DEFECT IN SOLUBLE TYROSINE AMINOTRANSFERASE IN SKIN FIBROBLASTS OF A PATIENT WITH TYROSINEMIA

LABORATORY INVESTIGATIONS

(Short title: Tyrosine Aminotransferase Defect in Tyrosinemia)

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SPECULATION

The demonstration of tyrosine oxidative enzymes in skin fibroblasts should make it possible to define enzyme defects in patients with congenital tyrosinemia without the need for liver biopsy.

Abbreviations

p-HPAA	p-hydroxyphenylacetic acid
p-HPLA	p-hydroxyphenyl lactic acid
p-HPHA	p-hydroxyphenylpyruvic acid
PLP	pyridoxyl phosphate
TAT	tyrosine aminotransferase
EDTA	ethylene diamine tetraacetate

INTRODUCTION

Since the first report of a patient with abnormal tyrosine metabolism by Medes (18) in 1932, several different disorders of tyrosine oxidation have been described (25).

The commonest is transient neonatal tyrosinemia due to partial impairment of p-hydroxyphenylpyruvic (p-HPHA) hydroxylase activity which converts p-HPHA to homogentisic acid in the catabolism of tyrosine. Hypertyrosinemia results and a number of unusual tyrosine metabolites are formed which, together with tyrosine, appear in the urine (tyrosyluria). These include p-HPHA, p-hydroxyphenyl lactic acid (p-HPLA) and p-hydroxyphenylacetic acid (p-HPAA). There are three inter-related causes of hypertyrosinemia in the newborn: 1. diminished amounts of p-HPHA hydroxylase apoenzyme in the liver, 2. high dietary intake of tyrosine, and 3. low intake of dietary ascorbic acid (20).

Two types of nontransient hypertyrosinemia have been described:

1. Type I or hereditary tyrosinemia. This manifests in an acute form with hepatic and renal dysfunction within the first six months of life and is usually rapidly fatal in untreated patients, or in a more chronic form with nodular hepatic cirrhosis, nephropathy causing the Fanconi syndrome, failure to thrive and hypoglycemia (14). Although the activity of p-HPHA hydroxylase is impaired, the cause of this type of tyrosinemia is unclear: The patients benefit from stringent restriction of dietary tyrosine intake (25).

2. Type II or hypertyrosinemia Oregon type or ocular-cutaneous tyrosinosis. We know of 9 reported patients who seem to conform to this type (1,2,6,8,10,15,24,29). There is some phenotypic variability but most have had mental retardation, keratosis plantarum, corneal dystrophy (Richner - Hanhart syndrome). In two patients cytosol tyrosine aminotransferase (TAT) deficiency in liver was reported (2,5,15). The disorder runs a non-progressive course with high plasma tyrosine levels and tyrosyluria. Restriction of dietary tyrosine intake reverses the biochemical abnormality and improves the skin and eye lesions (1,6,8,15,24,29).

Other reported cases of hypertyrosinemia, including that of Medes, showed the clinical features of neither Type I nor Type II (17,18,27).

We describe a patient with seizures and developmental delay, but with no other clinical abnormalities, in whom cytosol TAT activity has been shown to be abnormally low in skin fibroblasts.

CASE REPORT

A girl, born after a normal pregnancy and delivery, appeared normal at birth; weight 2.53 Kg. The parents, living in a remote area in northwestern Ontario, are native Canadian Indians and, as far as is known, there is no consanguinity. Their plasma tyrosine levels were normal. A second child, a boy, was born a year after the birth of the patient and is normal. He does not have tyrosinemia.

When five months old, the patient had gastroenteritis with dehydration and generalized seizures. Two months later there were further seizures, mainly confined to the right side of the body and associated with a febrile upper respiratory infection. Despite anticonvulsant therapy, afebrile right sided seizures continued intermittently and at age 8½ months she was admitted to the Children's Hospital.

Physical examination was normal; weight 7.7 Kg (10th percentile), length 70 cm (50th percentile). There was no evidence of liver or bone disease and there were no skin or eye abnormalities. Full neurological investigation, including skull radiographs, brain scan and electroencephalogram, was normal. The urine gave positive dinitrophenylhydrazine and nitrosonaphthol tests (21) and showed large amounts of tyrosine on thin layer chromatography. Plasma tyrosine level was 0.91 µmoles/ml and tyrosine metabolites were later demonstrated in urine (see below). Treatment with ascorbic acid (500 mg daily by mouth) and pyridoxine HCl (250 mg daily by mouth) had no consistent effect upon blood tyrosine levels (Fig. 1). At 50 weeks of age she was tested on the Yale Developmental Schedules. Her overall performance was at 44 weeks with many successes at 48 weeks. She thus appeared to fall into the low-normal range.

Anticonvulsant therapy with phenobarbital and dilantin was maintained and there were no further seizures. There was nothing suggesting the need for dietary treatment at this time and she was allowed home at 14 months of age.

During the next 10 months she remained well except for two brief afebrile grand mal seizures; growth continued to be normal. When re-assessed at 24 months, the blood tyrosine level was still greatly elevated (Fig.1) and a positive urine dinitrophenylhydrazine test indicated the continued presence of ketoacids. Physical examination was normal but she was hyperactive and her developmental quotient was now assessed as only 75%. The electroencephalogram was still normal. Because of this deterioration in development a diet low in phenylalanine (25-31 mg/Kg) and low in tyrosine (20-27 mg/Kg) was begun. Blood tyrosine levels during the next year are shown in Figure 1.

When last seen at age three years she showed significant delay in all areas of development -- motor and adaptive skills at the thirty month level, language and social skills at about the 12 month level.

Methods

Amino acids in plasma and urine were determined with a Perkin Elmer KLA-5 amino acid analyser. Plasma samples were deproteinized with sulfosalicylic acid prior to analysis (25). Organic acids were identified and quantitated by gas chromatography - mass spectrometry-computer techniques (7), (courtesy of Dr. O.A. Mamer of Montreal).

Tissue Preparation: Skin fibroblasts were grown from a skin biopsy taken, after informed consent, from the patient at age 12 months. Control fibroblast cultures were taken from 4 age-matched normal subjects. Between 2 x 10⁵ and 4 x 10⁵ cells from each fibroblast line were seeded into two separate 100 mm dishes (Falcon Plastics Oxnard, Ca) containing 5 ml of McCoy's 5a tissue culture medium (Grand Island Biological Co.), supplemented with 5% human cord serum (Microbiological Associates, Bethesda, MD) and antibiotics (penicillin, streptomycin-fungicillin). The dishes were then incubated at 37.5° in a water-jacketed incubator with 100% humid atmosphere of 5% CO₂ in air. The cells were subcultured twice a week. Sixteen fibroblast cultures each from the patient and age-matched controls were used in these experiments. The fibroblasts used were all of low passage numbers.

Cells were released from the dishes for treatment with 0.05% trypsin in Dulbecco's phosphate buffered saline (pH 7.2) for 7 - 10 min. at 37°. Trypsinization was stopped by the addition of a 5-fold excess of the growth medium. A sample was taken for cell enumeration. Cells were collected by centrifugation, washed thrice in 0.2 M phosphate buffer (pH 7.2) and 15 x 10⁶ cells from each culture were homogenized with 4 volumes of cold 0.14 M KCl by 6 strokes of the tightpestle of a Dounce homogenizer. Subcellular fractionation by differential centrifugation was carried out essentially according to Tulkens et al. (26). The 18,000 x g pellet of the post nuclear fraction was used as the mitochondrial fraction and the high speed (105,000 x g) supernatant as the cytosolic fraction. The protein content of this fraction usually was 500-600 µg per ml.

Assay of Tyrosine Aminotransferase: (L-tyrosine: 2 oxoglutarate aminotransferase, E-C 2.6.1.5): This enzyme was assayed in the cytosolic fraction by two different methods, the p-hydroxybenzaldehyde method of Diamondstone (3) and the method of Rosen et al (23) based on the formation of a coloured complex between p-HPHA and ammonium molybdate. In some experiments the Diamondstone method and in others the Rosen method were used. Both methods gave comparable assay results. The enzyme activity was assayed at four different concentrations of pyridoxal phosphate (PLP) added to the incubation medium - 0, 1.2, 10 and 20 mM. In initial experiments (Table I and Figure 2) the enzyme was assayed in the undialyzed cytosolic fraction. In later experiments the cytosolic fraction was dialyzed against 0.2M phosphate buffer (pH 7.4) containing 0.005 M mercaptoethanol and 0.001 M EDTA for three hours or passed through a Sephadex G-25 column and eluted with the same buffer to remove free pyridoxal phosphate of the cytosol. The mitochondrial enzyme was assayed only by the method of Diamondstone. (3) The lower limit of sensitivity of this procedure is the formation of 0.2 µmole p-HPHA.

Assay of p-Hydroxyphenylpyruvate Hydroxylase (p-hydroxyphenylpyruvate, ascorbate: O₂ oxidoreductase (hydroxylating), ED 1.14.2.2): The colorimetric assay method of Fellman et al. (4) was used.

Results

Amino Acids and Phenolic Acids in Plasma and Urine: Figure 1 shows plasma tyrosine levels from age 11 - 36 months in relation to vitamin and dietary treatment. Concentrations of other plasma amino acids, including methionine and phenylalanine, showed no consistent abnormality. Urine tyrosine levels before the institution of dietary treatment ranged from 13.4 - 27.6 mM/100g N (normal < 4.0). Plasma p-HPHA was 0.9 mg/l (normally undetectable (8)). Urinary organic acids (g/g creatinine) were: p-HPHA 0.8 (trace), p-HPLA 1.6 (< 0.01), p-HPAA 0.5 (< 0.02). Normal values (8) are in parentheses.

Growth and viability of the cultured fibroblasts: Fibroblasts from the patient and matched controls were cultured for several passages in presence of McCoy's 5a tissue culture medium supplemented with 5% human cord serum. This medium contained 0.5 mg per liter of pyridoxine and 0.5 mg per liter of pyridoxal. Thus, fibroblasts from both sources were cultured in a medium containing adequate concentration of vitamin B₆. There was no difference between the two fibroblast cultures in regard to the time required to achieve confluency as well as in the viability of the cells.

Cytosolic TAT: The results are given in Table I. In the absence of PLP added to the incubation medium there was a small amount of TAT activity with both the patient and control fibroblasts. Increasing the PLP concentration of control fibroblasts from zero to 20 mM led to a 15-fold increase in enzyme activity. A similar increase in PLP concentration of the patient's fibroblasts had approximately 20 percent the activity of controls. When the concentration of PLP was increased to 50 mM there was an inhibition of enzyme activity only in control cultures. At all the concentrations of PLP tested, the fibroblast cultures derived from the patient showed much lower activity of TAT.

Fig. 2 shows the cytosolic TAT activity of fibroblasts from patient and controls plotted against the concentration of the cytosolic protein with PLP concentrations of 1.2 and 10.0 mM. Although there was linearity in the response

Figure 1: Plasma tyrosine levels of the patient in relation to treatment given. The horizontal interrupted line indicates the mean levels for normal infants (2,17).

Conc. Pyridoxal phosphate added to incubation medium (mM)	TAT ^a ASSAY ACCORDING TO			
	Diamondstone (3)		Rosen et al (23)	
	Fibroblast Source:		Fibroblast Source:	
	Patient	Controls	Patient	Controls
Nil	7.6 ± 0.8 ^b	7.5 ± 1.6 ^b	6.2 ± 1.2 ^c	15.2 ± 3.8 ^c
1.2	15.0 ± 2.8 ^d	39.6 ± 3.3	18.3 ± 2.6 ^d	42.6 ± 5.3
10.0	24.2 ± 2.1 ^d	93.4 ± 5.8	21.2 ± 2.1 ^d	76.4 ± 4.8
20.0	22.4 ± 3.8 ^d	103.7 ± 6.2	20.3 ± 3.4 ^d	85.3 ± 7.5

^a enzyme activity expressed as μ moles p-HPPA formed/30 min/mg protein.

^b Mean ± standard deviation of 7 separate experiments in each of which the co-substrate was included in concentration range of 0–20 mM. The reaction mixture contained, in a total volume of 3.2 ml, the following: 2.6 ml of 0.2 M potassium phosphate buffer, pH 7.2 containing 19.2 μ moles of L-tyrosine; 0.1 ml of a buffered dithiocarbamate; and 0.2 ml of appropriate dilution of the fresh enzyme. The mixture was equilibrated at 37° and the reaction was started by the addition of 0.1 ml of a buffered solution of 0.3 M α -ketoglutarate. The reaction was stopped after 10 min. by the addition of 0.2 ml of 10 N NaOH and the tubes shaken immediately. After 30 min. at room temperature the solutions were read at 331 m μ against a zero time control prepared by the addition of NaOH before the α -ketoglutarate.

^c Mean ± standard deviation of four experiments in each of which the cosubstrate was included in concentration range 0–20 mM. The reaction mixture contained in a total volume of 2.9 ml the following: 1.9 ml of 0.2 M potassium phosphate buffer, pH 7.3 containing 30 μ moles of diethyl dithiocarbamate and 0.16 M sucrose; 0.3 ml of the cytosol and 0.1 ml of PLP solution (0–20mM). The mixture was equilibrated at 38° for 3 min. and the reaction started by the addition of 0.6 ml of 0.01 M tyrosine. The mixture was incubated for 10 min. at 38° and reaction stopped by the addition of 0.3 ml of 100% trichloroacetic acid. The mixture was filtered on a Whatman #1 filter paper. To 1 ml of the filtrate 2 ml of the color reagent (12 ml of 3% ammonium molybdate in 5 N HCL plus 20 ml of 1% KH_2PO_4 plus 32 ml distilled water) was added. Absorbance at 850 m μ was read after 1 hour of color development.

^d $p < 0.005$ with respect to controls.

Figure 2: Cytosol tyrosine aminotransferase activity in fibroblasts of the patient and controls plotted against concentration of enzyme protein in the assay with pyridoxal phosphate (PLP) concentrations of 1.2 and 10 mM.

at all protein concentrations tested the enzyme activity of patient's fibroblasts was about fifth of the activity of the control fibroblasts. In these experiments undialyzed cytosolic fractions used would have contributed to the final concentration of pyridoxal phosphate in the TAT assay medium.

In further experiments a dialysate or a sephadex G-25 filtrate of the cytosol of fibroblasts from the patient or age matched control was used to assay for TAT. Both cytosolic enzyme preparations, free from endogenous pyridoxal phosphate, had very little measurable TAT activity. Addition of pyridoxal phosphate to the enzyme preparation resulted in measurable TAT activity which was linear with respect to protein included in the assay (Fig. 3). The enzyme from control fibroblasts showed a linear response to the incubation time up to 30 minutes, after which it plateaued. The enzyme preparation from the patient's fibroblasts gave a linear response to the incubation time up to 40 minutes. The data presented in Fig. 3 are for a 5 minute assay. The enzyme from the patient's fibroblast was much less active, being about 20 percent of the activity of the enzyme from control fibroblasts. The profile of enzyme activity was the same regardless of the use of a dialyzed or undialyzed cytosol in measuring enzyme activity.

The kinetic characteristics of the cytosolic TAT of the two fibroblasts were determined using Lineweaver Burke plots. The results are compared in Table II. There was no significant difference between the enzyme from either source in terms of the V_{max} and the K_m for tyrosine. These values are in the range reported for TAT from other sources. V_{max} values ranging from 1.35 to 5.0 μ moles product formed per min per mg protein have been reported (9,22). The reported values for the K_m for tyrosine are in the range 1.2 to 1.7 $\times 10^{-3}$ M (9,11,19,28). However there is a significant difference between the enzyme from the patient's fibroblasts and control in regard to the K_m for pyridoxal phosphate. The K_m for pyridoxal phosphate of the control enzyme is in the range reported for enzyme from rat liver (16). The K_m of the enzyme from the patient's fibroblasts for pyridoxal phosphate was almost two orders of magnitude more than that for the enzyme from control fibroblasts. Thus the enzyme from the patient's fibroblasts has a significantly decreased affinity for pyridoxal phosphate.

Mitochondrial TAT: The means ± one standard deviation of five separate determinations were as follows: patient's fibroblasts, 29.5 ± 4.8 controls, 26.4 ± 8.1 μ moles p-HPPA formed/30 min/mg protein. Thus, there was no statistically significant difference in the activity of mitochondrial TAT in fibroblasts of patient and controls.

Cytosolic p-HPPA hydroxylase: There was no difference in p-HPPA hydroxylase activity in patient and control fibroblasts (Table III).

DISCUSSION

TAT and p-HPPA hydroxylase are the first two enzymes in the oxidative pathway of tyrosine. PLP is a cosubstrate for TAT and ascorbic acid a co-substrate for p-HPPA hydroxylase. TAT exists in at least two forms: a solu-

Figure 3: Tyrosine aminotransferase activity of dialysed cytosol of fibroblasts from patient and control fibroblasts plotted against concentration of enzyme protein in the assay with pyridoxal phosphate concentration of 1.2 mM. (●.....●), Control cytosolic enzyme; (■.....■), patient's cytosolic enzyme.

Table II
KINETIC CHARACTERISTICS OF CYTOSOLIC TAT
OF CULTURED FIBROBLASTS

Source of enzyme	No. of experiments	PLP removed by	V_{max} (μ moles p-HPPA formed/min/mg protein)	K_m for Tyrosine	K_m for PLP
Control fibroblasts	4	Dialysis	25.0 ± 4.1	1.1 ± 0.1 · 10 ⁻³ M	1.9 ± 0.2 · 10 ⁻⁶ M
	4	Sephadex G-25 filtration	22.7 ± 2.8	1.2 ± 0.1 · 10 ⁻³ M	1.3 ± 0.1 · 10 ⁻⁶ M
Patients fibroblasts	4	Dialysis	28.5 ± 1.3	1.1 ± 0.2 · 10 ⁻³ M	2.6 ± 0.2 · 10 ⁻⁴ M
	4	Sephadex G-25 filtration	25.0 ± 2.2	1.2 ± 0.1 · 10 ⁻³ M	2.5 ± 0.2 · 10 ⁻⁴ M

Enzyme preparation from fibroblast cultures and the assay of TAT by the method of Diamondstone (3) are described "Methods".

Table III
CYTOSOLIC p-HPPA HYDROXYLASE OF CULTURED FIBROBLASTS

Source of fibroblasts	p-HPPA hydroxylase ^a
Patient	0.82 ± 0.05 ^b
Controls	0.78 ± 0.09

a Enzyme activity expressed as μ moles product formed/hour/mg protein.

b Mean ± standard deviation of 5 determinations. The reaction mixture in a total volume of 2.0 ml contained 0.5 mM p-hydroxyphenylpyruvate, 100 mM potassium phosphate (pH 7.5) and enzyme extract which was activated by the addition of an equal volume of 2.5 mM 2,6-dichlorophenolindophenol and 52 mM glutathione. Tubes were incubated with shaking at 37° for 10 min. The reaction was stopped by the addition of 0.5 ml of 800 mM sulfosalicylic acid and the mixture centrifuged at 1000 g for 5 min. 1 ml of the supernatant was extracted twice with 2 ml ethylacetate. The combined ethylacetate extract were shaken with 1 ml of 200 mM potassium phosphate (pH 8.0). The ethylacetate was discarded and 0.2 ml of 1 M KBH₄ added. After 10 min. 0.1 ml of 5 M acetic acid was added and the tubes shaken to destroy excess of KBH₄; 0.2 ml of 200 mM cysteine and 3.0 ml of 50 mM potassium phosphate (pH 12.0) were then added and the color allowed to develop for 15 min. Tubes were well aerated and the absorbance determined at 390 nm.

ble form in the cytosol, another form in mitochondria. Both forms have been found in liver, heart, muscle, kidney and brain (5). p-HPPA hydroxylase, a cytosolic enzyme, has been reported only in liver, and to a lesser extent in kidney (13). We have shown that both cytosolic and mitochondrial TAT and p-HPPA hydroxylase are also present in skin fibroblasts, thus making it possible to define enzymatic defects in tyrosine oxidation without the need for liver biopsy.

The cytosolic TAT of fibroblasts had the following characteristics:

1) In the undialyzed cytosol, at the optimal pyridoxal phosphate concentration, the activity of the enzyme was about 20% of control levels on a unit weight of protein basis.

2) Small molecular weight factors present in the cytosol seem to decrease the affinity of the control enzyme for pyridoxal phosphate as an incremental rise in enzyme activity was seen with increases in pyridoxal phosphate concentration in the incubation medium.

3) In experiments where the dialyzed or undialyzed cytosolic enzyme from the patient was mixed with the corresponding control enzyme the resultant enzyme activity was additive, ruling out the presence of any protein inhibitor of TAT in patient's cytosol.

4) The Km of the patient's enzyme for the co-substrate, pyridoxal phosphate, is about two orders of magnitude higher than the Km of the control enzyme for pyridoxal phosphate. The patient's fibroblasts have a defective mutant enzyme with a considerably decreased affinity for pyridoxal phosphate.

The study of skin fibroblasts of the patient indicated the presence of normal levels of mitochondrial TAT and p-HPPA hydroxylase with a defective cytosolic TAT characterized by a low affinity for pyridoxal phosphate.

The enzyme abnormality in skin fibroblasts of this patient appears similar to those found in the livers of the "Oregon" or Type II tyrosinemia patients, but we do not know if it is identical (5). In these patients the nature of the defect is not known. TAT seemed to be either deficient (ie. absent protein) or the mutant enzyme was so defective that in the enzyme assay there was no response even to increments of enzyme protein (Kennaway and Buist Fig. 1 (12). These assays were done at a PLP concentration of 1.2 mM. In our patient the enzyme is present but defective in having a very high Km for PLP and would presumably behave in the same manner as that of Kennaway and Buist. Our patient had mental retardation but otherwise none of the clinical features described in the Oregon patient which included multiple congenital abnormalities, severe mental retardation, bullous eruptions on fingers, palms and soles, and stellate corneal ulcers. Since enzyme analyses were not performed in the other reported patients with hypertyrosinemia having one or more components of the Richner-Hanhart syndrome (keratitis, skin lesion, mental retardation) it is not known whether they had the same enzymatic defect as the Oregon patient. Although it is tempting to ascribe the clinical lesions in this condition to the high circulating levels of tyrosine and phenolic acids, particularly since the eye and skin lesions improved significantly in the 6 patients who received restricted tyrosine diets, the variability in clinical manifestations among the patients makes this simplistic theory unattractive. Nor does it explain why the two main types of hereditary tyrosinemia have completely different clinical manifestations. The benefits of dietary treatment in our patient have been uncertain except that she has had no further seizures.

The paradox of an error of metabolism in which there are elevated concentrations of both the substrate and the product of the defective enzyme, namely tyrosine and p-HPPA has been fully discussed by Scriver and Rosenberg (25).

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