Glycolytic Intermediates of Tissues in Enzymopathic Hemolytic Anemia

Susumu ODA
Third Division, Department of Internal Medicine, Yamaguchi University School of Medicine, Ube, 755, Japan
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INTRODUCTION

Since Valentine, Tanaka and Miwa[1] discovered hemolytic anemia with pyruvate kinase deficiency, a number of enzyme deficiencies in the Embden-Meyerhof pathway have been reported as a cause of hemolytic anemia. Among these enzyme deficiencies, phosphoglycerate kinase (PGK)[2,3,4] and triosephosphate isomerase (TPI) deficiency[5,6] in erythrocytes have been frequently associated with neuromuscular disorders. PGK deficiency has been found in muscle cells as well as in erythrocytes[7]. TPI deficiency has been demonstrated in erythrocytes, leukocytes, spinal fluid and skeletal muscle[8]. Thus, enzyme deficiency is expected to be a cause of clinical manifestations, such as neuromuscular disorder. Electrophoretic study of these enzyme deficiencies has been reported to be one of the good tools with which to clarify the character of abnormal enzymes. Among a number of reports concerning hereditary nonspherocytic hemolytic anemia associated with enzyme deficiency, the reason that PGK and TPI deficiencies have very frequently been associated with severe neuromuscular disorder remains unclear.

This report presents the results of measurement of glycolytic intermediates of tissues (muscle, liver and spleen) in hemolytic anemia due to enzyme deficiency (one case of phosphoglycerate kinase deficiency, two of glucosephosphate isomerase deficiency and two of pyruvate kinase deficiency). This may provide us information as to whether enzyme deficiency affects cells other than erythrocytes and whether it causes clinical symptoms in these organs.

MATERIALS AND METHODS

The patients examined here were one case of phosphoglycerate kinase (PGK) deficiency, two of glucosephosphate isomerase (GPI) deficiency (GPI"Narita" and GPI "Matsumoto") and two of pyruvate kinase (PK) deficiency. The clinical and laboratory data of all patients have been reported elsewhere.[4,10,11,12] Glycolytic intermediates in the muscle were estimated in all patients and glycolytic inter-

mediates in the liver and spleen were estimated in the patients who had received splenectomy. All specimens were obtained by biopsy or splenectomy. The specimens (about 250 mg.) were homogenized, using a Universal Homogenizer (Nihon Seiki Seisakusho Co., Tokyo, Japan) in 5 ml of cold 0.6 N perchloric acid immediately after weighing. Then, they were centrifuged at 26,800 G. for 20 min. in a refrigerated centrifuge (Automatic High Speed Refrigerated Centrifuge, Hitachi 20 PR, Tokyo, Japan), and the pH of the supernatant was adjusted to 7.4 with KOH. Determinations of glycolytic intermediates and adenine nucleotides were done enzymatically according to the method described by Minakami et al.[13] except for 2,3-diphosphoglycerate (2,3-DPG), which was assayed enzymatically by the method described by Rose and Liebowitz[14], and 1,3-diphosphoglycerate (1,3-DPG), which was assayed described by Nakashima et al.[15] Normal means of glycolytic intermediates of muscle, liver and spleen were calculated from three different samples. These samples were obtained at the time of splenectomy for gastric cancer without metastasis to liver and spleen, and from the patients with breast cancer or thyroid cancer.

Electrophoresis of PGK: Blood samples collected in ACD solution were examined. The red cells were washed three times with cold saline solution and the packed red cells were diluted three times with 0.01 M tris-citrate, pH 7.5. After the mixture was frozen and thawed three times, toluene was added to the hemolysate and it was shaken vigorously for five min.. The stroma was removed by centrifugation. Muscle specimens of PGK deficiency were obtained by biopsy and normal muscle, liver and spleen were the same samples for glycolytic intermediates assay. These tissue specimens were homogenized with 9 % sucrose in 0.01 M tris-citrate, pH 7.5 and the homogenate was centrifuged at 26,800 G. for 30 min. at 4°C. Preparation of these samples was done according to Beutler's method.[9]

Electrophoresis was carried out in a thin polyacrylamide gel system. A 0.1 M tris-citrate buffer, pH 6.85, at 25°C was used for the bridge buffer. Five mM of mercaptoethanol was added to the buffer. The acrylamide gel was prepared according to Imamura's description,[16] that is, 3.33 % acrylamide, 0.22 % N,N′-methylene bis acrylamide, 0.35 % N,N,N′,N′-tetramethylethylenediamine and 0.03 % ammonium persulfate and then equilibrated with the bridge buffer overnight. Electrophoresis was carried out for 4–5 hours at 20–25 V/cm. with a cooling system in a cold room (4°C). PGK activity was detected by the conversion of NADH to NAD, through which ultraviolet light could pass. Ten milliliters of reaction mixture which was the same as Beutler's, containing 100 mM tris-HCl, pH 8.0; 3 mM $MgCl_2$; 5.4 mM EDTA (neutralized); 4 mM 3-phosphoglycerate; 1.4 mM NADH; 4 mM ATP was warmed to 37°C. Then 3ml of the warmed 3 % agarose solution was cooled to about 50°C and added to the reaction mixture with 0.1 U/ml. glyceraldehydephosphate dehydrogenase. The mixture was poured into the framed gel. After the staining gel became solid, it was incubated at

37°C. Photographs were taken every fifteen min. with ultraviolet light (Manaslu-light, 2536 Å, Manaslu Chemical Ltd., Tokyo, Japan) by placing the photographic paper (Mitsubishi Photographic Paper, Gekko V-3, Japan) under the gel.

RESULTS

Fig. 1 shows erythrocytes and muscle glycolytic intermediates in phosphoglycerate kinase (PGK) deficiency, as reported previously.[7] Muscle glycolytic intermediates had pattern similar to that of erythrocytes, that is, increased fructose-1,6-diphosphate (FDP), dihydroxyacetone-phosphate (DHAP), 1,3-DPG and 2,3-DPG, which suggested the blockade of glycolysis at the step of PGK. The accumulation of 1,3-DPG in the muscle was more prominent than in the erythrocytes.

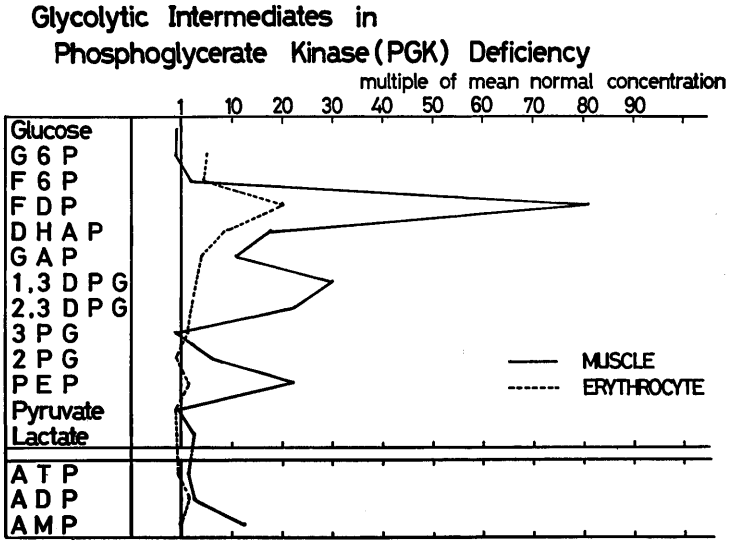

Fig. 1. Glycolytic intermediates in erythrocyte and muscle of phosphoglycerate kinase deficiencyy.
G-6-P: glucose 6-phosphate, F-6-P: fructose 6-phosphate, FDP: fructose 1,6-diphosphate, DHAP: dihydroxyacetone phosphate, GAP: glyceraldehyde 3-phosphate, 1,3-DPG: 1,3-diphosphoglycerate, 2,3-DPG: 2,3-diphosphoglycerate, 3-PG: 3-phosphoglycerate, 2-PG: 2-phosphoglycerate, PEP: phosphoenolpyruvate, ATP: adenosine triphosphate, ADP: adenosine diphosphate, AMP: adenosine monophosphate.

Glycolytic intermediates in the muscle with GPI deficiency and PK deficiency are shown in Fig. 2. Neither GPI deficiency nor PK deficiency followed the typical pattern for glycolytic intermediates, which suggested a blockade of glycolysis at the step of those enzymes.

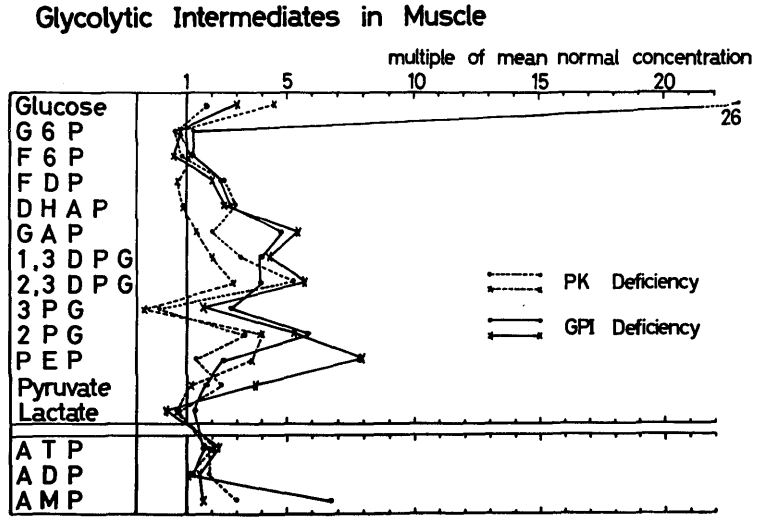

Fig. 2. Glycolytic intermediates in muscle
•———• GPI "Narita"
×———× GPI "Matsumoto"

Glycolytic intermediates in the liver are shown in Fig. 3. The cases of PK deficiency did not suffer disturbance of glycolysis. There was no finding which suggested a disturbance of glycolysis in GPI deficiency, although the level of glycolytic intermediates was in general high.

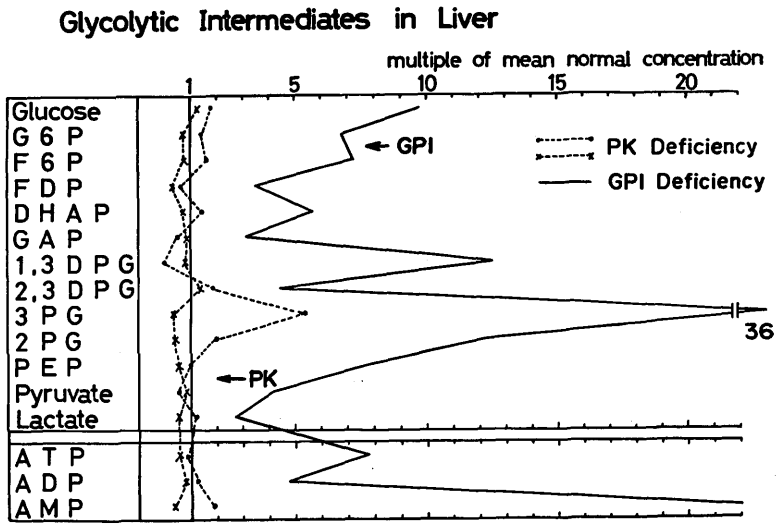

Fig. 3. Glycolytic intermediates in liver
•———• GPI "Narita"

Glycolytic intermediates in the spleen are shown in Fig. 4. Glycolytic intermediates in the spleen with GPI deficiency did not suggest a disturbance of glycolysis. In PK deficiency, 2-phosphoglycerate (2-PG) and 3-phosphoglycerate (3-PG) increased.

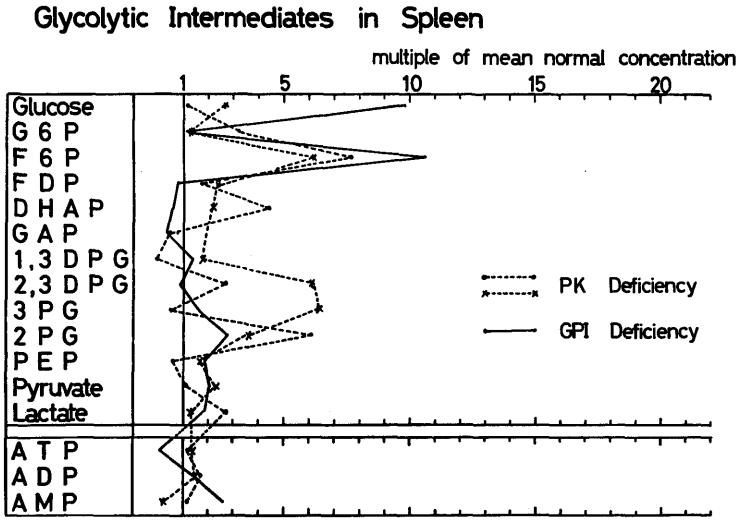

Fig. 4. Glycolytic intermediates in spleen
•———• GPI "Narita"

PGK electrophoreses by thin polyacrylamide gel are shown in Fig. 5 and 6. PGK electrophoreses of normal muscle, liver, spleen and erythrocyte are shown in Fig. 5. PGK mobilities in the electrophoreses among these tissues were very similar. Erythrocytes and muscle PGK with the enzyme deficiency developed a faint band which was of similar, if not identical, mobility to the normal control in our electrophoretic system. The parents and maternal grandmother of the PGK deficient patient had erythrocyte PGK which was very similar to the normal control. (Fig. 6)

DISCUSSION

Glycolytic intermediates in erythrocytes and muscle cells with PGK deficiency substantiated the blockade of glycolysis at the step of PGK. It appears that absence of the Rapoport-Luebering pathway in the muscle caused more marked accumulation of 1,3-DPG, which is a substrate of PGK, while 1,3-DPG in the erythrocytes was catalyzed to 2,3-DPG by diphosphoglycerate mutase. Erythrocyte and muscle PGK activity of PGK deficient patient were 4 % and 5 % of normal

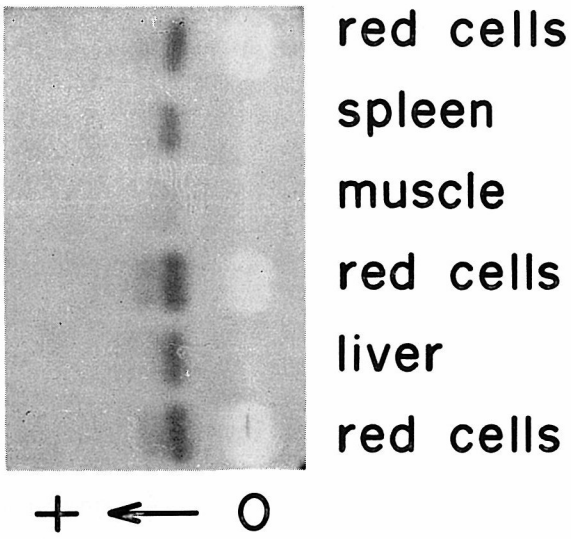

Fig. 5. Electrophoresis of PGK of normal muscle, spleen and erythrocytes.

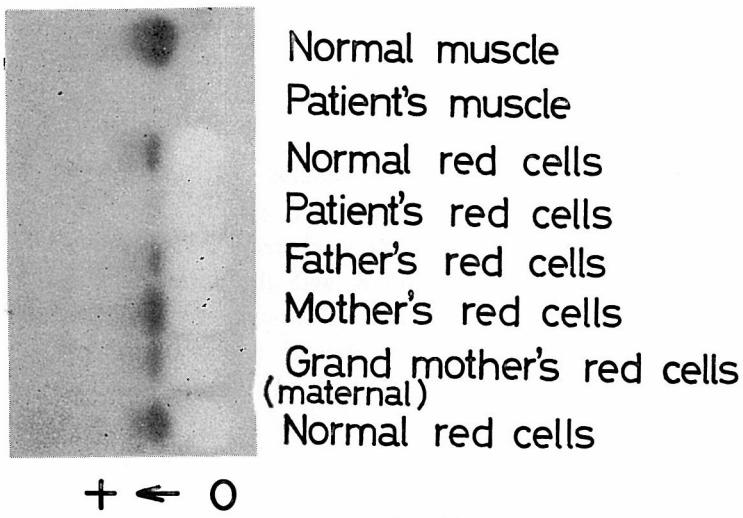

Fig. 6. Electrophoretic study of patient With PGK deficiency and his family.

control values, respectively.[4)7)] In addition, erythrocytes and muscle PGK were stained very faintly in electrophoresis with normal mobility (Fig. 6). With our method, the patient's PGK activity (about 5 % of normal value) developed a visible band. The results of glycolytic intermediates, assay of PGK activity and electrophoresis of the muscle all prove that PGK deficiency affects muscle as well as erythrocytes. Beutler[9)] reported that the electrophoretic mobility of PGK in the

liver, heart, kidney and skeletal muscle were all similar to that of erythrocytes. In our electrophoretic system, the pattern of PGK activity of the muscle, liver spleen and erythrocytes were also very similar. In addition, it is postulated that the structural locus for this enzyme is X-linked.[17][18] PGK in all organs may be controlled by a single control gene. So far, erythrocyte and muscle have been available for biochemical studies in our case with PGK deficiency. Clinically, the patient has symptoms of disturbance of the nervous system, such as ataxia and mental retardation, in addition to hemolytic anemia and atrophy of muscle. PGK deficiency associated with neuromuscular disorders has been reported previously.[2][3] PGK deficiency would be expected to cause dysfunction of tissue other than erythrocyte, although in our case, a possibility of association with other diseases cannot be excluded. TPI deficiency has also been associated with severe neuromuscular dysfunction. TPI deficiency was revealed in erythrocytes, leukocytes, muscle cells and spinal fluid,[8] and the electrophoretic patterns of these various organs have been reported to be similar.[19] It is conceivable that enzyme deficiency affects every organ and causes dysfunction of those organs if the enzyme has not a tissue-specific variant. On the other hand, glycolytic intermediates in the muscle, liver and spleen of GPI "Narita" did not reveal disturbance of glycolysis in spite of the fact that abnormal GPI "Narita" was found in erythrocytes, liver, spleen and muscle.[20] Even in erythrocytes, the pattern of the glycolytic intermediates did not clearly substantiate the enzyme deficiency, although glucose 6-phosphate was high and fructose 6-phosphate was within normal range. Payne et al.[21] reported that GPI in the various tissues seemed to be identical. In GPI "Matsumoto", abnormal GPI also surely affected tissues other than erythrocytes, but there were neither clinical symptoms except hemolytic anemia, nor the characteristic pattern of glycolytic intermediates for the enzyme deficiency. The reason that glycolytic intermediates in GPI deficiency did not reveal a characteristic pattern as PGK did, was probably due to the fact that the degree of GPI deficiency was 40% of the normal value and glucose 6-phosphate could metabolize through the pentose phosphate shunt. Abnormal GPI affected tissues other than the erythrocytes but only hemolytic anemia appeared clinically. It is assumed that mature red cells cannot produce enzymes because of the absence of ribosomes and that the abnormal enzyme (GPI "Narita" and GPI "Matsumoto") is unstable in vivo as indicated by thermostability tests in vitro,[20] resulting in critical deficiency of the enzyme for maintaining their function after red cell maturation. However, the cells of muscle, liver and spleen are able to produce enzymes and enzyme deficiency hardly reaches a critical level in those organs.

Miwa and Nishina[22] reported that accumulation of glycolytic intermediates such as phosphoenolpyruvate (PEP), 2-phosphoglycerate (2-PG), 3-phosphoglycerate (3-PG), 2,3-DPG were observed in erythrocytes with PK deficiencies, which apparently suggested a blockade of glycolysis at the step of PK. However, glycolytic inter-

mediates in muscle, liver and spleen of erythrocyte PK deficient patients did not reveal such characteristic patterns as did the erythrocytes. PK has different isozymes according to the organ. Liver has two types of PK isozyme, that is, L-type and M_2-type.[16] The livers of two erythrocyte PK deficient patients revealed lack of L-type PK. It is possible that erythrocyte PK and L-type liver PK are controlled by the same gene, but that M_2-type PK probably compensates the absence of L-type PK in the case which lacks L-type PK. PK of the muscle is composed of M_1-type and PK of the spleen is composed of M_2-type. The accumulation of 3PG, 2PG and 2,3-DPG in the spleen of PK deficient patients was probably caused by contamination of the blood. Thus, the PK deficient patient only suffers dysfunction of the erythrocytes because of the tissue-specific variant.

SUMMARY

1. Glycolytic intermediates of tissues with enzymopathic hemolytic anemia including one case of PGK deficiency, two of GPI deficiency and two of PK deficiency, were studied to evaluate whether the abnormal enzyme affected cells other than erythrocytes and caused tissue dysfunction clinically. The pattern of glycolytic intermediates in the muscle of the erythrocyte PGK deficient patient was similar to that in the erythrocytes. The electrophoretic pattern of PGK of the muscle, liver, spleen and erythrocytes were all very similar. It is assumed that the abnormal enzyme affects all organs of the body and causes dysfunction of these organs.

Glycolytic intermediates of tissues of GPI deficiency did not reveal disturbance of glycolysis, although it has been reported that GPI has no tissue-specific variant. GPI deficient erythrocytes had 40 % normal GPI activity and erythrocytes could metabolize glucose 6-phosphate through the pentose phosphate shunt, resulting in no characteristic pattern of glycolytic intermediates for enzyme deficiency. The reason that only dysfunction of erythrocytes appeared was apparently due to the inability of the mature red cells to produce enzymes and to the instability of abnormal enzymes. It follows that the GPI level in matured erythrocytes is critical for maintaining normal glycolysis. However, the cells of the muscle, spleen and liver are able to produce enzymes, thus no dysfunction of these tissues due to enzyme deficiency appears.

In PK deficiency, the characteristic pattern of glycolytic intermediates which indicates disturbance of glycolysis was not observed in muscle, liver and spleen. Only erythrocyte PK was affected, probably due to the fact that erythrocyte PK was controlled by a gene which differed from that of other organs.

2. Electrophoresis of PGK by thin polyacrylamide gel system was described. In this system, the PGK of the PGK deficient patient (about 5 % of normal PGK

activity) could develop a visible band after electrophoresis.

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