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ORIGINAL ARTICLE

Development and performance of an enzyme immunoassay to detect creatine kinase isoenzyme MB activity using anti-mitochondrial creatine kinase monoclonal antibodies

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Abstract

Objective: The MB fraction of creatine kinase (CK-MB) has long been used as a cardiac marker. It is known that the CK-MB immunoinhibition method lacks selectivity and accuracy, because the appearance of macro CK type 2, corresponding to mitochondrial creatine kinase (MtCK) in some patient serum may render CK-MB activity measured by conventional method abnormally high. Thus, to improve the specificity and accuracy of the CK-MB assay, we developed two types of monoclonal anti-MtCK antibodies against sarcomeric MtCK and ubiquitous MtCK, and present herein the performance of a new method using these antibodies. ***Material and methods:*** The performance of our test for detecting CK-MB activity was compared with other methods, and the range of CK-MB activities in normal human serum was investigated. ***Results:*** The two types of monoclonal antibodies developed by us were isoenzyme-specific to sMtCK or uMtCK. The correlation coefficients of our method and conventional method to electrophoresis were 0.973 and 0.873, respectively. The mean CK-MB activity in normal human serum by our method and the conventional method was 2.4 and 11.7 U/L, respectively. Thus, our data indicated that about 80% of CK-MB activity, determined using the conventional method, seems to correspond to the MtCK activity. ***Conclusion:*** Our method is novel in offering higher accuracy of measuring true CK-MB contents in human serum as compared to the conventional method. The possibility of accurately estimating CK-MB activity by our method which can inhibit MtCKs in healthy person and patient serum is likely to bring a break-through in clinical diagnostics.

Key Words: *Biological markers, MB creatine kinase, Macro-creatine kinase, Mitochondrial creatine kinase, myocardial infarction, diagnostics reagent kits*

Introduction

Creatine kinase (CK; EC 2.7.3.2) catalyses the reversible transphosphorylation between ATP and creatine phosphate, and plays a very important role in the energy metabolism of cell components such as the cytoplasm and mitochondria. Cytosolic CKs exist as dimers of the subunits M and B, and are distinguished as three isoenzymes: CK-MM, CK-MB, and CK-BB. On the other hand, CKs in mitochondria exist as two isoenzymes: sarcomeric mitochondrial CK (sMtCK) and ubiquitous mitochondrial CK (uMtCK) [1]. Both of the mitochondrial CK isoforms exist as dimer and octamer [2–4]. sMtCK has 80% amino acid sequence homology with uMtCK [1].

CK-MB is found in substantial amounts in the serum of heart infarct patients in the damaged myocardium as well as troponin I [5] and troponin T [6]. CK-MB activity assay is used as a clinical marker to detect myocardial infarction. However it is known that CK-MB activity assay lacks selectivity and accuracy, as compared with CK-MB mass assay [7,8], electrophoresis [9]. Because CK-MB activity determined using immunoinhibition method is abnormally increased when the CK-BB activity is also high in

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patient serum [10,11]. In addition, macro CK type 1 [12], and macro CK type 2 [13,14] are also found in the serum of patients. The anti-CK-M antibody included in CK-MB activity reagents can inhibit only CK-M subunit but not macro CK type 1 or macro CK type 2, corresponding to MtCK. Thus, the appearance of macro CK type 1 and MtCK in the serum renders the specificity of the immuno-inhibition method low in the CK-MB activity assay. Lee et al. reported high false-positive CK-MB activity levels in 136 of 8322 patients in whom CK-MB analysis was carried out [13]. There were 36 cases with macro CK type 1 (incidence: 0.43%), and 100 cases with MtCK (1.20%). This data indicates that the frequency of MtCK appearance in serum is much higher than that of macro CK type 1.

In order to improve the selectivity and accuracy of the conventional CK-MB assay, it seems important to inhibit not only CK-MM activity, but also MtCK activity in the serum concomitantly, using the anti-MtCK antibody. We show here, that immuno-inhibition of both CK-MM and MtCK results in more reliable values for CK-MB than conventional CK-MB assays and the improvement of selectivity of the conventional CK-MB activity assay here seems to contribute significantly to a more accurate clinical diagnosis of acute myocardial infarction. In the present study, we developed two distinct monoclonal antibodies, which are highly specific for human sMtCK and uMtCK. Thereupon, we used the conventional CK-MB activity reagent in combination with these highly specific anti-MtCK antibodies and estimated whether our method has comparable performance to the CK-MB mass assay and electrophoresis.

Materials and methods

Antigens

Human recombinant sMtCK and uMtCK were purified at the Institute of Cell Biology, ETH Zurich, according to published methods [15]. Both MtCKs were isolated from recombinant *Escherichia coli* cultures. The resulting two proteins had purities of 99%, according to SDS-PAGE. The purified human MtCK isoenzymes were stored at −80°C in phosphate buffer (50 mmol/L sodium phosphate (pH 7.0), 150 mmol/L sodium chloride, 0.2 mmol/L EDTA, and 2 mmol/L β-mercaptoethanol).

Monoclonal antibody

Hybridoma production: Seven-to eight-week-old BALB/c mice were injected intraperitoneally with 50 μg of human recombinant sMtCK or 50 μg of human recombinant uMtCK, each emulsified in an equal volume of Freund's complete adjuvant (PIERCE, IL, USA). Six booster injections were administered at an interval of 4 to 5 weeks. Antibody levels in the serum of immunized mice were determined periodically by the screening method of the enzyme-inhibition test, as given below. The spleens that had a high antibody titer and the highest specific antibody against MtCK were removed from the mice. Splenocytes were fused *in vivo* with mouse myeloma cells in the presence of 50% polyethylene glycol 3350 (M.W. 3350, Sigma-Aldrich Co., Tokyo, Japan), essentially as described by Köhler and Milstein [16]. After fusion, the obtained hybridomas were washed to remove polyethylene glycol 3350, and were resuspended in the cloning medium (S-Clone cloning medium CK-B, Sanko Junyaku Co., Ltd., Tokyo, Japan) with hypoxanthine-thymidine supplement and cultured in a 96-well plate (2.5×10^5 cells/wells) in a cloning medium containing hyopoxanthine-aminopterin-thymidine supplement. Hybridomas were cloned by limiting dilution in the above medium with hyopoxanthine-thymidine supplement and murine thymocytes ($0.5–1.0\times10^6$ cells/well) three times. Subsequently, single clones were picked and selected by investigating the specificity to CK isoenzymes (CK-MM, CK-BB, and CK-MB) and MtCKs (sMtCK and uMtCK). Consequently, a hybridoma 'mCKI-578' producing specific anti-sMtCK antibody was selected, and a hybridoma 'UI-1881' producing specific anti-uMtCK antibody was selected.

Determination of IgG fraction in the serum of immunized mice and selecting method for production of IgG Hybridomas clones: Determination of IgG fraction in serum of the immunized mice or selecting method for IgG hybridomas clones was performed as follows: 25 μL of 200 U/L sMtCK or uMtCK antigens were incubated with 25 μL of serum of the immunized mice or hybridoma supernatant fluid for 60 min at room temperature. The reaction was started immediately by addition of 200 μL of the reagent for determination of CK activity [17,18] containing 4700 U/L diaphorase (NADPH, *Bacillus megaterium*, Asahi Kasei Pharma Co., Tokyo, Japan) and 0.6 mmol/L nitrotetrazolium blue (Dojindo Laboratories, Kumamoto, Japan). After incubation in the dark for 60 min at 37°C, the absorbance of the sample was measured at 550/690 nm and performance of IgG fraction in serum of mice and hybridoma was estimated.

Production of anti-MtCK antibody: BALB/c adult mice were injected intraperitoneally with 0.5 mL of pristine (2,6,10,14-tetramethylpentadecane, Sigma-Aldrich Co.). Three days after injecting pristine, they were injected intraperitoneally with the hybridomas (10^7 cells/mL). Ascites fluid was then collected one to two weeks later, pooled, and stored at 4°C after adding 0.5 g/L sodium azide. The collected ascites fluid was purified using a Hyper D

protein A column. After sterile filtration, monoclonal anti-MtCK antibodies were stored in buffer (20 mmol/L HEPES (pH 7.0), 150 mmol/L sodium chloride, and 0.5 g/L sodium azide) at 4°C.

Reagents

In the conventional CK-MB activity assay, the CK-MB activity was assayed using a kit for determination of CK activity including anti-CK-M polyclonal antibody. In our new method for detrmining CK-MB activity, CK-MB activity was assayed using the conventional CK-MB activity kit including the two types of anti-MtCK monoclonal antibodies. The kit for determination of CK activity consists of two kinds of reagents. Reagent 1 contained 125 mmol/L imidazol (pH 6.6), 2.5 mmol/L ethylenediamine-N,N,N′, N′-tetraacetic acid (EDTA), 12.5 mmol/L magnesium acetate, 2.5 mmol/L ADP, 2.5 mmol/L $NADP^+$, 25 mmol/L glucose, 25 mmol/L N-acetyl-L-cysteine, 6.25 mmol/LAMP, 12.5 μmol/L P^1,P^5-di(adenosine-5′) pentaphosphate (Ap_5A), 1875 U/L glucose-6-phosphate dehydrogenase and 3750 U/L hexokinase. Reagent 2 contained 150 mmol/L phosphocreatine.

Anti-CK-M polyclonal antibody was obtained from Merck Ltd (Tokyo, Japan). ADP, $NADP^+$, and AMP were from Oriental Yeast Co, Ltd (Tokyo, Japan). N-acetyl-L-cysteine, EDTA, and phosphocreatine were from Dojindo Laboratories. Imidazole, magnesium acetate, and glucose were from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Ap_5A was from Roche Diagnostics KK (Tokyo, Japan). Glucose-6-phosphate dehydrogenase and hexokinase were from TOYOBO Co., Ltd (Osaka, Japan).

Samples

Correlation study was performed using 105 residual plasma samples from patients, after the completion of the requested laboratory tests in Itabashi Hospital, Nihon University (Tokyo). Residual samples were made anonymous and stored at −80°C until assayed. All procedures conformed to official practices of the Japanese Society of Laboratory Medicine [19].

The basal ranges of CK-MB as well as MtCK activities in normal human serum were estimated using samples from 114 healthy individuals in Sysmex Corporation and Itabashi Hospital, Nihon University. Blood samples were collected without anticoagulants from the volunteers. Collected blood samples were centrifuged at 1500*g* for 10 min, and the serum was separated. All samples were stored at −80°C until assayed.

Instruments

CK-MB and CK activities were assayed using H-7170S automated biochemical analyser (Hitachi High-Technologies Co., Tokyo, Japan). CK-MB mass was assayed on ADVIA Centaur Immunoassay system (Siemens K.K., Tokyo, Japan). The assay procedures for CK-MB activity, CK activity, and CK-MB mass were performed in accordance with the manufacturer's protocol.

Principle of CK-MB activity assay

Samples were incubated with reagents containing antibodies against CK-M subunit and MtCKs. CK-M subunit and MtCKs in samples were completely inhibited. Creatine phosphate was added to start the reaction, and residual CK-B subunit activity was measured [20–22].

Procedure of CK-MB Activity Assay: 12.9 μL of sample was incubated with 180 μL of reagent 1 for 2.5 min at 37°C, and the absorbance change per minute of blank sample was measured at 340/700 nm (ΔA_{blank}) using H-7170S automated biochemical analyzer. The reaction was started immediately by addition of 45 μL of reagent 2. After 2.5 min, the absorbance change per minute of sample at 340/700 nm (ΔA_{sample}) was measured. The absorbance change per minute of test was calculated by subtraction of ΔA_{blank} and ΔA_{sample}. CK-MB activity was determined from a calibration curve constructed using CK-MB calibrator, Calibzyme CK (MB) (Sysmex Corporation), which contains CK-MB derived from human cardiac muscle.

Procedure for analysis of CK isoenzyme by electrophoresis: Electrophoresis was carried out as follows: samples were applied to the cellulose acetate membrane (TITAN III-LIPO, Helena Co. Ltd., Saitama, Japan), such that the total CK activity applied was approximately 400 U/L per lane. A pack of Electra HR Buffer (Helena Co. Ltd.) was diluted with 900 mL of purified water, and was used as the electrophoresis buffer (67.3 mmol/L barbital and 5.8 mmol/L tris-hydroxymethylaminomethane, pH 8.8). Electrophoresis was carried out at 190V for 30 min under cool conditions. After electrophoresis, the samples were stained onto cellulose acetate strips with the kit for determination of CK activity containing 4700 U/L diaphorase, and 0.6 mmol/L nitrotetrazolium blue for 30 min at 37°C, and were determined at 570 nm using the densitometer (Cliniscan 2, Helena Co. Ltd.) after bleaching with 3% acetic acid.

Statistical Analysis

The reference intervals and means values for CK isoenzymes were calculated by a non-parametric analysis to estimate the upper 95 percentile of healthy

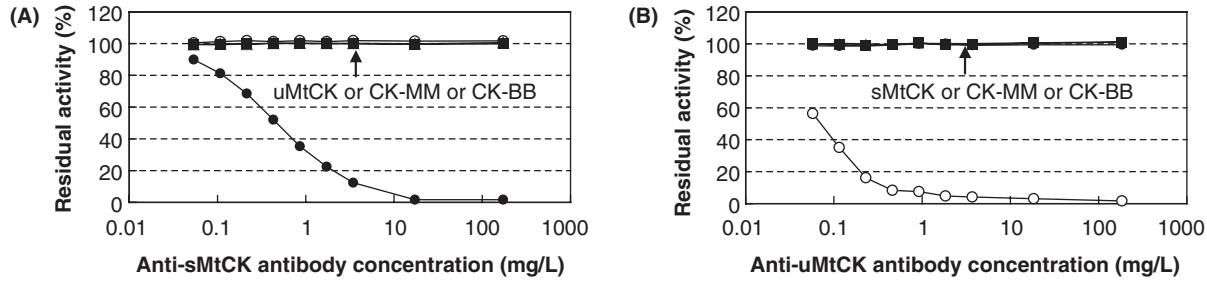

Figure 1. Inhibition of sMtCK, uMtCK, CK-MM, and CK-BB using anti-MtCK antibodies. (A) anti-sMtCK antibody; (B) anti-uMtCK antibody. •, sMtCK 149 U/L; °, uMtCK 104U/L; ▲, CK-MM 118 U/L; ■, CK-BB 125 U/L.

individuals, using Stat Flex ver.5.0 for Windows (ARTECH Co., Osaka, Japan). Other data was calculated using Microsoft Excel 2003 (Microsoft, Redmond, WA, USA).

Results

Characterization of the monoclonal antibody

The IgG concentration of anti-sMtCK antibody and anti-uMtCK antibody was 5.1 and 5.3 g/L, respectively, by calculating "$Abs_{280\,nm}=1.38$" as 1 g/L. We obtained a total of 41 mg of anti-sMtCK and 38 mg of anti-uMtCK antibody per mouse. Anti-sMtCK antibody had IgG1 subclass with kappa light chains, and the isotype of anti-uMtCK antibody was IgG2b subclass, which also had kappa light chains.

Specificity of anti-MtCK monoclonal antibody

Anti-sMtCK and anti-uMtCK antibodies were tested for their ability to inhibit sMtCK and uMtCK using the kit for determination of CK activity containing either anti-sMtCK antibody or anti-uMtCK antibody (Figure 1). Anti-sMtCK and Anti-uMtCK antibody of above 20 mg/L quantitatively inhibited sMtCK and uMtCK, respectively.

Anti-sMtCK and anti-uMtCK antibodies were tested for isoenzyme-specificity against sMtCK, uMtCK, CK-MM, CK-MB, and CK-BB. Anti-sMtCK antibody did not inhibit uMtCK at all. On the other hand, anti-uMtCK antibody recognized uMtCK but not sMtCK. Anti-sMtCK antibody inhibited sMtCK activity more than 98%. The same was true for anti-uMtCK antibody, which inhibited uMtCK activity more than 98%. Both the anti-MtCK antibodies however, did not inhibit CK-MM, CK-MB, or CK-BB (Table I). Figure 2 shows that the ability to inhibit the corresponding homologous MtCK isoenzyme form using both anti-MtCK antibodies was tested by electrophoresis. MtCKs treated with anti-MtCK antibody or non-treated MtCKs were separated by cellulose acetate membrane electrophoresis. MtCK bands in lane 3 and lane 7 disappeared, which indicates that MtCK activity in lane 3 and lane 7 was inhibited by anti-sMtCK antibody and anti-uMtCK antibody, respectively. On the other hand, sMtCK was not inhibited by anti-uMtCK antibody (lane 4), and uMtCK was not inhibited by anti-sMtCK antibody (lane 6). Thus, the two monoclonal anti-sMtCK and anti-uMtCK antibodies were fully MtCK isoform-specific. Electrophoretically, octameric uMtCK and sMtCK were migrating cathodic to CK-MM. Dimeric MtCK was found at an electrophoretic position that almost superimposed with CK-MM. Both the anti-MtCK antibodies inhibited the enzyme activity not only of octameric MtCKs but also of dimeric MtCKs.

Comparison with CK-MB mass assay and electrophoresis

Parallel measurements with our new CK-MB activity assay, conventional CK-MB activity assay, CK-MB mass, and electrophoresis were performed using 105 plasma samples from patients. The results are presented as a correlation plot in Figure 3. The equation for our new method compared with CK-MB mass and electrophoresis was $y=0.555x+4.5$; $r=0.977$, and $y=0.922x+4.3$; $r=0.973$, respectively. There was a good agreement between the results from this proposed method and each of the two reference methods. On the other hand, the equation for the conventional method compared with CK-MB mass and electrophoresis was $y=0.552x+26.0$; $r=0.857$, and $y=0.937x+24.9$; $r=0.873$, respectively. No good correlation was found between the conventional method and each method of CK-MB mass and electrophoresis. The discrepancy of CK-MB activity

Table I. Inhibition of anti-MtCK antibodies on sMtCK, uMtCK, CK-MM, CK-MB, and CK-BB.

CK isoenzymes	Activity (U/L)	Anti-sMtCK antibody inhibition (%)	Anti-uMtCK antibody inhibition (%)
sMtCK	498	98.2	2.0
uMtCK	392	2.1	98.2
CK-MM	3412	0.4	0.6
CK-MB	112	1.3	1.4
CK-BB	138	3.2	3.4

Inhibition of both the anti-MtCK antibodies against sMtCK, uMtCK, CK-MM, CK-MB, and CK-BB was investigated using a CK activity measurement kit, containing either 20 mg/L of anti-sMtCK antibody or 20 mg/L of anti-uMtCK antibody.

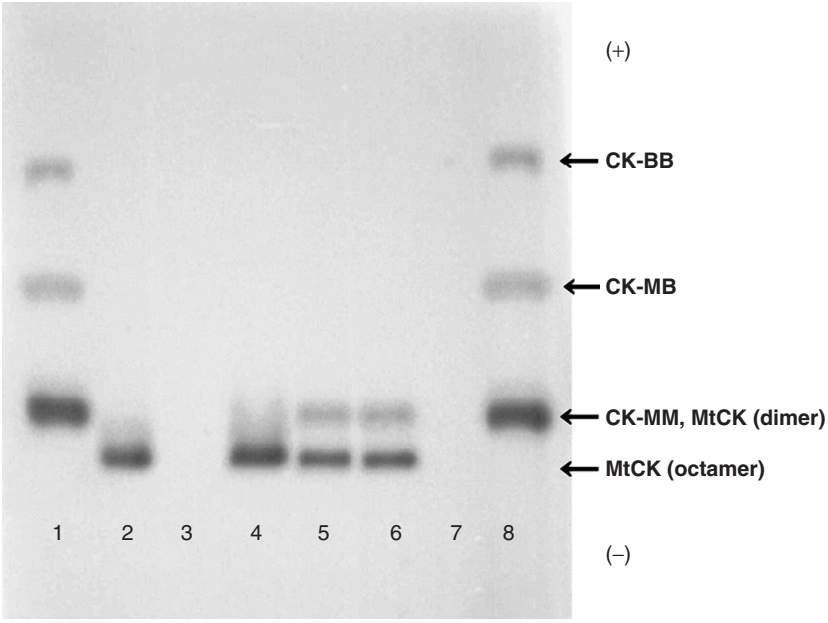

Figure 2. Specific binding of anti-MtCK antibody to MtCK on cellulose acetate membrane by electrophoresis. Lane 1, CK isoenzyme controls (CK-MM, CK-MB, and CK-BB); lane 2, non-treated sMtCK; lane 3, sMtCK treated with anti-sMtCK antibody; lane 4, sMtCK treated with anti-uMtCK antibody; lane 5, non-treated uMtCK; lane 6, uMtCK treated with anti-sMtCK antibody; lane 7, uMtCK treated with anti-uMtCK antibody; lane 8, CK isoenzyme controls (CK-MM, CK-MB, and CK-BB). An activity stain for CK was performed after electrophoresis on cellulose acetate membrane. Disappearance of MtCK bands indicates that MtCK activity was inhibited by anti-MtCK antibody. Although sMtCK was inhibitedly by anti-sMtCK antibody (lane 3), it was not inhibited by anti-uMtCK antibody (lane 4). Similarly, uMtCK was inhibited by anti-uMtCK antibody (lane 7), not but anti-sMtCK antibody (lane 6).

was recognized in samples assayed using electrophoresis and the conventional method. Figure 4 shows the CK isoenzyme electrophoretical analysis of a patient sample. CK-MB activity of this sample was not electrophoretically almost detected. But CK-MB activity determined using the conventional method was 114.4 U/L, and this method gave false positive. CK-MB activity assayed by our new method was 14.0 U/L, and this sample had a negative result. On the other hand, CK-MM and MtCK activity were detected by electrophoresis. Disappearance of MtCK bands indicates that MtCK activity was inhibited by anti-MtCK antibody. MtCK activity treated with anti-sMtCK antibody was detected (lane 4), which shows that no sMtCK exist in this sample. However, the MtCK band disappeared after treatment with anti-uMtCK antibody (lane 7), indicating that the serum of this patient contained uMtCK. In addition, MtCK and CK-MM band was not detected after treatment with anti-uMtCK antibody and anti-CK-M antibody (lane 8). Thus, the band recognized in lane 7 was CK-MM band.

Range and mean values of CK-MB activity in serum of healthy people

The range and means of serum CK-MB and CK activities in 114 healthy individuals is shown in Table II, and the frequency distribution in Figure 5. In the conventional method, the CK-MB values ranged from 6.6 to 17.2 U/L and the mean CK-MB activity in serum was 11.7 U/L. On the other hand, the values for CK-MB ranged from 0.8 to 5.3 U/L, and the mean CK-MB activity in new method was 2.4 U/L. It should be noted that the latter values determined by our new method involving blocking of MtCK by monoclonal antibodies are significantly lower than those determined by the conventional methods, indicating that appreciable amounts of MtCK activity may be present in the serum of even healthy people and that CK-MB values may be over estimated by the former conventional methods.

Discussion

Figure 3 showed that there were differences in CK-MB values obtained by the conventional method and electrophoresis, compared with our new method. Although some of the CK-MB values estimated by the conventional method were above 50 U/L, those determined by electrophoresis were 0 U/L (Figure 3D). Electrophoretical CK isoenzymes analysis indicated that CK-MB activity in one of the sample was actually almost non-existent (0 U/L) (Figure 4, lane 8). This discrepancy was attributed to the appearance of MtCK in plasmas, and seems to be present in most of samples from patients with malignant tumors. Therefore, the conventional method, which cannot inhibit MtCK gave high false positive CK-MB activity. There is no good correlation between the conventional method and each of CK-MB mass and electrophoresis tests. This is one of the likely reasons

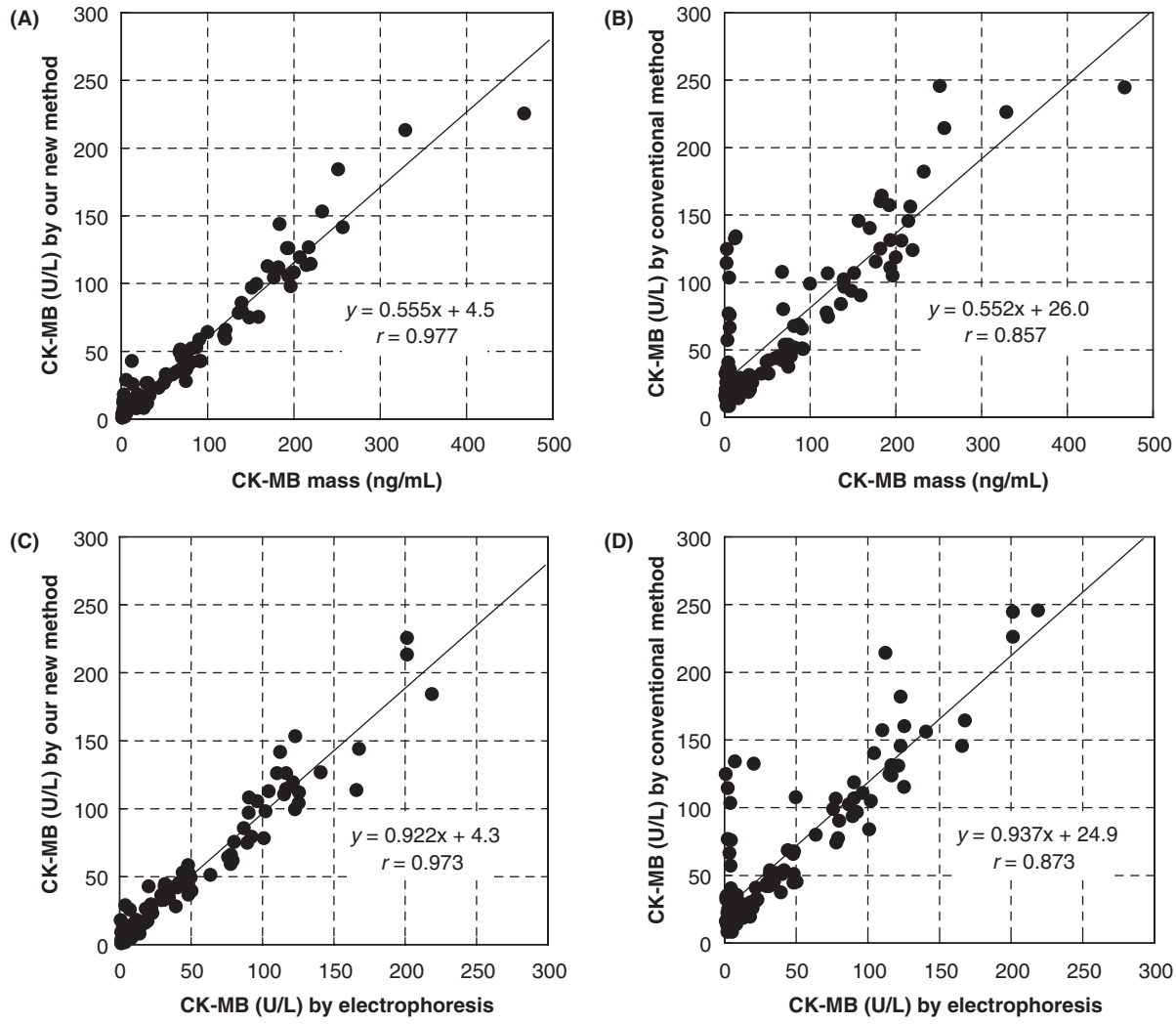

Figure 3. Correlation studies. (A) CK-MB mass assay vs. our new method. (B) CK-MB mass assay vs. the conventional method. (C) Electrophoresis vs. our new method. (D) Electrophoresis vs. the conventional method. In correlation studies, 105 samples of residual plasma after the completion of the requested laboratory test were used.

why the conventional method has lower specificity for CK-MB than these two reference methods. On the other hand, most of samples assayed by our new method were similar to those calculated by electrophoresis. Inhibition of MtCK by the two types of anti-MtCK monoclonal antibodies additionally contained in the reagent makes our new method more selective and specific for CK-MB compared to the conventional method, and similar in performance compared to the electrophoresis and CK-MB mass methods.

Figure 5 and Table II show the range of the serum CK-MB activity in healthy individuals. Hoshino et al. reported that the mean CK-MB activity in healthy human serum by electrophoresis was 2.4 U/L, with a range of 0.3 to 4.5 U/L [23]. The mean and range of CK-MB activity in healthy human serum determined with our new method was almost equal to their data obtained by the CK electrophoresis method, and this illustrates that our new method was more accurate than the conventional CK-MB immunoinhibition method using anti-CK-M antibodies.

By inhibiting not only CK-MM activity, but also MtCK activity, which has been shown to be present in the serum of healthy individuals, the mean CK-MB activity in healthy individuals was found to be 2.4 U/L with our new method, in contrast to 11.7 U/L obtained for CK-MB with the conventional method. This data shows that about 80% of the CK-MB activity determined without inhibiting MtCK activity in the serum by anti-MtCK antibodies using conventional reagent kits actually corresponds to MtCK activity that gets incorrectly measured as CK-MB activity. This led us to determine and estimate MtCK activity levels in the serum from healthy people (Figure 5C). MtCK activity in serum was calculated by subtracting CK-B activity determined by using our method from that determined by using the reagent without both anti-MtCK antibodies, and then dividing this difference by two, because CK-MB activity was measured by doubling the residual CK-B activity. The range of MtCK activity in the serum from healthy people was found to be 1.7–6.1 U/L (mean value, 3.8 U/L).

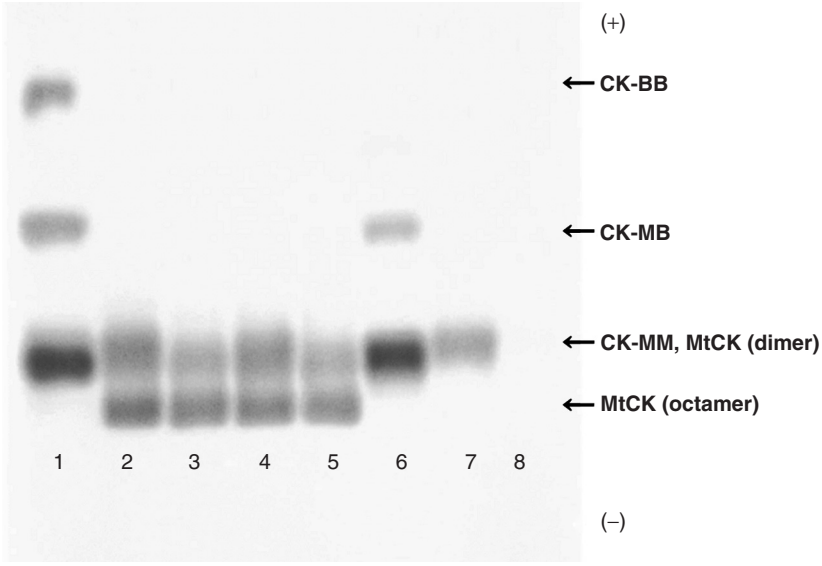

Figure 4. Electrophoretic pattern of patient serum, which contain CK-MB activity abnormally increased by the conventional immunoinhibition method. Lane 1, CK isoenzyme controls (CK-MM, CK-MB, and CK-BB); lane 2, non-treated sample; lane 3, sample treated with anti-CK-M antibody; lane 4, sample treated with anti-sMtCK antibody; lane 5, sample treated with anti-CK-M antibody, and anti-sMtCK antibody; lane 6, CK isoenzyme controls (CK-MM and CK-MB); lane 7, sample treated with anti-uMtCK antibody; lane 8, sample treated with anti-CK-M antibody and anti-uMtCK antibody. CK activity and CK-MB activity determined using the conventional method, our new method, and electrophoresis of the sample was 92.5, 114.4, 14.0, and 1.4 U/L, respectively. Although CK-MB contained in this sample was not electrophoretically almost detected, CK-MB activity was detected as high level by the conventional method. Disappearance of MtCK band in lane 7 indicates that MtCK activity was inhibited by anti-uMtCK antibody. This sample contain uMtCK not but sMtCK, since MtCK band was detected in lane 4 and disappeared in lane 7. No band detected after treatment with anti-uMtCK antibody and anti-CK-M antibody (lane 8). Therefore, CK-MM and uMtCK exist in this sample, and CK-MB and sMtCK was not contained.

Hoshino et al. reported that the range of MtCK activity in normal human serum was 0.9–6.4 U/L (mean value, 3.4 U/L) by electrophoresis [23]. Thus, our data was almost identical to those in their report. Our results corroborate that indeed MtCK activity exists even in normal human serum and the range of

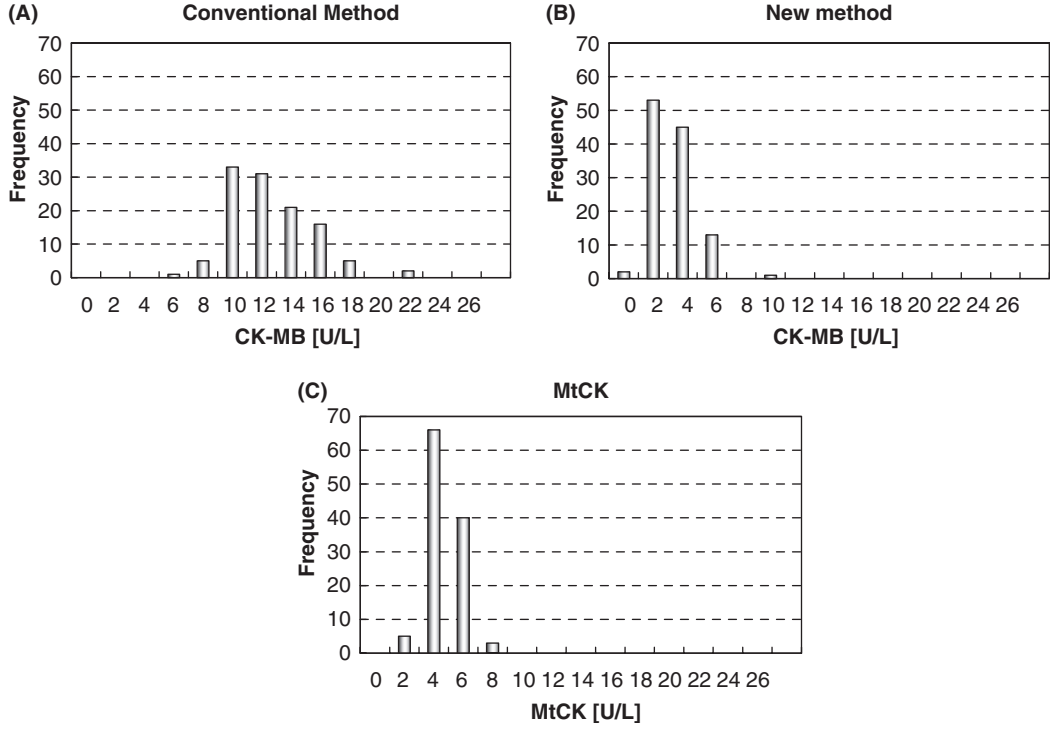

Figure 5. Range of CK-MB activity in normal human serum by the conventional method (A) and new method (B), MtCK activity (C). The frequencies of CK-MB and MtCK activities in the serum samples from 114 healthy individuals were plotted. Each column represents the frequency of CK-MB and MtCK activity indicated on the abscissa.

Table II. CK-MB and MtCK activities in serum from healthy individuals.

CK isoenzymes	*N*	Mean (U/L)	Range of CK isoenzymes* (U/L)
CK	114	101	39–237
CK-MB (Conventional method)	114	11.7	6.6–17.2
CK-MB (New method)	114	2.4	0.8–5.3
MtCK	114	3.8	1.7–6.1

*Data were calculated by non-parametric analysis with the use of Stat Flex ver.5.0 for Windows.

CK-MB activity determined by our new method was generally lower than that estimated by the conventional immunoinhibition method using anti-CK-M antibodies. By inhibiting MtCK activity, our new method clearly showed a higher specificity for CK-MB. Furthermore, it seemed likely that the cut-off level of CK-MB activity (25 U/L) was lower with our new assay compared to that of the conventional method.

MtCK in the serum exists as a dimer and octamer [24]. After electrophoresis on cellulose acetate membrane, the dimeric MtCK was found close to the electrophoretic position of CK-MM and the octameric MtCK was electrophoresed cathodic to CK-MM [25]. The octameric MtCK was gradually converted with time into dimeric MtCK after collecting blood from a donor [26]. In these zymograms, MtCK migrated very close to the CK-MM position and in fact overlapped with the CK-MM band sometime after collecting blood sample, while MtCK was detected as a band cathodic to the CK-MM position in a fresh blood sample. Therefore, this is a reason why the existence of MtCK in normal human serum had been unrecognized until recently [15], although there were several earlier reports describing the existence of MtCK in serum from patients with malignant tumors.

In conclusion, the possibility of being able to more accurately estimate the CK-MB content in serum by the use of our new method, which can inhibit MtCKs in both healthy person and patient serum, is likely to bring a break-through in clinical CK-MB diagnostics. Our data shows that CK-MB activity assay using anti-MtCK antibody, in combination with the conventional anti-CK-M antibody, seems to be more useful as a selective cardiac biomarker and thus superior to the conventional method.

It is known that the half-life of CK-MB is very short compared with Troponin. Therefore, our new method is useful in diagnosing not only acute myocardial infraction but also reinfarction and monitoring treatment after onset of acute myocardial infarction.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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