tested. When up to 200 µl was used only four subjects demonstrated binding, less than 92% of total, suggesting low tropomyosin levels varying from 1.4 to 2.85 ng/ml. The remaining subjects all had precipitation levels in excess of 92% of maximum binding which fell below the effective detection limit of the assay, indicating levels under 0.8–1.0 ng of tropomyosin/ml of serum. In all cases in which test serum was assayed an identical volume of test serum was assayed in the absence of immune serum to enable non-specific binding to be determined, as this differed from non-specific binding estimates calculated in the absence of both test and immune serum.

Acute myocardial infarction

Of the 18 patients studied, 14 suffered only single uncomplicated acute myocardial infarc-
The radioimmunoassay developed for the measurement of human cardiac tropomyosin levels in serum has been shown to be both sensitive and reliable. This was aided in part by the high and reproducible level of iodine incorporation into the protein and also by the fact that, owing to its tertiary structure, tropomyosin is a remarkably stable protein (Holzner et al., 1965). It also proved possible to raise antisera against tropomyosin that could be used at very high dilutions. As it is relatively easy to prepare up to 25 mg of highly purified tropomyosin/100 g of post-mortem myocardium, that is stable for long periods at -20°C, and as only very small volumes of antisera are used in each assay, several thousand assays can be carried out with only limited amounts of antigen and antibody. The radioimmunoassay was specific for tropomyosin, the other major contractile and regulatory proteins having no effect on the dose-response curve. Although purified skeletal muscle tropomyosin demonstrated some cross-reactivity with the human cardiac antiserum, this varied depending on the source of the skeletal tropomyosin. The precise tissue specificity of the antiserum must therefore await further in-

![Graph](image)

**Fig. 4.** Peak serum human cardiac tropomyosin levels in patients with uncomplicated acute myocardial infarction. (a) Tropomyosin levels in control patients; (b) tropomyosin levels in acute myocardial infarction (bar indicates the mean).

levels of total creatine kinase and creatine kinase MB isoenzyme fell in all patients after 25 h, usually reaching normal levels by 72 h after infarction, the human cardiac tropomyosin levels were still significantly raised above normal in all patients at this time. After 72 h tropomyosin levels were still 30–60% of their peak values and in the patient shown in Fig. 5 levels had still not fallen to normal more than 5 days after infarction. Preliminary investigations suggest that levels do not return to normal until 8–12 days after infarction depending on the severity of the infarct (P. Cummins, A. J. F. Page & W. A. Littler, unpublished work). In two patients who had very low levels of creatine kinase MB isoenzyme (less than 15 i.u./l at peak compared with normal levels of approx. 5 i.u./l), where diagnosis of infarction based on enzyme criteria alone would not have been satisfactory, human cardiac tropomyosin levels were significantly raised above normal.

Recurrent myocardial infarctions were suffered by four patients as diagnosed by ECG and enzyme criteria. In two cases the creatine kinase MB isoenzyme profile did not give a clear indication of recurrent infarction although this was always very apparent from the level of human cardiac tropomyosin.

**Discussion**

The radioimmunoassay developed for the measurement of human cardiac tropomyosin levels in serum has been shown to be both sensitive and reliable. This was aided in part by the high and reproducible level of iodine incorporation into the protein and also by the fact that, owing to its tertiary structure, tropomyosin is a remarkably stable protein (Holzner et al., 1965). It also proved possible to raise antisera against tropomyosin that could be used at very high dilutions. As it is relatively easy to prepare up to 25 mg of highly purified tropomyosin/100 g of post-mortem myocardium, that is stable for long periods at -20°C, and as only very small volumes of antisera are used in each assay, several thousand assays can be carried out with only limited amounts of antigen and antibody. The radioimmunoassay was specific for tropomyosin, the other major contractile and regulatory proteins having no effect on the dose–response curve. Although purified skeletal muscle tropomyosin demonstrated some cross-reactivity with the human cardiac antiserum, this varied depending on the source of the skeletal tropomyosin. The precise tissue specificity of the antiserum must therefore await further in-
vestigation of its tropomyosin subunit specificity. It did, however, appear that skeletal muscle damage, as might be expected from repeated intramuscular injections, did not interfere in the assay and was not responsible for the elevated tropomyosin levels as total creatine kinase levels returned to normal after 72 h. Moreover, Yazaki, Nagai, Yamaoki & Veda (1980) have demonstrated that cardiac specific contractile protein levels in serum remain elevated in experimental myocardial infarction for up to 7 days after infarction.

The assay was sensitive enough to be able to detect levels down to 1 ng/ml, which were present in four out of 15 healthy subjects. It may be that these represent the higher limit of normal levels in patients, but these could only be detected when four times the normal serum volume was assayed and fall near to the lower limit of the assay. Although at present the assay requires 15–24 h to perform, we believe it is possible eventually to shorten this to 3–4 h by using solid-phase-support antibody and enzyme-linked immunoassay.

Tropomyosin levels were significantly raised in all 18 patients with acute myocardial infarction and there were no false negative or positive results. The initial pattern of release of tropomyosin was very similar to that of creatine kinase, being detectable within 7–8 h after infarction, although tropomyosin reached peak values a few hours later. It proved possible to measure significant increases in tropomyosin levels even in patients where only small increases in creatine kinase levels were present. Moreover, the magnitude of tropomyosin release was enhanced particularly in the case of two patients who suffered recurrent infarctions. The reasons for this are currently being investigated. It may relate to the time course of release of this structural protein from the myocardial cell, its clearance from the body or the site of the infarct. Moreover, its stability could result in it being detectable at a time when other enzymes may be present in serum, but have lost their enzymic activity owing to denaturation and proteolysis. Certainly, it is possible to measure tropomyosin in serum samples that have been stored at −30°C for long periods without any change in level at a time when creatine kinase activity has disappeared. In all of the patients studied tropomyosin levels were still very high at a time when creatine kinase and creatine kinase MB isoenzyme levels had returned to normal. This could obviously prove of benefit in the assessment of those patients who present some days after their infarction.

Detection of human cardiac tropomyosin may also prove useful in the longer-term assessment of
patients who have suffered myocardial infarction. It may prove possible to detect ongoing cardiac cell damage even in patients with severe ischaemic heart disease. Research is currently being carried out to answer these questions and to define the kinetics of tropomyosin appearance and disappearance to obtain a reliable estimate of infarct size.

Acknowledgments

The collaboration of Dr A. J. F. Page and Sister S. Derry and the Staff of the Coronary Care Unit, East Birmingham Hospital in collecting blood specimens is gratefully acknowledged. We thank Mrs S. Gillette for skilled technical assistance in carrying out the creatine kinase assays and Dr R. N. Clayton and Mr R. Shakespeare, Department of Medicine, University of Birmingham for advice in establishing the radioimmunoassay. The help of Mr A. D. Barnes of the Renal Unit, Queen Elizabeth Hospital, Birmingham and his surgical colleagues in the provision of human myocardium is gratefully acknowledged. This research was supported by a grant from the British Heart Foundation.

References


CUMMINS, P., McGURK, B. & LITTNER, W.A. (1979b) Possible diagnostic use of cardiac specific contractile proteins in assessing cardiac damage. Clinical Science, 56, 30P.


