Early Diagnosis of Acute Myocardial Infarction Based on Assay for Subforms of Creatine Kinase–MB

Peter R. Puleo, MD, Philip A. Guadagno, MS,
Robert Roberts, BSc, MD, Mary V. Scheel, BS, Ali J. Marian, MD,
David Churchill, MD, and M. Benjamin Perryman, PhD

Thrombolytic therapy for patients with acute myocardial infarction (AMI) has produced the need for an accurate early diagnostic marker. We previously developed and assessed an assay for the creatine kinase (CK)–MB subforms; assay time is 25 minutes. Plasma MB2 (tissue subform) activity, MB1 (plasma-modified subform) activity, and MB2/MB1 ratio in 56 healthy individuals were 0.61±0.33 units/l, 0.63±0.33 units/l, and 0.94±0.39, respectively. Only one individual had both an MB2 activity greater than 1.0 units/l and an MB2/MB1 ratio of more than 1.5. Similar results were obtained in 50 hospitalized patients without cardiac disease; two of these patients had both an MB2 activity and an MB2/MB1 ratio greater than the cutoff values. Among 49 patients with AMI, MB2 activity and the MB2/MB1 ratio began to increase 2 hours after AMI; the ratio reached a plateau of 3.1 by 4–6 hours. The first available plasma sample was abnormal by the subform assay in 67% of patients and by a conventional MB assay in 27% of patients. Assay sensitivities in samples collected at 2–4, 4–6, and 6–8 hours after AMI were 59%, 92%, and 100% for the subform assay and 23%, 50%, and 71% for the conventional assay (p<0.03 versus subform assay at each time interval). False-negative results were obtained by the subform and conventional assays in 15 and 45 samples at a mean of 2.3 and 5.8 hours, respectively. Subform assay provides rapid and reliable diagnosis of AMI within 4–6 hours after the onset of symptoms, which is 6 hours before conventional CK-MB assays are accurate. (Circulation 1990;82:759–764)

Thrombolytic therapy is now standard initial treatment of patients with acute myocardial infarction (AMI). Recent data have confirmed that early implementation of therapy is required for maximum reduction in mortality rates1–3; consequently, delay of treatment to obtain confirmatory diagnostic information is precluded. However, accurate diagnosis of AMI in the early hours remains problematic. Chest discomfort and early electrocardiographic manifestations are nonspecific and do not distinguish transient myocardial ischemia from necrosis; only 30% of patients hospitalized with suspected acute necrosis are subsequently confirmed to have AMI.4 In addition, conventional assays for plasma creatine kinase (CK)–MB require at least 8–12 hours from the onset of symptoms to provide reliable diagnostic data.5,6 Because thrombolytic therapy carries a small risk of life-threatening hemorrhage,7 mandates subsequent heparin therapy, and necessitates prolonged monitoring in an intensive care setting, a diagnostic marker that is reliable within 6 hours of the onset of symptoms would be useful in detecting patients without AMI in whom treatment could be withheld or aborted. Such a marker would also be valuable in facilitating the accurate diagnosis and triage of patients with chest pain in the emergency department.

Several years ago, we purified the plasma subforms of the MM isoenzyme of CK and described the mechanism whereby plasma carboxypeptidase-N catalyzes the conversion of CK-MM released from tissue (CK-MM3) to the modified plasma subforms (MM2

See p 1073
and MM1). In the early hours of AMI, the ratio of the newly released tissue form of CK-MM to the plasma subforms is increased, providing a marker for the early diagnosis of myocardial infarction. However, this finding lacks specificity; a similar shift in MM subform ratio is observed in noncardiac muscle injury. Accordingly, we investigated the subforms of CK-MB, an isoenzyme specific to myocardial muscle, as a potentially more specific indicator for the early diagnosis of AMI. CK-MB, like CK-MM, exists as a single form in tissue (MB2) and is modified by the plasma enzyme carboxypeptidase-N, yielding the more electronegative subform MB1.

We postulated that the baseline plasma MB2/MB1 ratio is altered upon minimal release of the tissue form, so a significant change in the ratio occurs in the early hours of AMI, even before total plasma CK-MB activity surpasses the upper limit of normal. Until recently, available assays lacked the necessary sensitivity to detect the individual MB subforms in normal plasma, preventing the use of the MB subforms for early diagnosis of infarction. We have recently developed and validated an assay for MB2 and MB1 that is sensitive, precise, and reliable for both subforms at activities within the normal range for CK-MB.

The present study was performed to determine the sensitivity and specificity of this assay for early diagnosis of AMI in patients presenting within 12 hours of the onset of symptoms.

**Methods**

The study population consisted of 55 consecutive patients admitted to the Methodist Hospital in whom the diagnosis of AMI was established by a history of prolonged (more than 30 minutes) chest pain at rest; an abnormal electrocardiogram, with at least 1 mm of ST segment elevation or depression in at least two leads or evolution of new Q waves; and a typical rise-and-fall plasma CK-MB activity as measured by a conventional CK-MB isoenzyme assay (see below). The time of onset of symptoms was carefully recorded for each patient at the time of admission. Plasma samples were also collected from 56 healthy adult volunteers and 50 hospitalized adult patients with no histories suggestive of myocardial ischemia.

**Isoenzyme and Subform Assays**

Blood was collected from all patients on presentation to the emergency department and every 2–4 hours thereafter. Isoenzyme and subform data were obtained from the same plasma specimen. Samples obtained within 12 hours of the onset of symptoms were included in the study, except for samples obtained after the initiation of thrombolytic therapy. Samples were collected in vacuum tubes pretreated with 2-mercaptoethanol to produce a final concentration of 10 mmol/l and with ethylene glycol bis (β-aminophenyl ether)–EGTA to produce a final concentration of 30 mmol/l. EGTA inhibits in vitro carboxypeptidase-mediated subform conversion after sample collection. Samples were then centrifuged for 5 minutes at 1,500 rpm, and the plasma was removed for immediate assay or storage at –20°C. Plasma total CK activity was assayed by the method of Rosalki; the upper normal limit is 120 units/l at 30°C. Plasma CK-MB activity was determined with a sensitive and precise batch adsorption assay (Lancer MB CK Isoenzyme Separation System, Sherwood Medical, St. Louis, Mo.); this method is used for clinical CK-MB determinations at the Methodist Hospital. The upper normal limit by this assay in our laboratory is 14 units/l.

CK-MB subform activity was assayed by a recently developed quantitative system in which the sample is subjected to high-voltage electrophoresis on dynamically cooled agarose gels (Rep, Helena Laboratories, Beaumont, Tex.). The use of agarose with low electroendosmosis characteristics and the maintenance of the gel at a low temperature during electrophoresis result in rapid separation of the differentially charged subforms of CK-MB with excellent preservation of activity. This system measures both the absolute activity of the two subforms and the relative percent of each subform, so delay for independent MB assay is not necessary. The subform assay is accurate from 0.0 to 30.0 units/l and requires only 25 minutes to perform the assay and complete the data analysis.

Assay specificity was defined in the population of hospitalized patients without active heart disease by the following formula: Specificity = true-negative samples/(true-negative samples + false-positive samples) × 100. Among the patients with AMI, samples were grouped by 2-hour intervals after the onset of symptoms, and sensitivity for each time interval was defined as true-positive samples/(true-positive samples + false-negative samples) × 100. No patient contributed more than one sample within one time interval.

**Statistics**

Values are given as mean ± SD. Differences among the control groups and patients with AMI in plasma MB2 activity, MB1 activity, and MB2/MB1 ratio were assessed with the Mann-Whitney U test. Differences in diagnostic sensitivity between the MB subform assay and the conventional MB isoenzyme assay performed on the same plasma samples were analyzed by McNemar’s χ² test. A p value of less than 0.05 was considered statistically significant.

**Results**

**Subform Activity in Control Groups**

Subform assay performed on plasma samples obtained from 56 healthy volunteers showed a mean MB2 activity of 0.61 ± 0.33 units/l and a mean MB1 activity of 0.63 ± 0.33 units/l. MB2/MB1 ratio was 0.94 ± 0.39. In six of the 56 normal individuals, plasma MB2 activity was more than 1.0 units/l; in five of these six individuals, plasma MB1 activity was also elevated, so the MB2/MB1 ratio was less than 1.5.
The cutoff values established in the normal population were then evaluated in plasma collected from 50 hospitalized patients without active cardiac disease. Mean MB2 activity, mean MB1 activity, and MB2/MB1 ratio were 0.48±0.40 units/l, 0.42±0.29 units/l, and 1.09±0.39, respectively. The mean subform activities and relative subform ratios did not differ significantly among normal volunteers and hospitalized patients. Five of the hospitalized patients had a plasma MB2 activity of more than 1.0 units/l; two of these also had a plasma MB2/MB1 of more than 1.5—an 84-year-old woman with bacterial pneumonia and a 78-year-old woman with small bowel obstruction. Therefore, the definition of an abnormal result as having both an MB2 activity and an MB2/MB1 ratio in excess of the cutoff values provided a specificity of 98% in the normal individuals and 96% in the hospitalized patients.

Patients With AMI

All patients had an increase in plasma CK-MB activity (by the conventional assay) to levels of more than the upper normal limit within 24 hours of the onset of symptoms. Thirty-eight patients evolved electrocardiographic Q waves; three of these patients were unable to give an adequate history because of cardiogenic shock or intubation after resuscitation but had well-defined times of onset of symptoms. One patient presented with left bundle branch block of unknown duration, prolonged chest pain, and pulmonary edema and was grouped with the patients having Q wave AMI. Sixteen patients were determined to have non-Q wave AMI by the presence of typical chest pain, ST segment elevation or depression without Q wave evolution, and diagnostic enzymatic criteria, as described above. Six patients (four with Q wave and two with non-Q wave AMI) were hospitalized more than 12 hours after the onset of symptoms and are excluded from further analysis. Eight patients who received thrombolytic therapy during the study period had single baseline samples obtained before the start of therapy; samples obtained from these patients after the initiation of therapy were excluded. A mean of 3.1 samples per patient were obtained from the remaining patients (range, one to four samples). No patient had more than one sample removed within a 2-hour interval.

Subform Activity in AMI

Results of plasma subform assay performed on samples collected from patients with AMI are shown in Table 1. A total of 122 samples were collected from these patients during the first 12 hours after the onset of symptoms. A rise in mean plasma MB2 activity and subform ratio was evident in samples obtained between 2 and 4 hours after the onset of AMI. Plasma MB2 activity rose throughout the first 10 hours of AMI; mean plasma MB2/MB1 ratio quickly reached a plateau by 4 hours after AMI; and no subsequent decline in MB2/MB1 was seen during the 12 hours encompassed by this study. Plasma MB1 activity increased throughout the first 10 hours at a rate approximately 20% of that of the MB2 activity rise. By definition, the %MB1 declined reciprocally with the increase in %MB2 during the first 4 hours and then did not change significantly during the remainder of 12 hours after AMI.

Time-Dependent Sensitivity of Subform Analysis for Diagnosis of AMI

Using the cutoff values established in the normal population and confirmed in the hospitalized patients, analyses of the first available blood samples provided positive results in 33 patients (67.3%) by the subform assay and only 13 patients (26.5%) by the conventional assay (Table 2). The first sample was positive by the subform assay and negative by the conventional assay in 20 patients (40.8%); these samples were obtained a mean of 4.5 hours after the onset of symptoms. In 16 patients (32.6%), the first sample was negative by both assays; in these patients, the initial sample was obtained an average of 2.3 hours after the onset of symptoms. The remaining 13 patients (27%) had positive results from both

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<th>Table 1. Mean Subform Activity and Ratio in Control Subjects and Patients With Acute Myocardial Infarction</th>
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<td>Population</td>
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<td>Control groups</td>
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AM, acute myocardial infarction. Values are given as mean±SD.
*p<0.01 versus control groups.
assays in the first specimen. No patient had a negative subform assay result in a sample that was positive by the conventional assay. During the entire 12-hour sampling interval, false-negative results by the conventional isoenzyme assay were obtained in 54 samples from 34 patients; these samples were collected a mean of 5.8 hours after the onset of AMI. False-negative subform assay results were obtained in 16 samples from 16 patients; mean time of these results was 2.3 hours after AMI. No sample collected subsequent to the initial sample yielded a false-negative result by the subform assay.

The criteria for diagnosis of AMI by the subform assay outlined above were insensitive during the first 2 hours after the onset of infarction. The sensitivity of the subform assay increased to 59% in specimens collected 2–4 hours after the onset of AMI and to 92% in specimens collected 4–6 hours after the onset of symptoms. No false-negative results by the subform assay were obtained in specimens collected 5 or more hours after the onset of AMI. In contrast, the sensitivity of the conventional assay was less than 50% during the first 6 hours of AMI and did not exceed 90% until 10–12 hours after the onset of symptoms. Among the 16 patients with non–Q wave AMI, the sensitivity of the subform assay was 33% before 2 hours, 86% between 2 and 4 hours, and 100% thereafter. Due to the small number of samples in the non–Q wave group, the differences between Q and non–Q wave AMI did not achieve statistical significance.

**Discussion**

The present study documents the accuracy of the plasma CK-MB subform assay in the early hours of AMI, during the interval that conventional CK-MB assays remain in the normal range. The assay reliably detected 59% of patients with AMI in whom plasma samples were available 2–4 hours after the onset of symptoms and 92% of patients from whom samples were obtained at 4–6 hours. Corresponding sensitivity was not achieved by the conventional MB assay until 12 hours after the onset of symptoms. In 67% of the patients, the first blood sample was positive by the subform assay despite the short interval from the onset of symptoms to obtaining the first sample; the same specimen was positive by the conventional assay in only 27% of patients. The specificity of the subform assay was 98% among normal individuals and 96% in a population of hospitalized patients without active cardiac disease.

The recommended upper normal limit for plasma CK-MB activity of most conventional assays is severalfold more than the mean value for a normal population; this is necessary to achieve an acceptably low rate of false-positive results. Consequently, in most patients with AMI, CK-MB release from myocardium must be sufficient to increase the plasma activity to levels severalfold higher than the preinfarction value before CK-MB activity exceeds the established upper limit. The data presented here demonstrate that normal individuals have a 1:1 ratio of MB2 to MB1 in the blood; this permits recognition of the recent release of tissue CK-MB as an increase in the plasma MB2/MB1 ratio. As a result, recent release of CK-MB from tissue can be detected in a patient with a normal baseline plasma CK-MB activity after release of sufficient CK-MB2 to raise the plasma MB activity to only 2.0 units/l. On the other hand, individuals without AMI whose plasma CK-MB activities are in the high-normal range are correctly classified because the MB2/MB1 ratios are less than 1.5, despite relatively high absolute CK-MB values.

The parameters used for the cutoff values were defined in a normal population, and the specificity was confirmed in hospitalized patients without known active cardiac disease. We elected not to define the specificity of this algorithm in a population of patients with active coronary disease in whom the diagnosis of AMI had been excluded because of the problem inherent in defining a gold standard for AMI in this setting. Appropriate categorization of the substantial subset of our patients with prolonged ischemic symptoms who are currently regarded as having unstable angina, some of whom may release a small quantity of myocardial CK, would result in a significant bias against the subform assay. We are currently assessing the changes in plasma subform levels in patients admitted to the coronary care unit with unstable angina.

A potential limitation of the plasma subform assay is the limited duration of the shift in the plasma subform ratio. The plasma MB2/MB1 ratio remained above the cutoff value of 1.5 throughout the 12-hour study period. However, preliminary data from our laboratory suggest that this value declines during the subsequent 12 hours. Thus, the time window during
which the subform assay is reliable remains to be defined but may be confined to the initial 14–18 hours of AMI. After this period, the use of a conventional CK-MB assay might be preferable. We\textsuperscript{13} and others\textsuperscript{12} have observed a similar decline in plasma %MM3; the decline in %MM3 in these trials occurred earlier than that observed in the present study for MB2/MB1 ratio, suggesting that the time window during which the plasma MM subforms are diagnostic might be more limited than that of the MB subforms. We are currently assessing the subform clearance kinetics of both isoenzymes in patients as well as in the dog model.

This study represents the first clinical assessment of the CK-MB subforms as diagnostic markers. We have previously assessed the kinetics of the CK-MM subforms in AMI; however, the assay required 90 minutes and had a lower limit of detection of 60 units/l. To achieve rapid separation of the subforms, we developed an assay based on high-voltage electrophoresis that separates MB2 from MB1 in 12 minutes on thin-layer agarose gels. Gel surface temperature, determined by real-time infrared photography, is maintained at 25°C by thermostatically controlled peltiers; consequently, there is no loss of CK activity during electrophoresis.\textsuperscript{18} The rapid separation of the MB subforms that is achieved by high-voltage electrophoresis makes the system well suited for immediate analysis of specimens from patients with AMI. The assay is precise and linear for both subforms at activities ranging from 0 to 30 units/l. The precision at 1.25 units/l is ±15.4% (0.19 units/l) for MB2 and ±16.7% (0.21 units/l) for MB1,\textsuperscript{18} which permits accurate distinction between normal individuals and patients in the early hours of AMI. Because the extent of normal daily biologic variation in the plasma subform activity of healthy individuals is unknown and is probably less than the limits of error of our assay, it is possible that an assay providing further enhancement of the analytic sensitivity for the subforms would allow detection of AMI at even earlier time points than were possible in our study.

Several investigators have studied the subforms of the MM isoenzyme as early markers of AMI. Morelli and coworkers reported the first change in CK-MM subform activity to occur at 6 hours after onset of AMI.\textsuperscript{10} Wu et al used two different assays for MM subforms and reported a sensitivity of 90% within 7 hours with both assays.\textsuperscript{12} In 28 patients with AMI, Jaffe et al obtained a sensitivity of 86% in the first specimen.\textsuperscript{11} However, assay of total plasma CK activity without MB isoenzyme determination has resulted in a false-positive rate of 30%.\textsuperscript{22} Because the MM isoenzyme is present in high activity in skeletal muscle, a shift in the MM subform ratio is not specific for myocardial injury and has been shown to occur after trauma and in a variety of diseases that affect skeletal muscle. Implementation of an intramuscular delivery system for the administration of thrombolytic agents would be expected to further confound interpretation of diagnostic plasma MM subform data. Another proposed early marker of myocardial injury, myoglobin, appears in the blood as early as 2 hours after onset of AMI but, like CK-MM, is abundant in skeletal muscle and therefore is a nonspecific diagnostic marker. In contrast, CK-MB is present in high concentration only in myocardium and is rarely elevated in the blood in the absence of myocardial necrosis.\textsuperscript{23}

The difference that we observed in time to diagnosis between the subform and conventional assays was not due to diagnostic inefficiency of our clinical CK-MB assay. Lee et al observed a sensitivity of 77% during the first 12 hours of AMI using a conventional CK-MB isoenzyme assay,\textsuperscript{2} findings that are compatible with those observed in the present study. Similarly, Irvin et al found a sensitivity of 81% during the first 12 hours.\textsuperscript{4} Thus, our finding of a 93% sensitivity within 10–12 hours represents an improvement over previously reported results for a conventional MB assay.

The biochemical modification producing the MB1 subform has not yet been precisely characterized. The existence of MM and MB subforms was first described by Wevers and coworkers.\textsuperscript{24} Three MM and two MB subforms have been observed. We have purified and characterized the MM subforms and shown that conversion of the MM subforms is mediated by the sequential cleavage of the two carboxy-terminal lysine residues by the plasma enzyme carboxypeptidase.\textsuperscript{8,9} Billadello et al.\textsuperscript{25} using modified CK-MM and CK-BB as precursors, recently produced and characterized modified MB subforms in vitro that have both polypeptide chains modified. The modified forms could be distinguished from one another by fast protein liquid chromatography and agarose electrophoresis. The pattern of cleavage produced by carboxypeptidase-N on MB purified from canine myocardium in vitro was compatible with cleavage of lysine from the M chain only, which is the mechanism we observed for conversion of CK-MM. Despite the high resolution of our subform assay, which allowed distinction between two molecules having a difference of a single electron charge, we have never observed a third band of CK-MB, as would be expected if both polypeptide chains were modified.

The clinical implications of our findings remain to be defined. Although the subform assay can be performed in 25 minutes, even a brief delay in thrombolytic therapy for patients in whom there is a high clinical suspicion of AMI is unwarranted. However, negative subform assay results in samples obtained 5 or more hours after the onset of symptoms would make the diagnosis of MI very unlikely and permit prompt cessation of thrombolytic therapy and avoidance of heparin anticoagulation in patients without AMI. The finding of a high sensitivity within 2 hours after the onset of symptoms in patients with non-Q wave AMIs is consistent with the more rapid release of CK reported in this entity.\textsuperscript{26} If confirmed in a larger number of patients, these data would be
particularly important if the indications for thrombolysis are expanded to include patients with non-Q wave infarction, because electrocardiographic findings in this condition are entirely nonspecific, and the decision to implement therapy is potentially difficult. Recent data suggest that patients presenting more than 6 hours after the onset of symptoms may derive significant benefit from thrombolytic therapy, although clearly less so than when treatment is initiated sooner.\textsuperscript{3,27} In such a population, results of subform analysis would be definitive by the time of hospital presentation. In addition, the subform assay could substantially enhance the emergency department diagnosis of AMI. Patients for whom the suspicion of AMI is relatively low are currently admitted to coronary care units until the diagnosis of AMI can be excluded. Less than 30\% of these patients are subsequently shown to have sustained an infarction.\textsuperscript{4} Accurate diagnosis in the early hours of AMI would facilitate the triage of many such patients to units with a lower intensity of care, resulting in more efficient and economic use of the critical care facility.

The present study was not designed to assess the noninvasive detection of reperfusion with the MB subforms. Preliminary data from our laboratory on a subsequent series of patients suggest that successful reperfusion is characterized by a higher subform ratio than is present in the patients observed in this series and that this difference can be observed within 2 hours of initiation of therapy. However, these data require confirmation in a series of patients who have undergone early angiography for the assessment of therapeutic success.

References


**KEY WORDS** • isoenzymes • subbands • isoenzymes
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