Creatine Kinase Isoenzyme of High Relative Molecular Mass in Serum of a Cancer Patient

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We describe an atypical serum creatine kinase isoenzyme in the serum of a woman with cancer of the left breast. This isoenzyme migrated toward the cathode, closely following the MM isoenzyme on agarose gel electrophoresis. Its relative molecular mass was estimated to be about 325 000, fourfold that of normal creatine kinase. It is more heat-stable and is inhibited more by urea than the normal MM isoenzyme. Isoenzyme monomer B activity was observed to be 20 U/liter in the serum, as measured with use of an antibody against the M monomer. On anion-exchange column analysis, creatine kinase activity was observed only in the MM fraction, in spite of the fact that B activity was observed in the patient’s serum. Results of the immunological investigation make it unlikely that the atypical isoenzyme is linked to immunoglobulin or beta-lipoprotein. It may have been present as the result of modification of normal creatine kinase by the therapeutic radiation the patient was receiving.

Creatine kinase (EC 2.7.3.2) is separable into three different components by several methods (7–5). Each isoenzyme is a dimer composed of two subunits (B or M), each having a relative molecular mass of 40 000 (7). The brain isoenzyme (CK-BB) consists of two identical B subunits, the muscle isoenzyme (CK-MM) contains two identical M subunits, and the intermediate isoenzyme (CK-MB) is a hybrid containing both subunits. On electrophoresis on agarose gel, CK-BB, CK-MB, and CK-MM migrate closest to the positions of prealbumin, α2-globulin, and γ-globulin, respectively.

In recent years, many investigators have discussed diagnostic and clinical evaluation of CK isoenzymes in acute myocardial infarction (AMI). We have also examined CK isoenzymes in various pathologic conditions including AMI (6).

We describe here an atypical serum CK isoenzyme of high relative molecular mass, which migrates cathodically on agarose gel electrophoresis. Such an isoenzyme has never been reported before, and we refer to it in this report as “macro-CK.”

Case History

The patient, a 52-year-old woman, underwent radical mastectomy after diagnosis of cancer of the left breast in March 1977; telecobalt therapy (6000 R, or 1.55 Ci/kg) was administered over the left supraclavicular fossa and the mediastinum during five months. In July, metastasis of the cancer to the skin of the left anterior chest region was found, so the patient received irradiation over the anterior thoracic wall, as well as local injection with Picibanil, a streptococcal preparation, and telecobalt-therapy with 1800 R over the left axillary fossa. At the end of September, remarkable retention of pericardial fluid and jaundice was noted, and a grade I atrio-ventricular block was found on electrocardiography.

The patient died on October 12. No autopsy was performed. The main laboratory findings (normal range of enzyme activity in parentheses) at the end of September were as follows: total protein 56 g/liter, albumin/globulin ratio 0.8, IgG 15 g/liter, IgA 2.46 g/liter, IgM 2.07 g/liter, aspartate aminotransferase 200 U/liter (5–35 U/liter), alanine aminotransferase 34 U/liter (3–30 U/liter), lactate dehydrogenase 4896 U/liter (50–400 U/liter), CK 93 U/liter (10–60 U/liter), γ-glutamyl transferase 528 U/liter (0–30 U/liter), leucine aminopeptidase 605 U/liter (27–142 U/liter), alkaline phosphatase 19.3 U (0.5–3.5 U, Bessey-Lowry), acid phosphatase 0.79 U (0.1–0.5 U, Bessey-Lowry).

Materials and Methods

Serum and pericardial fluid from the patient were used for analysis. Total CK activity was determined at 37 °C by the method of Rosalki (7) with "CPK Stat-Pack" reagents (Calbiochem, Los Angeles, Calif.). An electrophoretic method for CK isoenzymes was carried out by a modification (6, 8) of the fluorescence technique of Somer and Kontinnen (2). Electrophoretic patterns were scanned with a densitron FR-1 (Jokoo Sangyo Co., Tokyo, Japan). CK isoenzymes were also separated on a mini-column of DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) as previously reported (5, 6, 9). Our elution procedure was similar to that described by Nealon and Henderson (4), but the serum was diluted with tris(hydroxymethyl)aminomethane buffer (50 mmol/liter, pH 7.0) before it was applied to the column. Thin-layer gel filtration on Sephadex G-200 superfine...
(Pharmacia Fine Chemicals) was developed for 2 h with phosphate buffer (67 mmol/liter, pH 7.4). A strip of TOYO filter paper No. 50 was placed on top of the gel surface to directly absorb the separated protein fractions. To detect CK activity, another strip of filter paper soaked in the reaction solution was then placed over the first sheet. After incubation for 60 min at 37°C, sites of CK activity could be seen under ultraviolet light (360 nm). Gel filtration was also performed on a Sephadex G-200 column (1.8 × 45 cm) at 4°C, which was eluted with phosphate buffer (67 mmol/liter, pH 7.4) at a flow rate of 9–10 ml/h. The relative molecular mass (Mr) of the patient’s serum CK was estimated by comparisons with standard proteins (Boehringer Mannheim GmbH Biochemica).

CK-B activity was determined by use of an antibody against the CK-M monomer. This antibody, which only recently became available (E. Merck, Merck-1-Test CK-MB 14301), was used according to the vendor’s instruction sheet.

Immunoelectrophoresis was carried out on agar gels against rabbit anti-whole human serum, anti-IgA, anti-IgM, and anti-β-lipoprotein (Behringwerke, Hoechst-Japan Co., Tokyo). After the agar plate was washed with isotonic saline, CK activity was detected both by the fluorescence method and by tetrazolium staining as in the methods used in electrophoretic CK isoenzyme analysis. The plates were then incubated for 60 min at 37°C.

The heat stability and response to urea, to 6 g/liter sodium deoxycylolate, to 50 mmol/liter ethylenediaminetetraacetic acid (EDTA), and to 10 mmol/liter β-mercaptoethanol were examined. All reagents were analytical grade.

Results

The atypical serum CK isoenzyme, “macro-CK,” migrated toward the cathode (Figure 1). This band does not appear when creatine phosphate is omitted from the incubation medium. “Macro-CK” was also present in the patient’s pericardial fluid.

Molecular size investigation. Thin-layer gel filtration patterns of patient’s serum and normal serum are shown in Figure 2. The position of the fractions was observed by ultraviolet fluorescence. Normal CK was found between the albumin and IgG fractions, while “macro-CK” was found between the IgG and IgM fractions, suggesting a greater relative molecular mass. Figure 3 shows the profile of Sephadex G-200 elution. The patient’s serum shows a peak of CK activity in fraction 23, as well as in the normal CK fraction, no. 31. This fraction 23, developed with thin-layer gel filtration, emitted a fluorescence, appearing between the IgG and IgM fractions. Figure 4 shows the regression line of the relationship between elution volume (Ve) and relative molecular mass of four proteins used for calibration purposes on the Sephadex
I. Estimate of relative molecular mass of patient's serum creatine kinase isoenzymes

Fig. 4. Estimate of relative molecular mass of patient's serum creatine kinase isoenzymes

Elution volume (Ve) of proteins of known relative molecular mass and patient's serum CK isoenzymes from Sephadex G-200 column (1.8 X 45 cm) are plotted vs. the logarithm of relative molecular mass. The four calibration proteins were: albumin, M₄ 67 000; aldolase, M₄ 158 000; catalase, M₄ 240 000; ferritin, M₄ 540 000.

Fig. 5. Elution pattern of patient's serum creatine kinase isoenzymes from DEAE-Sepharose CL-6B mini-column (5 X 60 mm)

The serum was diluted with tris(hydroxymethyl)aminomethane buffer (50 mmol/liter, pH 7.0) before it was applied to the column; we kept in mind that serum contains about 150 mmol of sodium chloride per liter. Sample was applied and four 1-ml fractions of 10 mmol/liter sodium chloride buffer were collected (fractions 2-5). Another three 1-ml fractions of 150 mmol/liter sodium chloride buffer were collected (fractions 6-8). Finally, eight 1-ml fractions (usually three 1-ml of 300 mmol/liter sodium chloride buffer were collected (fractions 9-18, usually 9-11)

G-200 column. The results indicate that the relative molecular mass of "macro-CK" is approximately 325 000. By this method, that of normal CK was estimated to be approximately 80 000.

Immunological investigation. CK-B activity was determined to be 20 U/liter by use of an antibody against CK-M monomer. This result suggests that "macro-CK" would have CK-B activity, because the lowest limit of visual detectability of enzyme activity is 5 U/liter by our electrophoretic technique.

Immunoelctrophoresis against anti-whole human serum, anti-IgG, anti-IgA, anti-IgM, and anti-β-lipoprotein failed to show any precipitin lines containing CK activity. Furthermore, to investigate the possibility that "macro-CK" might be linked to serum immunoglobulin, the serum was also incubated overnight at room temperature with rabbit antisera against IgG, IgA, and IgM. Increasing volumes of the patient's serum were added to 0.1 ml of the rabbit anti-serum according to the method of Harada et al. (10). The samples were centrifuged at 3000 rpm and the CK activity in the supernatant fluid was determined. After washing with saline three times, the precipitate was completely dissolved in glycine/HCl buffer (50 mmol/liter, pH 3.4). The protein concentration was determined by absorbance at 280 nm. However, the results showed no increase of protein precipitate or decrease in CK activity in the supernatant fluid.

Fig. 6. Inhibitory effects of temperature (56 °C) and urea on the patient's serum creatine kinase isoenzymes

The cathode is at the bottom of the figure. (1), (2), (3), and (4): treatment of patient's serum with 1, 2, 3, and 4 mol/liter urea, respectively. (5) and (6): the patient's serum after heating at 56 °C for 5 min and 10 min, respectively; (7): the patient's serum

Physico-chemical treatment. Inhibitory effects of temperature and urea are shown in Figure 6. The "macro-CK" retained 75% of its original activity after heating at 56 °C for 5 min, and 15% after heating for 10 min at the same temperature. Normal CK activity disappeared entirely after heating for only 5 min. Inhibition studies were also carried out with urea. Normal CK showed only slight inhibition by 2 mol/liter urea, whereas "macro-CK" was inhibited entirely by the same condition.

Treatment of patient's serum with 6 g/liter sodium deoxycholate, 10 mmol/liter β-mercaptoethanol, and (or) 50 mmol/liter EDTA had no effect on the apparent molecular mass or electrophoretic mobility of "macro-CK."

Discussion

In analyzing for CK isoenzymes by electrophoresis, non-specific reactions often create problems; e.g., the reaction catalyzed by myokinase (2, 8), nonspecific staining by tetrazolium (2, 8), or nonspecific fluorescent substances in the albumin zone (especially in the serum of patients on hemodialysis treatment) (8). However, it is apparent that "macro-CK" is not present as a result of a nonspecific reaction, because the cathodic band referred to in this paper does not appear without creatine phosphate.

CK isoenzyme bands having cathodic mobility in tissue homogenate have been described in association with the mitochondria and myofibrillar extracts of rabbit (11), beef (12), and rat (13) myocardium, and rat skeletal muscle (13). CK activity associated with the mitochondria (m-CK) is more heat stable and more inhibited by urea than is the enzyme in the supernatant fraction (11). The special features of "macro-CK" are that it is more heat stable and more inhibited by urea than...
is normal CK-MM (Figure 6). Such heat stability contrasts with that of m-CK.

There is some disagreement concerning the amount of m-CK and its relative molecular mass. Sobel et al. (11) reported that the molecular weight of m-CK is approximately 80 000, which is near to that of CK in the cytoplasm, whereas Farrell et al. (12) reported approximately 250 000. In a short communication previously published concerning an atypical cathodic band (14), we had estimated the molecular weight of m-CK according to Sobel et al. and our data confirmed their findings. As shown in Figure 4, the relative molecular mass of normal CK was estimated to be about 80 000, which is in good agreement with reports by other investigators (1, 11). But we estimated "macro-CK" to have a relative molecular mass of about 325 000. This finding suggests that "macro-CK" may be a tetramer of normal CK.

Increased serum enzyme activity after irradiation has been widely reported. MacWilliam et al. (15) suggested that this increase could originate from the damaged cytoplasm of heart muscle. In our case, the increase in CK activity was slight, so it could not reflect profound tissue damage. Taken together, these findings make it seem most unlikely that "macro-CK" originated from the mitochondria.

There have been a few reports (16, 17) concerning abnormal electrophoretic patterns of serum CK isoenzyme bands. In them all, an abnormal fraction has been found between the CK-MM and CK-MB position. It has been mentioned that a CK species with abnormal electrophoretic mobility gives rise to a false positive CK-MB in column chromatography. In the present investigation, it is very interesting that CK activity was observed only in the CK-MM fraction by ion-exchange column chromatography, in spite of the fact that CK-B activity was observed in the patient's serum by use of an antibody against the CK-M monomer. With our method, the possibility of CK-MB or CK-BB carryover into CK-MM can be neglected. This evidence suggests that the superficial charge of "macro-CK" may be similar to that of CK-MM or that it may carry a positive charge.

However, Witteveen et al. (18) reported an as-yet-unidentified CK, chromatographically behaving as CK-MM and immunologically indistinguishable from CK-MM in human heart tissue. They suggested that any CK, for example m-CK, or another CK-MM that has a different subunit structure (19), would be measured as CK-MB with the immunological method, because the antibodies only block the normal CK-M subunit. In our case, CK-B activity observed is believed to be due to CK-MB originating from the cytoplasm of the myocardium; however, it is not certain whether this patient's serum really showed CK-B activity.

In conclusion, the evidence thus obtained suggests that "macro-CK" may be a tetramer of normal CK. However, the possibility of CK combined with some substance (relative molecular mass about 240 000) carrying a positive charge cannot be entirely excluded. Furthermore, it is not certain whether "macro-CK" really involves CK-B activity. We are continuing our investigation of sera from identical cases in which the patient is exposed to radiation in the thoracic region.

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References