Early detection of myocardial infarction in conscious dogs by analysis of plasma MM creatine kinase isoforms*

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ABSTRACT To determine whether myocardial infarction could be detected early after onset by analysis of subforms of the MM isoenzyme (isoforms) of creatine kinase (MM CK) in plasma, we subjected eight conscious dogs to coronary occlusion and quantified isoforms in serial plasma samples by chromatofocusing. The fractions of MM₈ (isoelectric point [pI] = 7.91), MM₉ (pI = 7.74), and MMc (pI = 7.51) in plasma samples before coronary occlusion averaged 11.4 ± 4.8% (SD), 22.3 ± 5.5%, and 66.3 ± 9.6% of total MM CK activity. The fraction of MM₈, the isoform of MM CK found in myocardium, increased significantly in plasma 1 hr after coronary occlusion, reached a maximum of 49.7 ± 8.0% in 4.1 ± 1.3 hr, and returned to baseline in 12.0 ± 2.3 hr. The fraction of plasma MM CK activity attributable to MMc, an isoform formed slowly in plasma from MM₈ via MM₉ as an intermediate, decreased significantly within 1 hr, reached a minimum of 14.0 ± 4.1% in 4.8 ± 1.1 hr, and returned to baseline in 13.0 ± 2.9 hr after coronary occlusion. Total CK activity did not increase significantly until later, i.e., 5 hr after occlusion, and peaked at 1371 ± 530 IU/liter in 10.9 ± 1.9 hr. Within the first 4 hr after coronary occlusion, MM₈ consistently comprised more than 20% of plasma MM CK activity despite insignificant increase of total CK. Changes in isoform proportions were consistent and independent of peak total CK activity and of cumulative CK release over a 10-fold range. Thus initial CK release indicative of infarction is detectable within 1 hr after the onset of ischemia by quantification of plasma MM CK isoforms.


EARLY DIAGNOSIS and accurate timing of the onset of initial and recurrent myocardial infarction are useful for patient selection and assessment of interventions such as coronary thrombolysis designed to salvage jeopardized myocardium.¹ The diagnosis of infarction is frequently established by detection of elevation of the MB isoenzyme of creatine kinase (CK) (EC 2.7.3.2) in plasma. Characterization of total CK and MB CK time-activity curves has been useful for estimation of infarct size.²⁻⁴ However, plasma CK activity does not generally exceed the normal range for several hours after the onset of ischemia. Furthermore, time-activity curves vary with infarct size so that early detection and timing of infarction are difficult to assess by analysis of CK activity alone.

Recently, subforms of individual isoenzymes of CK (isoforms) distinguished by differences in isoelectric points (pI) have been found in plasma after myocardial infarction.⁵⁻⁹ Isoforms in plasma were soon recognized to reflect not only the form released into plasma from tissue but also conversion of one form to another in plasma in vitro or in vivo.¹⁰ Using a quantitative chromatofocusing assay that avoided intrapreparative denaturation, we have characterized the kinetics of conversion.¹¹ The isoform in either normal or necrotic myocardium was found to be MM₈ (pI = 7.91). The MM₉ (pI = 7.74) and MMc (pI = 7.51) isoforms evolved sequentially from MM₈ in a consistent, time-dependent fashion in plasma in vitro and in vivo. These forms appear to correspond to those separated by isoelectric focusing (IEF) that have been designated with numerical subscripts, but differ in apparent isoelectric points: MM₈ corresponds to MM₁ (pI = 6.86

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by IEF); MM\(_b\) corresponds to MM\(_a\) (pl = 6.49); and MM\(_c\) corresponds to MM\(_b\) (pl = 6.24).

Because the peak activity of tissue isoform in plasma after infarction has been found to occur several hours before the time of occurrence of peak total CK activity,\(^8\) it seemed likely that changes in the relative proportions of isoforms in plasma after myocardial infarction might occur before total CK activity became significantly elevated. This study was performed to determine whether or not analysis of changes of MM CK isoform patterns after coronary arterial occlusion in conscious dogs facilitated early detection of infarction.

**Methods**

**Animal preparations.** Fifteen male mongrel dogs weighing 16 to 23 kg were anesthetized with pentobarbital (30 mg/kg iv), and a left thoracotomy was performed aseptically via the fifth intercostal space. A snare made from 20 pound test nylon line within a polyethylene (PE240) tube was looped around the left anterior descending or left circumflex coronary artery distal to the first major branch. Its end was coiled in a subcutaneous pocket and the chest was closed. An external jugular vein was catheterized with silicone rubber (Silastic) tubing filled with 1000 U/ml heparin. The distal end of the catheter was exteriorized posteriorly.

Seven days after surgery, when plasma total CK activity had returned to normal (<100 IU/liter), 250 ml of blood was obtained through the jugular venous catheter and stored at 4°C in fresh acid citrate dextrose (ACD) (15 ml/100 ml blood of 80 mM sodium citrate, 35 mM citric acid, and 120 mM dextrose). To avoid hypovolemia, an equal volume of saline was infused intravenously.

Three days after collection of blood the dogs were supported in a sling, and electrocardiographic monitoring was initiated. The coronary snare was exteriorized through a small incision, lidocaine (30 mg iv) was given, and the snare was tightened to occlude the left anterior descending or left circumflex coronary artery distal to the first major branch. The cones were washed three times with 100 ml of chromatofocusing equilibration buffer (pH 8.95) to recover virtually all of the CK activity and the total volume (<1 ml) was applied to the chromatofocusing column. Blue Sepharose chromatography and concentration of the sample removed 80% to 90% of the total protein in the plasma aliquot without appreciable loss of CK activity.

When total CK activity in the plasma sample was less than 100 IU/liter, a 10 to 15 ml aliquot of plasma was applied to an 80 ml Blue Sepharose column. The pH 8.2 eluent was passed through a 5 ml protein A Sepharose column (CL4B; Pharmacia, Piscataway, NJ) affinity chromatography. A plasma volume with at least 1000 mIU of activity was diluted fivefold with 50 mM sodium phosphate buffer at pH 5.8 containing 2 mM \(\beta\)-mercaptoethanol, 0.4 mM EDTA, and 5 mM magnesium chloride. After centrifugation at 1900 g for 10 min, the supernatant fraction was applied to either 12 ml (1 to 2.5 ml of plasma) or 80 ml (3 to 10 ml of plasma) Blue Sepharose columns previously equilibrated with the pH 5.8 buffer.

Selective elution of CK protein from Blue Sepharose was accomplished with 50 mM sodium phosphate buffer at pH 8.2 containing 5 mM \(\beta\)-mercaptoethanol, 0.2 mM EDTA, and 5 mM magnesium chloride. Fractions containing CK activity were pooled in filtration membrane cones (CF25; Amicon, Danvers, MA) and concentrated to 0.6 to 0.7 ml by centrifugation for 30 min at 950 g (4°C). The cones were washed three times with 100 ml of chromatofocusing equilibration buffer (pH 8.95) to recover virtually all of the CK activity and the total volume (<1 ml) was applied to the chromatofocusing column. Blue Sepharose chromatography and concentration of the sample removed 80% to 90% of the total protein in the plasma aliquot without appreciable loss of CK activity.

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CK fractions were pooled and concentrated in filtration membrane cones to a volume of less than 1 ml for chromatofocusing. Protein A Sepharose removed 50% to 60% of the non-CK protein remaining after Blue Sepharose chromatography. Recovery of CK activity exceeded 90% and was comparable for each MM CK isoform.

After reduction of the non-CK plasma proteins and concentration of the CK activity, pH of the sample was adjusted to 8.8 and the sample was applied to the chromatofocusing column as previously described. Fractions of each of the three MM CK isoforms were expressed as percentages of total MM CK activity in the original plasma sample. Because specific enzyme activity is the same for each MM isoform and because the release of MB and BB CK from heart is minimal in dogs undergoing infarction, absolute enzyme activity of each MM isoform in the plasma sample was calculated by multiplying measured total CK activity by the percentage of MM CK attributable to each MM isoform.

**Accuracy and precision.** Hemodynamic changes associated with frequent blood sampling were minimized by concomitant infusion of equal volumes of autologous, whole blood anticoag,
ulated with ACD. Because ACD binds calcium, we excluded possible effects of the concentrations of ACD in transfused blood on the rate of isof orm conversion in vitro. Purified MM_A CK was obtained from canine myocardium as previously described and incubated at 37°C with canine plasma obtained before or 5 hr after coronary arterial occlusion. The 5 hr plasma sample was obtained after serial transfusions of ACD-treated blood (160 ml total volume). During 4 hr of incubation the proportions of isof orms in control plasma and in plasma containing ACD were indistinguishable.

To determine whether the Blue Sepharose or protein A Sepharose procedures affected isof orm proportions in each of the three purified MM CK isof orms detected in samples, plasma supplemented with known amounts of each isof orm was applied to Blue Sepharose and protein A Sepharose columns. For both columns, the percentages of isof orm portions in the eluent were unchanged in comparison with values in the sample applied to the column. We have shown previously that chromatofocusing per se does not affect isof orm proportions.

The reproducibility of the isof orm analytical system combining chromatofocusing with one or both of the affinity chromatography steps was tested over several days by repeated analyses (two to five per sample) of samples with high (1260 IU/liter), moderate (623 IU/liter), or low (<100 IU/liter) CK activity.

Results

Among 15 dogs subjected to coronary arterial occlusion, five survived for at least 48 hr. Ten died because of ventricular fibrillation occurring 10 min to 17 hr after occlusion. Data from three dogs that died only after total CK activity had reached a maximum are included in the results. Complete coronary occlusion and recent myocardial infarction were verified at autopsy in each case.

The reproducibility of the isof orm analyses was documented in repeated assays over several days of samples containing the entire range of total CK activities encountered in this study. Maximal differences encountered for the same sample analyzed sequentially were less than 5%. No isof orm-specific differences were evident.

CK time-activity curves. Total CK activity in plasma samples before occlusion was consistently less than 100 IU/liter (62 ± 16, range 46 to 90) and did not differ significantly from CK activity in separate, normal, unoperated dogs (69 ± 22 IU/liter, n = 7). Total CK activity began to exceed 100 IU/liter (taken as the upper limit of normal based on the mean plus 2 SDs of values in samples from normal dogs) between 2 and 5 hr after coronary occlusion. Time-activity curves of total CK exhibited considerable but expected variation from dog to dog (figure 1) because of differences in the extent of infarction sustained. Thus cumulative CK release varied over a 10-fold range. Total CK activity peaked to 421 at 1873 IU/liter, 8 to 14 hr after coronary occlusion (table 1).

Time-activity curves of MM CK isof orms showed a consistent sequence of changes despite differences in infarct size. Thus the activity of MM_A increased promptly and reached a maximum early. Changes in activities of MM_B and MM_C followed sequentially. In keeping with the association of increases in MM_A activity and total CK release, it is not surprising that the configuration of MM_A time-activity curves reflected the configuration of the total CK time-activity curves (figure 1). Consequently, configurations of MM_B and MM_C time-activity curves were reflections also of the configuration of the total CK time-activity curves.

FIGURE 1. Activities of total CK and MM_A and MM_C CK isof orms after coronary occlusion in eight dogs. Numbers on each line identify individual dogs corresponding to those listed in table 1. Because the contribution of MB CK was modest (<9% of total CK activity) and because BB CK was not detectable in plasma samples analyzed by electrophoresis, the activities of isof orms were expressed as a fraction of total CK activity.
TABLE 1  
Total CK and MM CK isoform profiles after coronary occlusion

<table>
<thead>
<tr>
<th>Dog</th>
<th>Time to CK &gt; 100 IU/l (hr)</th>
<th>Time to maximal CK (hr)</th>
<th>Maximal CK activity (IU/l)</th>
<th>Cumulative CK released (IU/l)</th>
<th>Time to maximal MMₐ (hr)</th>
<th>Maximal MMₐ (IU/l)</th>
<th>Time to minimal MMₐ &gt; 20% (hr)</th>
<th>Minimal MMₐ (IU/l)</th>
<th>Time to maximal MMₐ &gt; 50% (hr)</th>
<th>Minimal MMₐ (IU/l)</th>
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<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>12</td>
<td>1773</td>
<td>5989</td>
<td>5</td>
<td>53.6</td>
<td>11</td>
<td>14.2</td>
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<tr>
<td>2</td>
<td>2</td>
<td>9</td>
<td>1857</td>
<td>6362</td>
<td>2</td>
<td>52.1</td>
<td>8</td>
<td>6.2</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>8</td>
<td>1055</td>
<td>2504</td>
<td>4</td>
<td>40.1</td>
<td>10</td>
<td>16.8</td>
<td>10</td>
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</tr>
<tr>
<td>4</td>
<td>3</td>
<td>14</td>
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<td>7658</td>
<td>4</td>
<td>51.7</td>
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<td></td>
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<td>5</td>
<td>5</td>
<td>10</td>
<td>421</td>
<td>682</td>
<td>6</td>
<td>35.4</td>
<td>12</td>
<td>19.5</td>
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<td></td>
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<tr>
<td>6</td>
<td>4</td>
<td>11</td>
<td>1320</td>
<td>3159</td>
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<td>50.5</td>
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<td>12.5</td>
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<td>4</td>
<td>12</td>
<td>1723</td>
<td>4375</td>
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<td>54.6</td>
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<tr>
<td>8</td>
<td>4</td>
<td>11</td>
<td>943</td>
<td>2874</td>
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<td>59.6</td>
<td>14</td>
<td>12.0</td>
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</table>

Mean ± SD 3.5 ± 1.1 10.9 ± 1.9 1371 ± 530 4200 ± 2329 4.1 ± 1.3 49.7 ± 8.0 12.0 ± 2.3 4.8 ± 1.1 14.0 ± 4.1 13.0 ± 2.9

MM CK isoform curves. In contrast to the absolute activities of individual MM CK isoforms, the time-dependent changes in the relative proportions of isoforms were similar in each dog and independent of total CK activity (figure 2 and table 2). The proportions of MMₐ, MMₐ, and MMₐ in control samples before coronary occlusion were 11.4 ± 4.8% (SD, n = 5), 22.3 ± 5.5%, and 66.7 ± 9.6%. In each dog, MMₐ was not greater than 17% of total MM CK in the control sample nor was MMₐ less than 54%. The proportion of MMₐ increased quickly and significantly exceeded control as early as 1 hr after coronary occlusion while total CK activity remained within the normal range (figure 3). The fraction of MMₐ reached a maximum of 49.7 ± 4.0% (SD) of total MM CK 4.1 ± 1.3 hr after occlusion, i.e., 6.8 ± 2.1 hr earlier than the time of occurrence of maximal total CK activity. The proportion of MMₐ in plasma remained elevated significantly throughout the first 8 hr after coronary occlusion (figure 3) and returned to control (<20%) in 12.0 ± 2.3 hr (table 1). The proportion of MMₐ exhibited a mirror-image time course in comparison with that of MMₐ, with a phase lag reflecting the slow generation of MMₐ from MMₐ through the MMₐ intermediate. The fraction of MMₐ began to decrease 1 hr after coronary occlusion and reached a minimum of 14.0 ± 4.1% in 4.8 ± 1.1 hr. The proportion of MMₐ remained significantly decreased for 10 hr after coronary occlusion (figure 3) and returned to control (MMₐ > 50%) in 13.0 ± 2.9 hr (table 1). Changes in the

FIGURE 2. Proportions of MMₐ and MMₐ after coronary occlusion in eight dogs.

TABLE 2  
Lack of dependence of proportions of isoforms on total CK activity

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent MMₐ and total CK activity</td>
<td>.190</td>
</tr>
<tr>
<td>Time to maximal MMₐ and time to maximal total CK</td>
<td>.601</td>
</tr>
<tr>
<td>Maximal MMₐ and maximal total CK</td>
<td>.560</td>
</tr>
<tr>
<td>Maximal MMₐ and cumulative CK released</td>
<td></td>
</tr>
<tr>
<td>Percent MMₐ and total CK activity</td>
<td>.201</td>
</tr>
<tr>
<td>Time to minimal MMₐ and time to maximal total CK</td>
<td>.487</td>
</tr>
<tr>
<td>Minimal MMₐ and maximal total CK</td>
<td>.328</td>
</tr>
<tr>
<td>Minimal MMₐ and cumulative CK released</td>
<td></td>
</tr>
</tbody>
</table>

*All correlations were statistically insignificant (n = 8).*
plasma occur rapidly, with a significant rise of MMₐ and a significant decrease of MM₈, evident as early as 1 hr after coronary occlusion. These early changes reflect ingress into the circulation of MMₐ from myocardium. The modest increase of total CK activity accompanying early ingress into plasma of MMₐ is not necessarily sufficient to elevate total CK above the upper limit of normal.

Chromatofocusing was used to quantify MM CK isoforms in this study because we previously found that it separated the isoforms without denaturing CK or producing artifactual sub-bands that may be encountered with procedures such as isoelectric focusing because of heating and ampholyte-protein complexing. Blue Sepharose and protein A Sepharose affinity chromatography efficiently removed non-CK protein from plasma and permitted separation of isoforms in samples with low CK activity. The reproducibility of the isoform analyses was satisfactory over the entire range of CK activities encountered.

Irreversible myocardial injury, manifest histologically, may occur as early as 20 min after the onset of ischemia. Thus initial release of MMₐ CK into the circulation may begin soon after the onset of coronary occlusion. Because the net ingress of MMₐ into the circulation is modest initially and because concomitant clearance of circulating CK tends to blunt any increase in total plasma CK, it is likely that the most prominent early manifestation of MMₐ ingress would be an increase in the proportion of MMₐ. This proportion is particularly sensitive to ingress of MMₐ because the fractional contribution of MMₐ to total MM is low in control plasma as a result of the prevailing conversion of circulating MMₐ to MM₈. Because the formation of MM₈ occurs slowly from MMₐ via MM₈ with a considerable time lag while MM₈ continues to be cleared from the circulation, the small initial increase of MMₐ produces a large change in the fractional contribution of MMₐ to total MM CK activity with an associated decrease in the fractional contribution of MM₈. As MMₐ release from the heart increases and begins to exceed the rate of CK clearance from blood, total plasma CK activity increases more strikingly. However, the relative contribution of MMₐ to total MM in plasma decreases after peaking within 2 to 6 hr and returns to baseline within 8 to 14 hr (table 1). Thus recurrent ingress of MMₐ into the circulation associated with early, recurrent infarction may be detectable before detection of a secondary rise in total CK activity.

Although total and MB CK activities exhibit time-dependent changes after myocardial infarction, they do not provide precise estimates of the time of onset of

**FIGURE 3.** Mean plasma total CK activity and proportions of MMₐ and MM₈. Points represent means ± 1 SD (n = 5). *p < .01 compared with control (time 0) by Tukey's range test.

fractional contribution of MM₈ to overall MM activity paralleled changes in the fraction of MMₐ, with the more modest maximal deviation from control resulting in a blunted peak. MM₈ reached a maximum of 44.6 ± 2.3% in 6.5 ± 2.4 hr after coronary occlusion and returned to baseline in 15.0 ± 3.5 hr.

The times to maximal MMₐ or minimal MM₈ proportional values were entirely independent of the time to maximal total CK activity and of cumulative CK release (table 2). Thus the time course and configuration of curves of percentages of MMₐ or MM₈ were independent of enzymatically estimated infarct size.

**Discussion**

The results of this study indicate that changes in the relative proportions of the three MM CK isoforms in
infarction. Absolute values at any interval after onset are dependent on infarct size.4 In contrast, the temporal changes in proportions of MM CK isoforms in plasma observed in this study are consistent regardless of enzymatically estimated infarct size (table 2). Their pattern reflects the consistency of kinetics of isofrom conversion over a wide range of total CK activity.11 Once MM₆ has been released from the myocardium into the circulation, it is converted to MM₇ and MM₈ according to kinetics that do not depend markedly on prevailing levels of CK activity.

MM CK in the dog heart consists primarily of MM₆ (95%). Myocardial MM₆ is not converted to either MM₇ or MM₈ in normal myocardium or ischemic tissue undergoing necrosis.11 Thus the time course of appearance of MM₆ in the circulation may provide an index of the duration of release of MM₆ from the myocardium.

Our findings indicate that proportions of MM₆ in plasma exceeding baseline (>20%) but associated with normal total CK activity (<100 IU/liter) reflect an onset of infarction within 4 hr (table 1). Conversely, baseline values of MM₆ associated with elevated total CK activity reflect an onset of infarction more than 14 hr previously (figure 3). The combination of elevated MM₆ and elevated total CK reflects an onset of infarction within 2 to 14 hr. Marked MM₆ elevations (>40%) associated with increased total CK reflect an onset of infarction of 2 to 7 hr previously. With an onset of infarction more than 8 hr before acquisition of the blood sample, MM₆ was consistently less than 40% of total MM CK in each dog (figure 2). Thus analysis of MM CK isoforms and total CK activity in a single plasma sample provides an index of the time of onset of infarction.

Control plasma samples exhibited predominantly MM₇ and small amounts of MM₆ and MM₈ compatible with previous findings with canine9 and human plasma.6, 8, 18 The presence of some MM₆ probably reflects release of CK from skeletal muscle and other tissues associated with physiologic turnover. The high proportions of MM₇ and MM₈ appear to reflect persistent conversion from MM₆ and differences in rates of clearance of individual isoforms from plasma in vivo. Clearance is fastest for MM₆ and slowest for MM₈.11, 19

The time-activity curves of the MM CK isoforms in this study resemble those reported in patients with acute myocardial infarction.6 This is not surprising since MM₆ is the dominant isofrom in human as well as in canine myocardium.6, 8, 18 Isoform conversion in human plasma in vitro is approximately 50% as rapid as conversion in dog plasma, but the sequence of conversion (MM₆ to MM₈ via an MM₇ intermediate) and its irreversibility are similar in the two species (unpublished observations). Thus it is likely that estimation of the time of onset of myocardial infarction can be accomplished in patients by analysis of the proportions of MM CK isoforms and total CK activity in one or a small number of plasma samples. On the other hand, the specificity of MM₆ or of any MM CK isofrom as a criterion for the diagnosis of myocardial infarction is less than that of the MB CK isoenzyme because MM CK may be released from tissues other than the heart. Nevertheless, because analysis of plasma MM CK isoforms appears to permit very early detection of irreversible myocardial injury, development of rapid methods of isoform analysis suitable for routine clinical analyses should facilitate early diagnosis of myocardial infarction.

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References
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