



Pyruvate Kinase Deficiency as a Model of Red Cell Enzymopathies Associated with Hereditary Nonspherocytic Hemolytic Anemia

Shiro Miwa

I. Discovery

The designation of hereditary nonspherocytic hemolytic anemia (HNSHA) was introduced in 1950 by Crosby, followed by a detailed discussion by Dacie *et al.* in 1953. In 1954, Selwyn and Dacie classified HNSHA into two types, type 1 and type 2, based on the distinction that the addition of glucose did not correct the increased rate of autohemolysis in type 2. This suggested a disturbance in the Embden-Meyerhof glycolytic pathway in type 2 patients. DeGruchy *et al.* reported that patients with type 2 HNSHA had increased red cell 2,3-diphosphoglycerate (2,3DPG) levels, suggesting a block of glycolysis distal to the 2,3DPG production step⁽¹⁾. Finally, in 1961, Valentine, Tanaka and Miwa^(2,3) directly assayed various red cell glycolytic enzymes in patients with type 2 HNSHA and discovered pyruvate kinase (PK) deficiency. Several key observations were made in the original reports. Heterozygotes had approximately one-half normal red cell enzyme activity; an autosomal recessive mode of inheritance became clear through studies of a large family; no inhibitors of PK were found; glucose utilization by red cells was approximately normal, but less than that of red cells from individuals with a comparable degree of reticulocytosis; and leukocyte PK activity was normal.

Leukocyte enzyme activity was about 300-fold that of normal red cells, which implies that leukocyte contamination in a red cell suspension could easily mask PK deficiency. Valentine *et al.* were successful in uncovering PK deficiency because, in connection with their earlier work on leukocyte enzymes, they had made a point of meticulously separating red cells and leukocytes in their preparations and even calculated the amount of remaining leukocyte contamination⁽⁴⁾.

II. Clinical Aspects

A. Prevalence and Clinical Features

PK and glucose 6-phosphate dehydrogenase (G6PD) deficiencies are the most prevalent deficiencies in HNSHA. Both

deficiencies have been reported in over several hundred families, while other rare red cell enzymopathies with HNSHA, such as glucose phosphate isomerase, phosphoglycerate kinase, pyrimidine 5'-nucleotidase or triose-phosphate isomerase, were each found in about 15 to 40 families. Approximately 80% of red cell enzymopathies (erythroenzymopathies) with HNSHA seem to be accounted for by the two common enzyme deficiencies⁽⁵⁾. So far, there appears to be no remarkable predominance in either race or geographic distribution.

PK deficiency is an autosomal recessive inherited disorder, and patients are usually either compound heterozygotes or homozygotes. In most instances, heterozygotes have no clinical manifestations, although some clinically affected cases have been reported.

Clinical severity varied considerably from mild and fully compensated to transfusion-dependent severe cases but was usually moderate. Anemia of PK deficiency is in general well tolerated, mainly because PK deficiency causes accumulation of 2,3DPG, which lowers the affinity of hemoglobin for oxygen, reducing the burden on the patient's heart.

PK deficiency shows normocytic and often macrocytic anemia, reflecting accompanied reticulocytosis. Splenectomy characteristically induces a paradoxical increment of reticulocyte counts as high as 40–90%. Specific changes in erythrocyte morphology are not observed. However, particularly in severe cases, echinocytes (dehydrated and spiculated red cells) are often observed. This morphological change becomes more prominent after splenectomy. The osmotic fragility of fresh red cells is usually normal, but the incubated osmotic fragility test may show abnormal value to a variable extent.

B. Diagnosis and Treatment

Diagnosis of PK deficiency depends on red cell enzyme assay^(6,7). Measurement of red cell glycolytic intermediates is helpful though not conclusive. A quantitative decrease of enzymatic activity can be determined by spectrophotomet-

ric assay of red cell lysates, which are prepared by carefully removing leukocytes and platelets. Since leukocytes contain as much as 300-fold red cell PK activity per cell and the distinct PK isozyme, the M_2 -type, is expressed in both leukocytes and platelets, contamination of these cells leads to false negative results at the enzymatic diagnosis.

The PK activities of clinically affected individuals usually range from less than 10% to 25% of the normal mean value; however, sometimes subnormal activity might be shown, or else higher than normal values might be indicated. A number of PK variants with lower affinities for phosphoenolpyruvate (PEP) have been biochemically determined. To avoid false negative results, examination of the enzyme at low substrate concentrations is preferable whenever the high K_m [PEP] variant is suspected. Marginally normal PK activity with concomitant rise of other age-dependent enzymes, such as hexokinase and G6PD, may indicate a family study or measurement of glycolytic intermediates for confirmation of PK deficiency. Heterozygous carriers show 40–60% PK activities of normal controls.

Methods of enzymatic characterization of variant PK were standardized by the International Committee for Standardization in Haematology (ICSH) in 1979⁽⁸⁾.

No curative therapy has been found, and nonspecific supportive therapies such as red cell transfusion or splenectomy have been utilized. Splenectomy is not curative but mostly achieves appreciable clinical improvement of anemia, usually 20–30g/l elevations of hemoglobin level, as well as growth and development for affected infants. In contrast to hereditary spherocytosis, expected response to splenectomy is not definite, suggesting that an indication should be based on severity of hemolysis. Retrovirus-mediated PK gene transfer into murine hematopoietic cells has been performed experimentally⁽⁹⁾ and anticipated as future therapy for PK deficiency.

III. Biochemistry and Molecular Genetics

Four isozymes of mammalian PK, designated as M_1 , M_2 , L and R-type, have been established^(10,11). All isozymes are active as tetramers and consist of identical subunits of about 60 kilodaltons (kDa). M_1 -PK is expressed mainly in muscle and brain; M_2 -PK is the only isozyme that is active in early fetal tissues and also almost ubiquitously expressed in most adult tissues including leukocytes and platelets; L-PK is a major hepatic isozyme and detected as a minor component in kidney, small intestine, and pancreas; and the R-PK is exclusively expressed in red cells.

All PK isozymes except M_2 -type are allosterically regulated, and fructose-1,6 diphosphate (F-1,6-DP) is known to act as an allosteric effector. Structural analysis by X-ray crystallography has been achieved by use of cat muscle M_1 -PK⁽¹²⁾, rabbit muscle PK⁽¹³⁾, *E. coli* type 1⁽¹⁴⁾ and yeast PK⁽¹⁵⁾, the latter two of which are allosterically regulated. A subunit is composed of four distinct domains: N-terminal and A, B and C domains⁽¹²⁾. The active site of PK lies in the pocket between domains A and B⁽¹²⁾, and the allosteric site is entirely located in the C domain⁽¹⁵⁾.

Kinetic, electrophoretic, and immunological properties suggest that both L-type and R-type differ from M_1 -type and M_2 -type and that these two kinds of isozymes are under the control of different genes, L gene⁽¹⁶⁻¹⁸⁾, located at chromosome 1q21⁽¹⁹⁾, and M gene⁽²⁰⁾, located at 15q22.2-22.3, respectively. Both human M and L gene have 12 exons and 11 introns; L gene spans about 9.5 kb and M gene about 32 kb. The primary transcript generated from the M gene is alternatively spliced, generating two mature transcripts, M_1 -type and M_2 -type, which specifically include the exon 9 and 10 sequence, respectively. The L gene has two promoters that direct tissue-specific transcription; the R-type promoter is localized upstream of exon 1 and highly and exclusively active in erythroid lineage of hematopoietic cells⁽²¹⁻²³⁾. In turn the L-type promoter in intron 1 has shown to be transcriptionally active in hepatocytes^(24,25) as well as pancreatic β cells. Recent observations concluded that in the rat system, the 5'-upstream enhancer was required for driving transgenes at a similar transcription level as the endogenous PK gene in erythroid cells of the fetal liver⁽²⁶⁾. The human and rat R-PK promoter as well as the rat erythroid-specific enhancer contain multiple GATA motifs; in addition the transactivation experiment demonstrated an involvement of GATA-1 for activation of rat R-PK promoter, leading to the conclusion that GATA-1, the key regulatory nuclear protein for erythroid-specific genes, is also an essential trans-acting factor for tissue-specific expression of the R-PK. In the enhancer located 3.5-kb upstream of the R-PK promoter, multiple in vivo footprints have been revealed, one of which shared similarity with NF-E4, a stage-specific erythroid transcription factor.

Alteration of isozyme composition from M_2 - to R-PK in the process of erythroid differentiation/maturation have been demonstrated by several strategies. Immunologically the R-PK can be detected at the basophilic erythroblast stage by isozyme-specific antibody⁽²⁷⁾. Both M_2 and R-PK are detectable in isolated CFU-E, and M_2 -PK activity disappears during erythroid differentiation induced by erythropoietin⁽²⁸⁾. Switching of erythrocyte isozyme is partly attributable to changes in rates of degradation as well as synthesis⁽²⁹⁾. Persistent expression of M_2 -PK has been noticed in mature erythrocytes of several PK-deficient subjects⁽³⁰⁾ as well as in animal models^(31,32). One may consider that the M-gene is reactivated or conserved in order to compensate for decreased R-PK activity; however, the existence of M_2 -PK in erythrocytes did not correlate with severity of hemolysis nor any specific gene mutations, and the mechanisms and significance of the phenomenon still remains unknown.

IV. Red Cell Metabolism in PK Deficiency

The glycolytic rate of PK-deficient red cells is reduced, and erythrocyte ATP is usually decreased, but some patients with extremely elevated reticulocyte counts show normal or even high ATP concentrations. High ATP in spite of impaired glycolysis is explained by the fact that PK-deficient reticulocytes almost entirely rely on mitochondrial oxidative phosphorylation. Glycolytic intermediates proximal to the PK

step, such as 2,3DPG and 3-phosphoglycerate (3PG), accumulate in affected erythrocytes, and markedly elevated 2,3DPG levels shift the oxyhemoglobin dissociation curve to the right. Because of this, a severe degree of anemia is tolerable for PK-deficient patients.

V. Molecular Abnormalities and Structure-Function Relationship

So far, over one hundred mutations have been described⁽³³⁾. Although some specific mutations are frequently seen, such as 1529A in Europeans and 1468T in Japanese, PK gene mutations responsible for HNSHA are quite heterogeneous, so that entire coding sequences as well as exon-intron junctions and the 5'-, 3'-flanking region must be examined.

As described earlier, a subunit of PK consists of four domains. The amino acid residues important for comprising the active site as well as the allosteric site have been proposed by X-ray crystallographic studies of cat muscle and yeast PK^(12,15). The amino acids comprising the active site are located in seven clusters, which are encoded in exons 5, 7, 8 and 9 of human R-PK. The majority of missense mutations are found inside or nearby these clusters⁽³³⁾. The single amino acid substitutions may alter hydrophobicity or secondary structure in the vicinity of mutated sites, resulting in disturbed contacts of either substrate or cations for the subunit⁽³⁴⁾. These structural changes are recognized as elevated Michaelis constants for PEP, which is measurable by the standardized procedure recommended by the ICSH⁽⁸⁾. True homozygotes of 941C (Ile314Thr; PK Hong Kong)⁽³⁵⁾, 1151T (Thr384Met; PK Tokyo⁽³⁶⁾, Nagasaki⁽³⁷⁾, and Beirut⁽³⁸⁾ as well as the murine PK gene mutation (1103A, Gly338Asp)⁽³¹⁾ are categorized in this group. The most frequently identified homozygous mutation in Japanese PK deficiency is 1261A, causing Gln421Lys in $\alpha 8$. This amino acid substitution causes a dramatic increase of hydrophobicity and may affect not only the active site but also the allosteric site, since the region is close to the $C\alpha 1$, where the tetramer interface in close proximity to the F-1,6-DP binding site is located. Consequently, the mutant PK shows decrease in allosteric activation by F-1,6-DP as well as impaired substrate affinities. The 1276T (Arg426Tyr, PK Naniwa)⁽³⁹⁾ and 1277A (Arg426Gln; PK Sapporo)⁽⁴⁰⁾ are considered to be classified in this group. The 3'-half of the exon 10 region encodes the amino acids essential for the allosteric regulation of PK. Two human R-PK mutations have been implicated as allosteric mutants: 1403T (Ala468Val; PK Hadano)⁽³⁵⁾ and 1436A (Arg479Pro; PK Amish⁽⁴¹⁾, PK Shinshu⁽³⁵⁾).

Approximately 70% of all the PK gene mutations so far elucidated are missense mutations. In addition to these, point mutations, deletions or insertions that induce more drastic structural changes have been reported. They include the splice site, frameshift, and nonsense mutations, and some of them abolish catalytic activity as expected. Curiously, several homozygous PK null mutations have been discovered. For example PK Beppu is a variant with one base deletion at 434C⁽³⁹⁾ causing frameshift and premature ter-

mination of translation. The mutant subunit encodes the 144 N-terminal amino acids of R-PK plus an aberrant 33 amino acids. Another example is PK Gypsy.⁽⁴²⁾ Uneven crossing over between the homologous sequence of introns 10 and 11 is thought to be a cause of deletions flanking exon 11 of this mutant, resulting in a 1149-bp deletion that generates the skipping of a 182bp exon 11 sequence of the mutant R-PK mRNA, with premature termination of its translation at 513 amino acids and an inclusion of aberrant 35 amino acid residues at the C-terminal. Interestingly, a canine PK variant, the Basenji, has been found to have one base deletion of R-PK at the corresponding site⁽³²⁾. Although the "null" R-PK expression of each variant has not been intensively established, survival of these PK variants raises the question of whether the PK L-gene is indispensable. Historically, the continuation of M_2 -PK expression in mature erythrocytes of some PK variants led us to suspect the quantitative anomaly of R-PK expression, and these variants were cited as "classical" PK deficiency⁽³⁰⁾. PK Beppu is one of the classical PK variants, and the M_2 -PK can be exclusively detected in zymograms of mature erythrocytes. These observations allow us to speculate that the extinction of the M gene along with erythroid maturation may be inhibited, and that this ameliorates the PK deficiency of the patient.

VI. Murine PK Deficiency

Murine PK deficiency was discovered in an inbred colony of the CBA strain by our group^(31,43,44). A homozygous missense mutation G1013A, which causes Gly338Asp single amino acid substitution, has been identified. 337Arg was reported to be important for substrate binding. A similar homozygous missense mutation at the catalytic domain has been identified in a human PK variant, PK Hong Kong, T941 C, Ile314Thr⁽³⁵⁾. Although both 1013A and 941C give rise to an amino acid change adjacent to the active site and may interfere with substrate binding to the subunit, the degree of anemia was much more severe in the human case. The erythroid-progenitor cell number was increased in the spleen of the PK-deficient mice to a level approximately 66 times higher than that of normal CBA mice, suggesting that compensatory extramedullary erythropoiesis in the spleen of the mutant mice, but not in the human variant, might account for the observed difference in the phenotype. The mice grew well and have normal reproductive ability, giving birth to 100% homozygous PK-deficient mice. Hence, this mutant mouse should be a useful animal model for studying the pathophysiology of PK deficiency and for developing new therapeutic methods to correct PK deficiency.

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