0273-2289/01/90/0097/$12.25

Cytochrome-*c* Detection

A Diagnostic Marker for Myocardial Infarction

Trevor Alleyne,* Jerome Joseph, and Valerie Sampson

Biochemistry Unit, Faculty of Medical Sciences,
The University of the West Indies, St. Augustine,
Eric Williams Medical Sciences Complex, Uriah Butler Highway,
Champs Fleurs, Trinidad and Tobago, E-mail: trevnmar@tstt.net.tt

Received May 28, 1999; Revised July 12, 2000;
Accepted October 1, 2000

Abstract

Following a myocardial infarction (MI) cells die or are damaged and their contents leak into the blood circulation, resulting in elevated serum levels of various enzymes, proteins, and organic molecules. Over the past few decades, it has become standard practice to employ the detection of these elevated substances as markers for the confirmation of MIs and to monitor MI patients' response to treatment. Although it has previously been shown that cytochrome-*c*, a small respiratory protein, is among those elevated, the lack of a suitable detection system has prevented its routine use in the diagnosis of MIs. We present a preliminary study in which chemiluminescence was employed to detect elevated levels of cytochrome-*c* in the serum of MI patients. The technique, which is specific for *c*-type proteins, is approx 30 times more sensitive than the traditional Coomassie blue stain and can detect as little as 0.03 µg of protein. It also has potential for diagnostic use in other diseases that are characterized by mitochondrial damage.

Index Entries: Cytochrome-*c*; myocardial infarction; myocardial marker.

Introduction

A myocardial infarction (MI) is accompanied by a variety of intracellular and extracellular changes that are influenced by the intensity and duration of the infarct. In an acute MI, there may be death of a localized region of cardiac muscle or death of small, diffuse regions of the heart as the blood supply to these regions is interrupted *(1)*. On reperfusion of the affected tissue, there is usually some incomplete reduction in oxygen, leading to the generation of free radicals, which precipitate further damage to

*Author to whom all correspondence and reprint requests should be addressed.

the tissue and cells *(2–4)*. The damaged cells and their components (e.g., mitochondria) become leaky, allowing their contents to flow into the surrounding fluids, resulting ultimately in elevated plasma levels of organic compounds, enzymes, and other proteins *(5)*.

Since the 1950s the detection of elevated levels of a number of blood serum components (mainly enzymes) has been employed to confirm the diagnosis of MIs and to monitor patient response to treatment. The principal diagnostic enzymes in current use are creatine kinase, lactate dehydrogenase, and aspartate amino transferase. Unfortunately, however, these do not always allow the confirmation of an MI in the initial stages of the event *(6–8)*. A technique that is particularly useful in early confirmation is the electrocardiogram (ECG), but this too has its limits. It has been reported that nearly 50% of all MI patients present with nondiagnostic ECGs by the time they are hospitalized *(9)*. As a result of these difficulties *(6–9)*, there has been an ongoing search for new methods and markers that could be used in the early diagnosis of MIs. Within the last 5 yr, several new markers have emerged, including myoglobin *(10)*, tropin I, and troponin T *(10,11)*. In this paper, we present a preliminary study on a new approach to the use of cytochrome-*c* as a marker for MI.

Cytochrome-*c* is a small (12-kDa) respiratory chain protein located on the periphery of the inner mitochondrial membrane *(12)*. This robust redox protein is responsible for the transfer of electrons from complex III to complex IV of the respiratory chain *(13)*. Although it has previously been demonstrated that there is an increase in the serum concentrations of this protein in the event of an MI *(14–16)*, its detection has not been routinely utilized in diagnosis primarily because of the lack of a suitably efficient detection method.

In 1993 Vargas et al. *(17)* demonstrated that the technique of chemiluminescence could be employed to detect the presence of *c*-type cytochromes. The technique, which is based on a peroxidase reaction with heme groups following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the proteins, yields negative results for all non-*c*-type heme proteins (e.g., hemoglobin and myoglobin) because those hemes that are not covalently bound are lost during electrophoresis. Compared to the traditional Coomassie blue stain for the detection of proteins, this procedure is approx 30 times more sensitive and can easily detect as little as 0.03 µg of cytochrome-*c*. We have now adapted the method of Vargas et al. *(17)* for use in the diagnosis of MIs. The method also has potential for diagnostic use in other diseases that are characterized by mitochondrial damage.

Materials and Methods

Diagnostic Procedure

Seven serum samples from six confirmed MI patients were obtained from the coronary intensive care unit of the Eric Williams Medical Sciences Complex. Blood samples (10 mL) were obtained from each of 44 normal

individuals. The red blood cells from the latter samples were removed by centrifugation at 4500*g* for 10 min, and the resulting blood serum was used as controls. Individual serum samples were mixed with twice their volume of sample buffer (pH 6.2) that contained 124 m*M* Tris-HCl, 5% SDS, and 20% glycerol. This protein mixture was denatured for 15 min at 42°C. Horse heart cytochrome-*c* type VI (0.6 mg/mL) was denatured in the same manner and used as a marker. Cytochrome-*c* was purchased from Sigma.

The serum samples or the cytochrome-*c* markers, 50 µL in each case, were loaded onto a 5.6% SDS polyacrylamide stacking gel and electrophoresed on a 15% SDS polyacrylamide separating gel as described by Laemmli *(18)*. Electrophoresis was conducted in duplicate, on either 12-cm (midi) or 5-cm (mini) gel systems.

After electrophoresis one gel was stained with Coomassie blue to visualize the protein bands, and the proteins from the duplicate gel were transblotted onto a nitrocellulose membrane of 0.45-µm pore size and the presence of cytochrome-*c* was established by the process of chemiluminescence as described by Vargas et al. *(17)*. In this method a permanent record of the chemiluminescence results is obtained on X-ray film. The X-ray film was analyzed by densitometry using a Bio-Rad 620 Video densitometer, and the concentration of cytochrome-*c* present on the original gel was deduced from standard curves.

Standard Curve

Serum was prepared from the blood of a normal (non-MI) subject, and 50-µL aliquots were mixed with 10 times its volume of denaturing buffer. Samples were injected with increasing concentrations of commercial cytochrome-*c*, up to a maximum final concentration of 0.60 mg/mL. Samples were denatured as previously described and 50-µL aliquots (i.e., 0.07–30 µg of protein) applied to the SDS gel. Following electrophoresis, which was performed in duplicate, one gel was stained for protein and the other used for the detection of cytochrome-*c* by chemiluminescence as previously described. In the latter case, following densitometry of the developed X-ray film, a standard curve of absorbance vs quantity of protein (milligrams/milliliter) was generated.

Results

The results obtained when serum from a normal (non-MI) subject was injected with increasing concentrations of cytochrome-*c* and the proteins were separated by electrophoresis are shown in Fig. 1A–C. Figure 1A shows the SDS gel that was stained with Coomassie blue to visualize the protein bands, and Fig. 1B shows an X-ray film that recorded the chemiluminescence results of the duplicate gel. Lane 1 of Fig. 1A contains 0.06 mg/mL of myoglobin. Lane 2 contains a normal serum sample, with no added cytochrome-*c*, which was used as a control. Lanes 3–9 are serum samples containing increasing concentrations of cytochrome-*c*: 0.013, 0.025, 0.06,

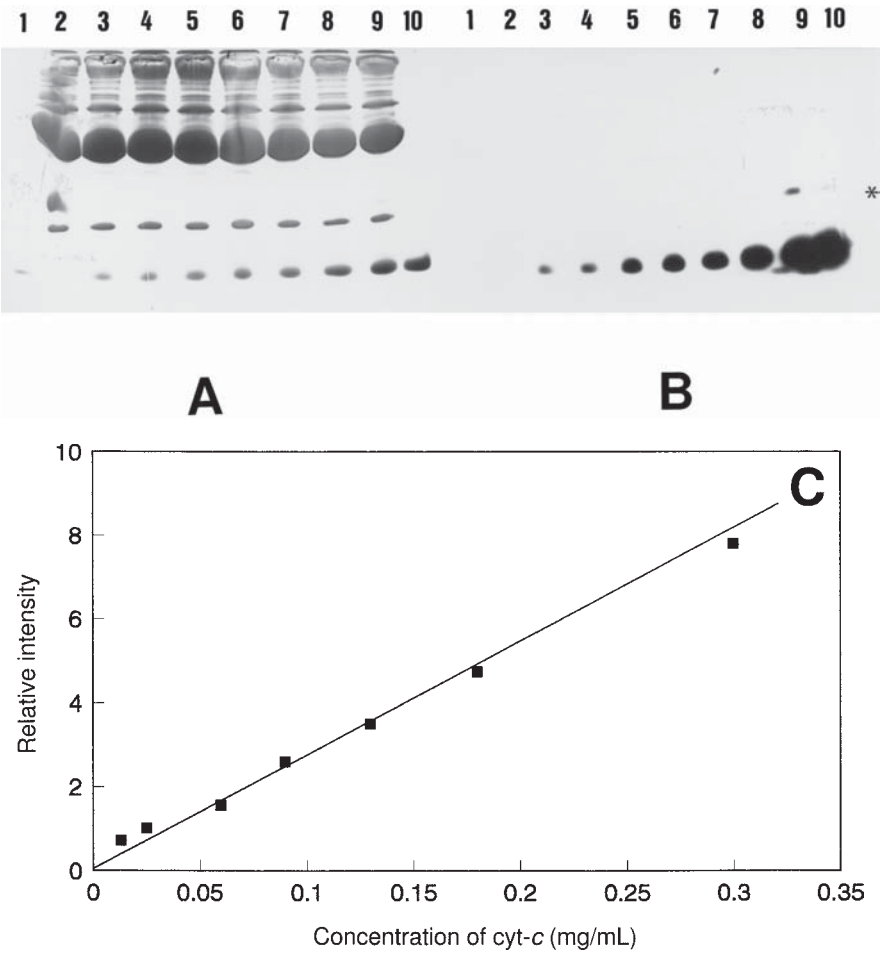

Fig. 1. SDS-PAGE of serum from a normal (non-MI) subject in the presence and absence of added cytochrome-*c*. Following the addition of cytochrome-*c* to the serum from a normal (non-MI) subject, the serum proteins were resolved by SDS-PAGE on duplicate gels. Lane 1 contains 0.06 mg/mL of myoglobin; lane 2 contains a serum sample with no added cytochrome-*c*; lanes 3–9 show serum containing cytochrome-*c*, 0.013, 0.025, 0.06, 0.09, 0.13, 0.18, 0.30, and 0.60 mg/mL, respectively; lane 10 contains pure cytochrome-*c*, 0.60 mg/mL, as a marker. **(A)** Gel stained with Coomassie blue; **(B)** X-ray film showing the chemiluminescence results of the duplicate gel. The native cytochrome-*c* in lane 10 and the serum samples with added cytochrome-*c*, lanes 3–9, but not the samples in lanes 1 and 2, produced fogging of the X-ray film. The intensity of the fogging increased with cytochrome-*c* concentration. The small proportion of cytochrome-*c* dimer, lane 9 (*), was undetected by Coomassie blue stain. **(C)** Densitometric results presented in the form of a standard curve of band intensity vs cytochrome-*c* concentration.

0.09, 0.13, 0.18, 0.30, and 0.60 mg/mL, respectively. Lane 10 contains 0.60 mg/mL of cytochrome-*c* used as a marker. On the X-ray film (Fig. 1B), no fogging was observed for lanes 1 and 2, which corresponded to the myoglobin and the control serum sample, respectively. For lanes 3–9, which

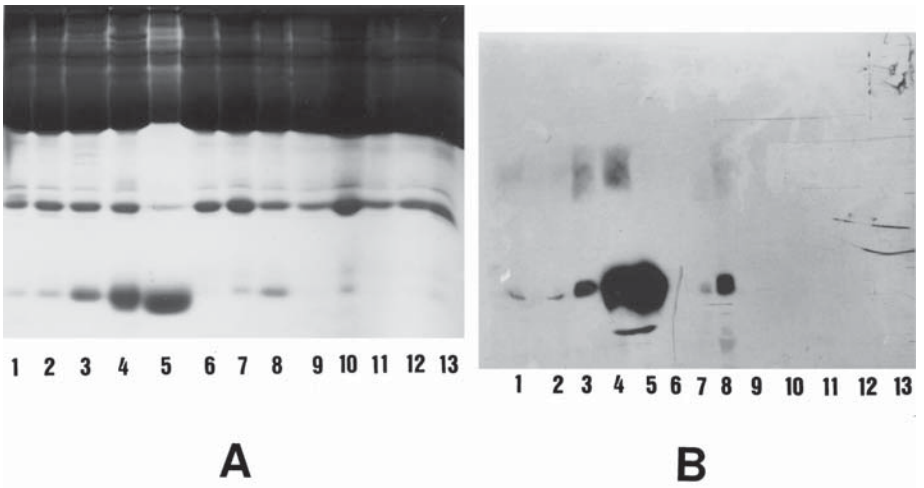

Fig. 2. Comparison by SDS-PAGE on duplicate gels of the protein composition of serum from nine normal subjects and four MI patients. Lanes 1–4 contain serum from MI patients; lane 5 contains a normal serum sample injected with cytochrome-*c* to a final concentration of 0.5 mg/mL; lanes 6–13 contain serum from normal subjects. **(A)** Gel stained with Coomassie blue; **(B)** X-ray film showing the chemiluminescence results of the duplicate gel.

contained serum treated with increasing concentrations of cytochrome-*c*, the X-ray film showed a single dark band in each lane; the exception was lane 9, which showed an additional minor band. There was also a single dark band in lane 10.

Because the only protein present in lane 10 was the cytochrome-*c* marker, we conclude that the dark band in lane 10 of the X-ray film resulted from this protein. Also, it can be deduced that the major dark bands in lanes 3–9 of Fig. 1B were owing to cytochrome-*c*. First, it was observed that these bands resulted from a protein that had the same relative mobility as cytochrome-*c*. Second, the densitometric analysis, Fig. 1C, shows that the intensity of the bands increased proportionally with increasing cytochrome-*c* concentration. Finally, in contrast, lane 2 of Fig. 1B, which also contained serum but with no added cytochrome-*c*, did not produce darkening of the X-ray film. The minor dark band seen in lane 9, which was not detected by Coomassie blue stain, most probably represents a small proportion of dimerized/polymerized cytochrome-c; this protein is known to dimerize/polymerize at high concentrations *(19)*. The main conclusion to be drawn from these results is that the technique of chemiluminescence when combined with SDS-PAGE can selectively detect cytochrome-*c* from among a host of other serum proteins. Moreover, the technique seems capable of detecting as little as 0.03 μg of cytochrome-*c*.

The stained SDS gels shown in Figs. 2A and 3A compare the protein composition of 13 of the 44 normal subjects studied with the 7 samples obtained from the 6 MI patients. Figures 2B and 3B show the X-ray results that were generated from the respective duplicate gels.

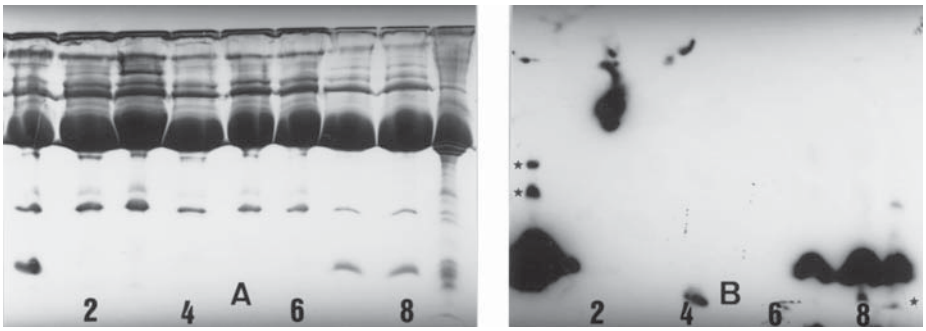

Fig. 3. Comparison by SDS-PAGE on duplicate gels of the protein composition of serum from five normal subjects and three MI patients. Lane 1 contains a normal serum sample injected with cytochrome-*c* to a final concentration of 0.5 mg/mL; lanes 2–6 contain serum from five normal subjects; lanes 7–9 contain three MI samples. **(A)** Gel stained with Coomassie blue; **(B)** X-ray film showing chemiluminescence results of the duplicate gel.

For Fig. 2A,B, lanes 1–4 contained serum from four of the seven MI samples. Lane 5 contained a normal sample that was inoculated with 0.5 mg/mL of cytochrome-*c*, and lanes 6–13 contained serum from eight normal subjects.

In Fig. 3A,B, lane 1 shows a normal serum sample containing commercial 0.5 mg/mL of cytochrome-*c*. Lanes 2–6 contain serum from five normal subjects and lanes 7–9 contain three MI serum samples obtained from two patients: samples 7 and 8 were obtained from the same patient but at different times.

It was observed that the seven MI serum samples, Fig. 2A (lanes 1–4) and Fig. 3A (lanes 7–9), contained a protein band that had the same relative mobility as commercial cytochrome-*c* (*see* Fig. 2, lane 5 and Fig. 3, lane 1). We concluded that at least a fraction of this protein was cytochrome-*c* because they produced fogging of the X-ray film during chemiluminescence (Figs. 2B and 3B). Cytochrome-c_1, the only other *c*-type protein present in mitochondria, is three times heavier.

The positive results obtained for three of the MI samples, Fig. 3 (lanes 7–9), are particularly interesting. For these three samples, serum was prepared from blood that was left standing at 4°C for close to or just over 2 wk, and by this time there was considerable hemolysis. For two of these (the samples in lanes 8 and 9, but particularly for the sample in lane 9), there was evidence of cytochrome-*c* degradation. The latter sample gave rise to a number of low molecular weight protein fragments, one of which produced a chemiluminescence signal.

The amount of cytochrome-*c* present in the serum of the MI patients as estimated from the standard curve was significant, between 0.06 and 0.25 mg/mL, for five of the MI samples (Fig. 3, lanes 7–9 and Fig. 2, lanes 3 and 4). Much less cytochrome-*c* was detected, approx 0.01–0.015 mg/mL for the two remaining MI samples (Fig. 2, lanes 1 and 2, respectively).

The comparatively weak chemiluminescence signals produced by the MI samples in Fig. 2 compared with those in Fig. 3 suggest that in the former, there was comigration of a protein with the same molecular weight as cytochrome-*c*. A protein of similar molecular weight was also observed in a number of the 44 controls studied (data not shown), but only two of these (Fig. 4A,B, lanes 7 and 8) were found to be cytochrome-*c*, initiating a chemiluminescence reaction; this result represents a 4.5% false positive.

Discussion

The peroxidase activity of heme groups, previously described by Welton and Aust *(20)* and McDonnel and Staehelin *(21)*, forms the basis of the chemiluminescence technique employed in the present study. The reaction is catalyzed by hemes and therefore can be initiated by all heme proteins. When combined with SDS-PAGE, however, the technique becomes specific for *c*-type heme proteins *(17)*. The reason is that the heme group of this class of proteins, unlike that of others, is covalently linked to the polypeptide chain and therefore travels with the polypeptide during electrophoresis. For other heme proteins, the noncovalent heme moiety dissociates from the polypeptide chain during denaturation and migrates off the gel during electrophoresis.

There are two *c*-type cytochromes present in mitochondria: cytochrome-*c* and cytochrome-c_1. Although the two proteins can be easily distinguished on the basis of molecular weight—cytochrome-c_1 is approximately three times heavier than cytochrome-*c*—the appearance of either in the serum would be indicative of mitochondrial damage. However, whereas there have been a number of reports of elevated serum cytochrome-*c* following MI *(14–16)*, elevated levels of cytochrome-c_1 have not been reported. The reason for this is almost certainly linked to their different location within the mitochondria. Cytochrome-*c* is an extrinsic protein and therefore readily leaks out of damaged mitochondria. Cytochrome-c_1, on the other hand, is intrinsic and is firmly embedded in the inner membrane of the mitochondria. Moreover, this protein exists in the form of a tight complex with another membrane-bound protein, cytochrome-*b*, so that simple rupture of the outer mitochondrial membrane will not permit its escape. We did not detect the presence of cytochrome-c_1 in the serum of the MI patients.

In agreement with the findings of Vargas et al. *(17)*, we found that the intensities of the bands, which resulted from the application of chemiluminescence, were significantly enhanced compared with those resulting from Coomassie blue stains: we observed a 30-fold enhancement. In fact, the technique was able to detect as little as 0.03 μg of cytochrome-*c*, and it picked up small proportions of cytochrome-*c* polymers, which were undetected by Coomassie blue stain. Thus, in addition to being specific for *c*-type cytochromes, the technique significantly extends the detection limits for this class of proteins. Our finding that the serum from the MI patients

contained detectable levels of cytochrome-*c* (0.01–0.25 mg/mL) whereas that from normal subjects did not is consistent with reports by Andrianova and Sidorova *(14)* and Gvatua et al. *(16)*.

Two factors—the severity of the MI and elapse time between the infarct and sample collection—determine the concentration of the marker molecules found in serum. These factors would account for the different levels of cytochrome-*c* found in the serum of the seven MI samples. For three of the samples (Fig. 3A,B, lanes 8 and 9), the serum was not isolated until 2 wk after blood collection. The fact that despite this long period of standing and despite extensive hemolysis, high levels of cytochrome-*c* were still detected is a major plus for the technique.

Like myoglobin, a marker currently in use *(22)*, cytochrome-*c* is not known to be tissue specific. Thus, several conditions other than MI, such as muscle or hepatic damage, would be expected to precipitate elevated serum levels of the identical form of this protein. It is very likely that one of these other conditions was responsible for the elevated cytochrome-*c* seen in the serum of the two positive controls. It is unlikely, however, that "false" positives will create major difficulties in the clinical setting because the patient's presentation and, when possible, his or her history are normally combined with ECG and other laboratory results in arriving at a final diagnosis.

Acknowledgments

We thank Dr. K. Itiaba for supplying the MI serum samples, A. Lalla for technical assistance, and Caribbean Health Research Council for its financial support.

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