Citrullinemia : Quantitative Deficiency of Argininosuccinate Synthetase in the Liver

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OYANAGI, K., ITAKURA, Y., TSUCHIYAMA, A., NAKAO, T., NAKANO, K. and SAEKI, T. *Citrullinemia : Quantitative Deficiency of Argininosuccinate Synthetase in the Liver*. Tohoku J. exp. Med., 1986, **148** (4), 385-391 — Two cases of citrullinemia were reported. Case 1 was an one month old female. Her clinical course and findings were different from the fluminant type of neonatal citrullinemia reported in predominantly Caucasian countries. Our patient was well controlled under a low protein diet and essential amino acids till 9 months of age, but unfortunately she died of Reye's like syndrome. Case 2 was 31 year old male (at the time of death). He was admitted to our hospital because of hyperammonemia and mental retardation. By subsequent laboratory investigations he was diagnosed as having adult type of citrullinemia and died of hepatoma. Enzymological analysis revealed that argininosuccinate synthetase (ASS) activities in the liver tissues of the patients decreased to 40% (Case 1), 20% (Case 2) compared with those in control liver tissues. The other urea cycle enzyme activities were all within normal range. ASS activities in the kidney and brains of the two cases were within normal range. The kinetic constant values of ASS for three substrates in the tissues of liver and kidney were all normal. Results of immunochemical analyses indicated that citrullinemia in our patients was caused by a quantitative deficiency of ASS associated proteins of the liver and kidney tissues as to the molecular weight. ——— citrullinemia; hyperammonemia; argininosuccinate synthetase

Citrullinemia is an inherited metabolic disorder of the urea cycle which is caused by abnormalities of argininosuccinate synthetase (ASS) (McMurry et al. 1962). At least three clinical phenotypes associated with ASS deficiency have been recognized (Walser 1983). (1) A neonatal form; occurs within the first week of life, with vomiting, coma and early death, (2) subacute form; a gradual onset of symptoms and later mental retardation, (3) late onset form; suffers from disturbance of consciousness during their adult age. Intelligence remained normal till onset (Takamizawa et al. 1973; Saito et al. 1976; Kooka et al. 1977).

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Citrullinemia has been explained as having both qualitative and quantitative abnormalities of ASS by enzymological and immunochemical analysis (Saeki et al. 1981).

Most of the citrullinemia cases in Japan are of the adult form and also having quantitative deficiency of ASS in the liver tissues.

We present here a variant type of neonatal citrullinemia found by a neonatal mass screening test and a typical form of the adult type. Reduced activities of ASS can be demonstrated in the liver tissues of the patients whereas ASS activities in the kidney and brain tissues were normal in both cases.

CLINICAL ASPECTS

Case 1 was the third child born to nonconsanguineous Japanese parents. Pregnancy and delivery were uneventful. She suffered from failure to thrive. At first, hypermethioninemia was discovered by a mass screening of neonates. However quantitative analysis of serum amino acids revealed that the serum levels of citrulline were elevated to 529 $\mu\text{mole/liter}$ (control 10-43). Blood ammonia levels were always within normal limits, but they were clearly elevated after oral loading test of ammonium chloride compared with those of controls. Dietary treatment (protein 1.0 g/kg and essential amino acids) was started at 2 months of age. Histological findings of the liver of the patient obtained by needle biopsy in the early period indicated a fatty liver, but the lesions were improved gradually under the treatment. After the treatment her weight gain was good. At 9 months of age she suffered from fever, diarrhea, frequent vomiting and convulsions and then died suddenly. Laboratory findings shortly before her death indicated that her blood ammonia levels were elevated to 345 $\mu\text{g}/100\text{ ml}$ and her serum concentrations of citrulline were slightly elevated to 85.6 $\mu\text{mole/liter}$ (control 10-43). These findings indicated that her death was not due to the crisis of citrullinemia.

Case 2 was born after a normal pregnancy and delivery in 1951. His family history was unclear because he was an adopted son. Until 22 years of age he developed normally, physically and mentally. He suffered from unconsciousness at age 27. Subsequent laboratory findings showed that his serum level of citrulline was elevated to 447 $\mu\text{mole/liter}$, other amino acids were within normal range and blood ammonia levels were always elevated to 150 to 300 $\mu\text{g}/100\text{ ml}$. Serum concentrations of ceruloplasmin and α -fetoprotein were normal. Histological findings of liver tissue revealed liver cirrhosis. He was treated by a low protein diet (30 g/day) since he was diagnosed as an adult type of citrullinemia.

He was relatively well controlled under the low protein diet but his liver dysfunctions were always progressive and he died of hepatoma at the age of 31.

MATERIAL AND METHODS

Autopsies were performed 2 hr after death. Liver, kidney, and brain tissues of the patients were frozen immediately and stored at -80°C for subsequent analyses. Mainly the cortex of the kidney and frontal lobe of the cerebrum of the brain were sampled.

Determinations of enzyme activities and K_m values; the activities of carbamyl phosphate synthetase, argininosuccinase in the liver tissues were assayed by the methods of Brown and Cohen (Brown and Cohen 1956). The activities of ornithine transcarbamylase and arginase were assayed by the methods of Shimke (Shimke 1962). ASS in the liver was measured at pH 7.5 and 25°C using the radiochemical procedure of Shimke (Shimke 1964), but those of brain tissues were determined at pH 8.5 and 37°C . For the determinations of K_m values, the concentrations of citrulline and aspartate varied from 0.02 to 50 mmol/liter and that of ATP was 0.09 to 3.6 mmol/liter with the concentration of each substrate fixed

at 5 mmol/liter and 0.9 mmol/liter, respectively.

Immunochemical analysis; to identify the amount of enzyme proteins in the liver tissues by single radial immunodiffusion according to the method of Mancini (Mancini et al. 1965) anti-rat ASS serum was used, which was confirmed to precipitate the human liver ASS as well as the rat liver ASS completely in the Oucheterlony double immunodiffusion method (Saeki et al. 1981).

Immunobinding assay of ASS associated protein after electrophoretic transfer from acrylamide gel to nitrocellulose sheet (Towbin et al. 1979); liver tissues were homogenized with 9 volumes of solution containing 1% SDS, 1% β -mercaptoethanol, 10 mM Tris HCl pH 6.8 and 20% glycerine (V/V) and then heated. 5 μ l of homogenated samples were applied to microslab SDS PAGE for 2 hr. The proteins were transferred from the gel to nitrocellulose membranes by electroblotting overnight. The membrane bound proteins were washed with TST (10 mM Tris HCl pH 7.5; 0.9% NaCl; 0.05% Tween 20) for 30 min and incubated in a blocking solution (TST containing 10% FCS). After that, the membrane reacted with Fab'-peroxidase conjugates (anti-rat liver ASS) during the night, which was carried out according to the method of Imagawa et al. (1982). After washing the nitrocellulose sheets 4 times with TST gently, they were immersed into the solution for color development. The solution was mixed with 15 mg of 4-chloro 1-naphthol in ice cold methanol 5 ml and 30% H_2O_2 10 μ l in cold TST 25 ml. The final procedure was performed in a dark place.

RESULTS

ASS activities in the liver of Cases 1 and 2 were reduced to about 40% and 20% of the mean control values respectively, while the other urea cycle enzymes were within normal range (Table 1). The ASS activities of brain and kidney tissues of the two cases were not different from controls (Table 2). The K_m values for three substrates of ASS in the two cases' liver and kidney tissues were normal (Table 2). The results of single radial immunodiffusion methods are shown in Fig. 1.

The amounts of enzyme protein in the liver tissues of the patients, which were presented by the size of the precipitation ring, were plotted against those activities. The curve obtained with the samples from patients corresponded with those of the controls (Fig. 1). Immunoblotting assay revealed that cross reactive

TABLE 1. *Activities of urea cycle enzymes in liver tissues (μ mole/min/mg protein)*

Enzyme	Case 1	Case 2	Control	
			(n)	Range
Carbamyl phosphate synthetase	0.019	0.028	(8)	0.0063–0.033
Ornithine transcarbamylase	1.08	1.02	(8)	0.33–1.7
Argininosuccinate synthetase	0.0035	0.0016	(5)	0.0062–0.0091
Argininosuccinase	0.023	0.021	(8)	0.0085–0.050
Arginase	1.3	2.2	(8)	1.8–5.1

TABLE 2. *ASS activities and Km values in the liver, kidney and brain*

	ASS activity (mU)				Km values (mM)		
	Case 1	Case 2	Control (n=5)		Case 1	Case 2	Control
Liver	3.5	1.6	7.9 $\pm$ 1.7	Citrulline	0.059	0.071	0.049
				Aspartate	0.020	0.015	0.033
				ATP	0.125	0.115	0.240
Kidney	4.5	2.0	3.3 $\pm$ 1.5	Citrulline	0.037	0.022	0.029
				Aspartate	0.010	0.045	0.017
				ATP	0.125	0.129	0.240
Brain	0.18	0.16	0.19 $\pm$ 0.056			n.d.	

U = μ mole/min/mg protein.

n.d. = not determined.

materials (CRM) were clearly evident in the liver and kidney tissues of the patients and also no difference of the molecular weight was observed in liver and kidney tissues of patients, controls, and purified rat ASS (Fig. 2).

DISCUSSION

Citrullinemia was reported initially by McMurry et al. (1962) as one of the inherited metabolic disorders caused by the abnormality of ASS. There are

Fig. 1. Determination of the amount of ASS protein in liver tissues of Case 1 and 2 by single radial immunodiffusion method. Diameter indicated the amount of ASS protein according to the sample activities respectively. ($\blacktriangle$), Case 1; ($\bullet$), Case 2; ($\circ$), ($\square$) controls.

Fig. 2. Immunobinding assay of ASS associated protein after electrophoretic transfer from polyacrylamide gel to nitrocellulose sheets: Sample tissues were homogenized with buffer containing 1% SDS and homogenized samples were applied micro slab SDS PAGE for 2 hr. After that, the protein of gels were transferred to nitrocellulose sheets by electroblotting. ASS associated protein was detected with anti-ASS Fab'-horseradish peroxidase conjugate. Lanes 1 and 6, control kidney; Lanes 3 and 8, control liver; Lanes 5 and 10, purified rat liver ASS; Lanes 2 and 4, kidney and liver of case 1 respectively; Lanes 7 and 9, kidney and liver of case 2 respectively.

several heterogeneities from both clinical and enzymological aspects (Walser 1983). Fulminant type of neonatal citrullinemia in whom, the first symptoms of the disease appeared during the first week with the elevated concentrations of citrulline to about 100-fold of controls. Most of the cases resulted in a rapid fatal course. The results of ASS assay showed extremely reduced enzyme activities in the liver tissues and cultured skin fibroblasts (McMurry et al. 1962; Zee et al. 1971). In some cases, abnormal Km values of enzyme for citrulline and aspartate were described (Saeki et al. 1981; 1982). The late onset type of this disease has been found in Japan indicating a milder elevation of serum citrulline than the neonatal type and intelligence was preserved till onset (Kooka et al. 1977), as observed in Case 2. ASS activities in the liver tissues were defective but activities of ASS in other tissues and the cultured skin fibroblast remained normal (Saeki et al. 1982).

Recently two types of abnormalities, qualitative and quantitative defects of this enzyme were illustrated by the kinetic and immunochemical analyses (Saeki et al. 1981).

We reported on a female neonatal citrullinemia found by a mass screening of neonates as hypermethionemia initially, but serum amino acids analyses revealed that the concentrations of citrulline were always 20-25 folds higher than those of controls. But its elevation was milder compared with the fulminant type of neonatal citrullinemia reported in other countries. Although ASS activities in the kidney and brain tissues of our patients were normal, ASS activities in liver tissues were reduced to 20% and 40% of the controls. However the ratios of ASS

activity of liver tissues and enzyme proteins were the same compared with those of controls, and the K_m values for the three substrates of ASS both in the liver and kidney tissues were normal.

Su et al. (1982) analysed ASS enzyme protein of cultured skin fibroblasts of citrullinemia and reported that CRM was not detectable in 9 of 11 citrullinemic cell line and in one cases CRM was present but smaller than normal. Our immunobinding assay revealed that CRM was present in citrullinemic liver and kidney tissues, and was of the same molecular weight.

From these results, it is speculated that expressions of the gene of ASS in liver and kidney tissues might be from the same origin. Therefore the cause of citrullinemia in our patients might be the result of an abnormality of the regulatory gene or a disturbance in the translation of mRNA. Case 1 was found at neonatal period and the youngest one of the quantitative deficiency of enzyme protein. Among the adult type of citrullinemia in Japan, some patients may develop this disease without any apparent clinical manifestations.

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