Carbonic Anhydrase III in Serum in Muscular Dystrophy and Other Neurological Disorders: Relationship with Creatine Kinase

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We measured with a radioimmunoassay the concentrations of carbonic anhydrase III (CA-III, EC 4.2.1.1) in sera from 68 patients with muscular dystrophy, 10 carriers of Duchenne muscular dystrophy (DMD), and 63 patients with other neurological disorders. The values obtained were compared with those for creatine kinase (CK, EC 2.7.3.2). Serum CA-III was strikingly increased in patients with DMD (mean, 274.4 μg/L) and congenital (Fukuyama-type) (182.8 μg/L) and limb-girdle (203.7 μg/L) dystrophies and positively correlated with the activities of CK in patients with DMD. CA-III concentration decreased with the subjects' age and the severity of the disease, similar to the tendency observed between age or severity and the concentration of CK. We found moderately increased CA-III in patients with polymyositis, myotonic dystrophy, amyotrophic lateral sclerosis, spinal progressive muscular atrophy, or Kugelberg–Welander disease and in carriers of DMD.

Additional Keyphrases: radioimmunoassay · age-related effects · heritable disorders

Creatine kinase (CK; EC 2.7.3.2) is widely used for the monitoring and diagnosis of Duchenne muscular dystrophy (DMD) and is thought to be the most sensitive index of muscle breakdown.4 Of the other enzymes in use for studying DMD (e.g., enolase, aldolase, pyruvate kinase, lactate dehydrogenase), none are as sensitive or as specific.

In 1980, Carter et al. (1) first reported an isoenzyme of carbonic anhydrase III (CA-III; carbonate dehydratase, EC 4.2.1.1) that was found in significant activities only in skeletal muscle and was markedly increased in sera from patients with certain neurological diseases, in particular in patients with DMD (2). They also reported that CA-III concentrations might be useful for making prenatal diagnoses of DMD and diagnoses of adult muscular diseases.

CA-III in human skeletal muscle is located mainly in type I fibers (3), whereas CK (4) is found in type II fibers. Therefore, the concentration of CA-III in serum might reflect a type I fiber abnormality more sensitively than CK would. We evaluated the applicability of CA-III measurements to the early detection and monitoring of the progress of muscular diseases, to evaluate whether the measurements of both enzymes might be useful as specific markers of fiber abnormalities. We recently developed a sensitive radioimmunoassay for human muscle CA-III (5) and tested it on a large number of serum samples taken from patients with various neurological diseases, including muscular dystrophy. We also compared the CA-III concentrations with those of CK, the enzyme widely used as an indicator of muscular diseases.

Materials and Methods

Subjects: We examined the sera from 68 patients with muscular dystrophy (48 with DMD, 15 with congenital (Fukuyama-type), and five with limb-girdle), 10 carriers of DMD (all are mothers of DMD patients), 63 patients with various neurological and muscular disorders (nine with polymyositis, eight with myotonic dystrophy, 14 with amyotrophic lateral sclerosis (ALS), nine with spinal progressive muscular atrophy (SPMA), three with Kugelberg–Welander disease, 10 with myasthenia gravis, four with neuro–Behçet’s disease, and six with polynuropathy), and 106 normal controls. Carriers of DMD were classified into three groups according to the concentration of CK and the family history criteria of Thompson et al. (6). Serum samples were kept at –80 °C until used. Hemolyzed sera were not used for this assay.

Purification of CA-III: CA-III was purified from the gastrocnemius muscle of amputated human limbs by the modified method of Nishita and Deutsch (7). All purification procedures were carried out at 4 °C. Muscle was homogenized in phosphate-buffered saline (phosphate, 10 mmol/L, pH 6.6, containing NaCl, 150 mmol/L), 2 mL/g of muscle. After centrifuging the homogenate at 10,000 × g for 30 min, we applied the supernate to a 2.2 × 45 cm column of CM-Sephadex C-50 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with phosphate-buffered saline. Protein was eluted by use of a linear gradient of NaCl from 0 to 100 mmol/L in phosphate-buffered saline. Fractions containing CA-III activity were pooled and concentrated, after which they were passed through a 2.2 × 118 cm column of Sephadex G-75 (Pharmacia) equilibrated with 10 mmol/L phosphate buffer, pH 6.6. CA-III was then separated by Ampholyte displacement chromatography (Pharmacia). The purified CA-III showed a single band on sodium dodecyl sulfate–gradient polyacrylamide gel electrophoresis. The concentration of CA-III present was estimated by the method of Lowry et al. (8) with bovine serum albumin as the standard.

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4 Nonstandard abbreviations: CK, creatine kinase; CA, carbonic anhydrase; DMD, Duchenne muscular dystrophy; SPMA, spinal progressive muscular atrophy; ALS, amyotrophic lateral sclerosis.

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Enzymatic determination of CA-III: We measured the activity of CA-III according to the method of Wilbur and Anderson (9), using a saturated CO₂ solution and 0.02 mol/L barbital buffer. The enzyme activity was calculated by measuring the time required for the pH to decrease from pH 8.0 to 6.3 (the end point).

Preparation of specific antiserum to CA-III: Anti-CA-III antisera were raised in rabbits by six subcutaneous injections of 0.5 mg of purified CA-III emulsified with an equal volume of Freund's complete adjuvant, given at weekly intervals. Antisera were collected 10 days after the last injection.

Radioimmunoassay procedure for CA-III: CA-III was radiolabeled by the Chloramine T method (10). The specific activity of the ¹²⁵I-labeled CA-III utilized in the assay was about \(20 \times 10^{12}\) counts/min per gram. We measured the concentration of CA-III with a double-antibody RIA as follows: Dilute the test and standard serum samples 20-fold with 10 mmol/L phosphate buffer, pH 7.0, containing 1 g of bovine serum albumin per liter. Mix 50 µL of 100-fold-diluted nonimmune rabbit serum and 100 µL of 20,000-fold-diluted anti-CA-III antiserum with 100 µL of the standard or diluted test sample, then incubate at 4 °C overnight. Add ¹²⁵I-labeled CA-III and again incubate the mixture at 4 °C overnight. Add 100 µL of 100-fold-diluted goat anti-rabbit γ globulin, incubate for 2 h at 37 °C, then centrifuge the sample tubes at 1500 × g for 10 min. Wash the pellet twice with isotonic saline, then count its radioactivity with a gamma counter. In our hands the assay was reproducible, with within- and between-run CVs of ≤5.1% and ≤7.5%, respectively.

CK assay: CK activity was spectrophotometrically assayed with an N-acetylcysteine-activated kit (Boehringer, Mannheim, F.R.G.). The normal upper activity concentration of this enzyme was 80 U/L in males and 70 U/L in females (n = 53 each). CA-III and CK were determined simultaneously on the same serum samples.

Results

We developed an RIA method for detecting CA-III (5). The optimal conditions for this assay have been described elsewhere (11, 12). In our RIA, as little CA-III as 100 pg/tube could be detected quantitatively. Nonspecific binding was <2% of the total binding. There was no cross-reaction with any of the other known CA isoenzymes (CA-I and -II) derived from erythrocytes, up to a concentration of 100 µg/L.

CA-III concentrations in sera from 106 healthy controls are shown in Figure 1. There was no significant difference for the concentration of CA-III by age and sex in these healthy controls. CA-III values >27 µg/L (the mean for the normal controls plus 3 SD, 12 + 15 µg/L) were considered positive for DMD. Values for the healthy controls were within the normal range, except for two who had borderline values (32 and 33 µg/L). The relationships between age and activity of CA-III or CK for patients with DMD or congenital types of muscular dystrophy are shown in Figure 2. In DMD, the increases in CA-III were most pronounced in those in the younger group, being about 10-fold those for the control groups. Thus the highest CA-III values were present at the early onset of the disease and tended to decrease with age. A less-obvious tendency was found for subjects with congenital muscular dystrophy, and a similar tendency was observed between age and the concentration of CK.

The positive correlation between the concentrations of CA-III and CK in the 68 patients with DMD is shown in Figure 3.

Serial determinations (over as long as two years) of the CA-III and CK concentrations in individual patients with DMD are shown in Figure 4. In seven of 10 patients, the concentrations of both enzymes clearly decreased with age. In two cases there were sporadic variations, and only one showed a concomitant increase for a short period; in no instance was a marked clinical change observed during this period.

The CA-III and CK concentrations in sera from 10 carriers of DMD and 63 patients with other neurological disorders are shown in Figure 5. Carriers of DMD had
The frequency of abnormal CA-III was higher than that of CK. In contrast, patients with myasthenia gravis, neuro–Bechterew’s disease, or polyneuropathy had normal values.

**Discussion**

Carbonic anhydrase in human tissue is present in three isoforms (CA-I, II, III), with high concentrations of CA-III being located in skeletal muscle (3, 13).

To assess the clinical value of CA-III, measurement, we developed a sensitive radioimmunoassay to quantify CA-III, using purified CA-III from human skeletal muscle (5). Sera from patients with muscular dystrophy showed markedly high concentrations of CA-III, the increases being more marked in the younger age group of patients with DMD, as reported previously (2, 14, 15). The observed enzyme activities show the destruction of muscle that results in loss of ambulation and the flattening of the enzyme-disappearance curves at about age 12 years, when little muscle is left (Figure 2). Patients with DMD are generally confined to a wheelchair by age 12 years, a turning point for DMD patients. From the serial determinations in individual DMD patients, we observed that CK activities increase early in life, reach a maximum at age five or six years, decline rapidly until 11–13 years, and then slowly decrease from thereon. This trend was also reported previously (16). The decreases in CA-III with age were similar, as was the correlation between CA-III concentrations and clinical severity. Presumably, these changes reflect either a decrease of the muscle mass of the patients with age or a change in the disease activity. The concentrations of CA-III in serum correlated well with the concentrations of CK in muscular dystrophy.

CA-III and CK concentrations in sera from patients with other neurological diseases and carriers of DMD do not depend on age (data not shown), unlike the case of patients with DMD. However, we noted that abnormal concentrations of CA-III are frequent in carriers of DMD. In patients with SPMA and ALS, CA-III was a more sensitive indicator of disease than CK was. These results probably reflect the type I fiber abnormality more predominantly observed in SPMA and ALS, considering that the CA-III is located mainly in type I skeletal muscle fibers, whereas CK is found in type II fibers. The enzymes have different molecular masses (28 kDa for CA-III, 82 kDa for CK). The extent of the release of these enzymes may be affected by the permeability of the muscle membrane. Hence, the specificity of CA-III for skeletal muscle is an advantage in diagnosing.

Measurement of CA-III in serum appears to be about as valuable as that of CK for the diagnosis of muscular dystrophy and other neuromuscular diseases. The CA-III determination also is of particular value in neurological disorders, in which the CK is normal.

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References