Red Cell Enzymes

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Mutations leading to red cell enzyme deficiencies can be associated with diverse phenotypes that range from hemolytic anemia, methemoglobinemia, polycythemia, and neurological and developmental abnormalities. While most of these mutations occur sporadically, some such as common glucose-6-phosphate dehydrogenase (G6PD) mutants are endemic and rarely cause disease. Common G6PD mutants likely reached their prevalence because they provide some protection against severe malarial complications. In this review G6PD, pyruvate kinase, 5′ nucleotidase, and cytochrome b5 reductase deficiencies will be discussed in greater detail. Limitations of commonly used screening tests for detection of these disorders will also be emphasized, as well as emerging knowledge about non-enzymatic function of the glycolytic enzymes.

Due to the loss of the nucleus, mitochondria, and ribosomes, mature erythrocytes are unable to carry out oxidative phosphorylation and protein synthesis. However, they still must sustain an active metabolism to maintain the flexibility and integrity of the red cell membrane as well as to preserve hemoglobin in its functional form to ensure adequate oxygen delivery. Red cell enzymes allow erythrocytes to meet these tasks by supporting two important metabolic pathways: glycolysis, and the pentose shunt. Other erythrocyte enzymes, e.g., pyrimidine 5′ nucleotidase, participate in nucleotide degradation and salvage and are essential for the removal of nucleotide precursors that may be toxic to erythrocytes.

Abnormalities of red cell enzymes result in diverse phenotypes, including hemolytic anemia, methemoglobinemia, polycythemia, and non-erythroid effects such as neurological and developmental abnormalities. The growing recognition that many glycolytic enzymes have non-enzymatic functions, including transcriptional regulation, stimulation of cell motility, and control of apoptosis, may explain some of the non-erythroid effects of mutations of the glycolytic enzyme genes.

On the other hand, some endemic enzyme abnormalities, such as the common glucose-6-phosphate dehydrogenase (G6PD) mutants, rarely cause disease. Deficiencies of red cell enzymes may also provide a protective advantage to external challenges. The common G6PD mutants likely reached their prevalence because they provide some protection against severe malarial complications.

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Glucose-6-Phosphate Dehydrogenase Deficiency

Biology

G6PD deficiency is one of the most prevalent disease-causing mutations worldwide (reviewed in [1,2]). However, most of the G6PD isoenzymes with decreased activity are associated with only moderate health risks without a significant effect on longevity. G6PD is a housekeeping enzyme essential for basic cellular functions, including protecting red cell proteins from oxidative damage. Oxidant damage of hemoglobin leads to the precipitation of hemoglobin, which may be morphologically recognized as Heinz bodies. The enzymatic activity of G6PD generates NADPH that is utilized for glutathione reduction. Reduced glutathione restores hemoglobin to the soluble form. Thus, maintaining a high ratio of reduced-to-oxidized glutathione represents the major defense against oxidative damage of hemoglobin. Reticulocytes have five times higher G6PD enzyme activity than the oldest erythrocyte subpopulation.

G6PD is encoded on the X chromosome and thus is subject to dosage compensation by X chromosome inactivation, a phenomenon that was discovered by studies of carrier females for G6PD deficiency. Further investigations of this phenomenon have played a pivotal role in our understanding of the hierarchy of hematopoiesis, clonality of malignant neoplasms, and the mechanism of X chromosome inactivation.

Clinical phenotypes of G6PD deficiency

More than 300 G6PD variants have been defined; most are sporadic but some occur at a high frequency. G6PD variants can be divided into three categories based on the type of hemolysis they cause. Most common are those variants associated with acute intermittent hemolytic anemia; some of these variants are endemic. In contrast, the variants associated with chronic hemolytic anemia are very rare and the severity of hemolysis is highly variable, ranging from mild to transfusion dependent. The third type of variant is associated with no obvious risk of hemolysis.

In individuals with a G6PD variant, the generation of NADPH and reduction of glutathione is variably impaired depending on the type of the G6PD mutation. Mutations associated with chronic hemolysis tend to cluster in the vicinity of the NADP-binding domain of the *G6PD* gene, while those associated with acute intermittent hemolysis or no hemolysis are scattered throughout the gene.

G6PD B is the wild-type allele. G6PD A+, a high frequency African-specific polymorphic allele, has almost normal enzyme activity and is not associated with hemolysis. Of the G6PD deficient variants that occur at a high frequency, the best known are African G6PD A– and the Mediterranean variants (referred to as G6PD Mediterranean); several variants are also present in Southeast Asia. All of these variants cause acute intermittent hemolysis. G6PD A+ has a gene frequency of 11%, compared to that of 20% for G6PD A– among African Americans. The *G6PD A–* mutation (202G→A) arose on a *G6PD A+* chromosome (376A→G).

Acute hemolysis

In a subject with an acute intermittent hemolytic G6PD deficient variant, there is no clinical or laboratory evidence of hemolysis unless the individual is exposed to oxidants (drugs), infections or fava beans. Predictably, exposure to the oxidants that cause hemolysis in the acute hemolytic G6PD variants further exacerbates hemolysis in patients with chronic hemolytic G6PD variants. The most common offending agents are drugs (**Table 1**)**,** infections, or fava bean ingestion. In spite of continuation of the drug or persistence of infection, hemolysis is short lasting, presumably due to the elimination of a subpopulation of red cells with very low G6PD activity. The younger red cells and reticulocytes, which have higher G6PD activity, are typically not hemolyzed in mild variants such as G6PD A–.

During the hemolytic episodes, either normal red cell morphology or nonspecific abnormalities (anisocytosis, polychromasia) may be observed. The morphological sequelae of the oxidative denaturation of hemoglobin, i.e., Heinz bodies, may be seen either directly on microscopic evaluation of the blood film or after the red cells are preincubated with oxidants such as phenylhydrazine. "Bite cells" are often cited as pathognomonic for G6PD deficiency; however, this has not been substantiated by any rigorous study known to these authors and the authors of this chapter have never seen "bite cells" during either acute hemolytic crisis or in the rare G6PD variants that are associated with chronic hemolytic states. In contrast we have seen rare "bite cells" in other hemolytic anemias of known and unknown cause wherein we carefully excluded G6PD deficiency. Thus, unless specific association data are provided we consider these cells as non-specific morphological abnormality that is falsely misinterpreted as diagnostic of G6PD deficiency

Table 1. Substances to be avoided in G6PD deficiency.

Acetanilid
Isobutyl nitrite
Methylene blue
Naphthalene
Nitrofurantoin (Furadantin)
Phenazopyridine (Pyridium)
Phenylhydrazine
Primaquine
Sulfacetamide
Sulfamethoxazole (Gantanol)
Sulfanilamide
Sulfapyridine

Evolutionary benefit of G6PD deficiency

The geographic distribution of populations with a high gene frequency of deficient variants overlaps closely with the prevalence of malaria, suggesting that G6PD deficiency might be protective against malaria. However, the mechanism of protection is unknown. Early studies of females heterozygous for G6PD deficiency showed higher levels of malaria parasites in normal compared to G6PD-deficient red cells. Although malaria invasion of the cells was similar, the growth of the parasites in the G6PD-deficient cells was inhibited.[3,4] Different results were found, however, in studies of the Mediterranean variant, where no differences in malaria invasion and growth of G6PD-deficient and non-deficient cells were found.[5] Similarly, conflicting results have been reported about whether hemizygous G6PD-deficient males have protection against malaria.[3,6,-7]

The most likely mechanism of malarial protection may be increased phagocytosis of G6PD-deficient erythrocytes containing the early ring-stage parasites. In the ring-stage parasite-infected cells, the level of reduced glutathione was lower in the G6PD-deficient cells compared with normal red cells, leading to membrane damage of deficient cells containing parasites that may be preferentially targeted for destruction.[3]

Diagnosis

The enzymatic activity of G6PD can be assessed by a rapid fluorescent screening test or by quantitative spectrophotometric analysis (the most useful description of the assays and details of techniques may be found in [8]). However, false-negative results are possible in milder forms of G6PD deficiency, especially if enzymatic analysis is performed shortly after resolution of acute hemolytic episodes when young erythrocytes, which have much higher enzymatic activity, predominate and if a screening test rather than a quantitative spectrophotometric analysis of the enzyme activity is used. Females heterozygous for G6PD deficiency are particularly difficult to diagnose by enzymatic assays, but now that the nucleotide substitutions of many G6PD-deficient isoenzymes have been established, molecular di-

agnostic methods can be used for the diagnosis of females who are heterozygous for common variants.

Therapy

Drugs that are known to precipitate hemolysis in G6PD-deficient subjects should be avoided. In subjects with G6PD A– deficiency, hemolysis is typically short-lasting in spite of continuous use of the offending agent. This is not always the case in the more severe Mediterranean variant of G6PD deficiency, and the precipitating agent should always be withdrawn. When anemia is severe and symptomatic, blood transfusion may be necessary. Folate supplementation should be provided in those patients with chronic hemolysis.

Pyruvate Kinase Deficiency

PK isoenzymes and molecular biology

The molecular biology of pyruvate kinase (PK) is complex. Four different PK isoenzymes are generated by the use of alternative promoters of 2 distinct genes (*PK LR* and *PK M*) that have variable expression in different tissues. The R isoform is unique to erythrocytes and gradually replaces the M_2 isoform found in early erythroid and myeloid progenitors. PK converts phosphoenol pyruvate to lactate, generating ATP. The activity of PK is increased by an interaction with fructose diphosphate (FDP) that changes the conformational structure of PK.

Clinical presentation and therapy

PK deficiency is the most common erythrocyte enzyme defect causing chronic congenital hemolytic anemia. The severity of hemolysis in PK-deficient patients is highly variable, ranging from life-threatening transfusion-requiring hemolytic anemia present at birth to a mild fully compensated hemolytic process without anemia. Affected individuals are either homozygous for the same mutation or compound heterozygotes for two different PK defects. PK deficiency is distributed worldwide, but it has been reported that the gene is more common among people of northern European extraction and perhaps Chinese and certain other ethnic and racial groups. A high frequency of this disorder has been well-documented among Pennsylvania Amish.

Patients with severe hemolysis may be chronically jaundiced and may develop the clinical complications of chronic hemolytic states, including gallstones, transient aplastic anemia crises (often due to parvovirus infection), folate deficiency, and, infrequently, skin ulcers. Splenomegaly is frequently seen but not invariable. The beneficial effect of splenectomy on hemolysis is well documented; typically, the degree of hemolysis and anemia is ameliorated and in severe cases the transfusion requirement is generally abolished. Unless the patient is transfusion dependent, it is advisable to delay splenectomy until after the age of three years when the risk of pneumococcal, *Hemophilus influenzae,* and meningococcal infections declines.

Mechanism of hemolysis and physiologic implications

The mechanism of hemolysis is not clear. Although it has been postulated that the defect in ATP generation contributes to the hemolytic process, this explanation is insufficient because ATP deficiency is difficult to demonstrate in many patients, and other disorders with more severe ATP deficiency are not associated with significant hemolysis. More than 100 mutations of *PK* have been reported. These mutations are scattered throughout the coding regions of the genes,[9] and there appears to be no correlation with the location of the mutation and the severity of the hemolytic anemia.[10]

Patients with hemolytic anemia who undergo splenectomy, with a resultant decrease in the hemolysis and improvement of anemia, have a higher number of reticulocytes than they did before the splenectomy. This perplexing observation indicates that our knowledge of the regulation of erythropoiesis and reticulocyte kinetics remains incomplete; however, it has been suggested that reticulocytes, having some mitochondria, have the capacity to make mitochondrial ATP, but as soon as this capacity is lost they are destroyed. The metabolic disturbances in PK deficiency affect both the survival of red cells and the maturation of erythroid progenitors (so far studied only in splenic progenitors). This results in their premature cell death, i.e., apoptosis, as demonstrated in a splenectomized PK deficient patient.[11] The same group made a similar observation in a PK deficient mouse. [12] It remains to be established whether the apoptosis of erythroid progenitors in PK deficiency extends to marrow erythroblasts, if this observation accounts for the previously unexplained post-splenectomy reticulocytosis, and if PK activity has any role in the apoptotic pathway in general.[13]

Malaria protection

PK deficiency is associated with protection against malaria in mice.[14] Evidence for a similar effect in humans remains to be proven; however, there is no indication of a positive selection pressure in the geographic distribution of PK deficiency.

Diagnosis

There are no specific clinical findings or morphological abnormalities in PK deficiency and no routinely available laboratory measurements aid in diagnosis. A screening test utilizing crude hemolysate with one concentration substrate has been employed for detection of pyruvate deficiency, but it may miss those exceedingly rare PK variants characterized by increased K_M and those more common PK variants with abnormal FDP interaction. Specialized laboratories can perform quantitative PK enzyme analysis with various concentrations of substrate with and without FDP. Due to the large number of the mutations and their low prevalence, it is difficult to replace these tests with molecular diagnostic methods.

Pyrimidine 5′ Nucleotidase (cN-III) Deficiency

Red cell pyrimidine 5′ nucleotidase is a member of a family containing at least 7 genes encoding 5′ nucleotidases.[15] Its proper designation is cytosolic 5:Nucleosidase II (cN-III). Other members of this family are responsible for activation of antiviral compounds (cN-II), and ecto-5′ nucleosidase (eN), also known as CD73, which plays a role in T-cell activation and cell adhesion. Because of the familiarity of the term pyrimidine 5′ nucleotidase to hematologists, cN-III will be referred to here as pyrimidine 5′ nucleotidase.

Inherited deficiency of pyrimidine 5′ nucleotidase is the third most common enzymatic deficiency resulting in hemolysis. Pyrimidine 5′ nucleotidase participates in RNA degradation in reticulocytes. The accumulation of pyrimidines in the red cells is presumed to be toxic and a cause of hemolysis. However, recent evidence suggests that deficiency of pyrimidine 5′ nucleotidase is at least in part compensated in vivo by other nucleosidases or perhaps other nucleotide metabolic pathways.[16] Pyrimidine 5′ nucleotidase deficiency is inherited in an autosomal recessive fashion and is the only congenital hemolytic anemia due to a red cell enzyme deficiency that has a specific, consistent morphological abnormality—basophilic stippling. Lead is a powerful inhibitor of pyrimidine 5′ nucleotidase and determination of lead levels should be included whenever the constellation of hemolytic anemia, pyrimidine 5′ nucleotidase deficiency, and basophilic stippling is found. Lead-induced acquired pyrimidine 5′ nucleotidase deficiency is treatable, unlike the congenital deficiency for which no therapy is available. Several clinical reports of the co-inheritance of hemoglobin E and pyrimidine 5′ nucleotidase deficiency leading to severe hemolytic anemia suggest that this enzyme is particularly susceptible to oxidative damage resulting from the instability of hemoglobin E.[17]

Cytochrome b5 Reductase

Methemoglobin

Methemoglobin is the derivative of hemoglobin in which the iron of the heme group is oxidized from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state. The ferric hemes of methemoglobin are unable to bind oxygen and, in addition, the oxygen affinity of the accompanying ferrous hemes in the hemoglobin tetramer is increased. As a result, the oxygen dissociation curve is left shifted and oxygen delivery is impaired. Methemoglobin is generated physiologically as a consequence of deoxygenation, but endogenous enzymatic hemoglobin reduction mechanisms reduce the methemoglobin to maintain a very low steady-state blood methemoglobin level (1% or less of total hemoglobin). Increased levels of methemoglobin above this steady state, termed *methemoglobinemia*, result from either enhanced methemoglobin production or decreased methemoglobin reduction. The primary reaction that reduces methemoglobin back to hemoglobin is catalyzed by the reduced form of nicotinamide-adenine dinucleotide (NADH)-cytochrome b5 reductase (b5R). Electrons are transferred from NADH (generated by glyceraldehyde 3-phosphate in the glycolytic pathway) to an enzyme, NADH cytochrome b5 reductase, and then to cytochrome b5. In hemoglobin-containing red blood cells, cytochrome b5 transfers electrons directly to methemoglobin to reduce it to hemoglobin. In nucleated cells and reticulocytes, cytochrome b5 transfers electrons to stearyl-CoA desaturase.

Cytochrome b5R deficiency

The most common cause of congenital methemoglobinemia is due to deficiency of the b5R enzyme. These individuals have a decreased ability to reduce the methemoglobin that is formed continuously at physiologic rates. b5R is a constitutively expressed enzyme, a product of a single gene that produces multiple transcripts. There are two types of deficiency. Because the b5R enzyme is encoded by a single gene, the suggested explanation for the two types of b5R deficiency is that in type I the abnormal gene product is produced at a normal rate but is unstable; as a result, only mature red cells, which cannot synthesize proteins, are affected. By contrast, when mutations cause underproduction of the enzyme or an enzyme with decreased enzymatic activity, the b5R deficiency is generalized to all cell types (type II).

Inherited in an autosomal recessive pattern, type I b5R deficiency is found worldwide, but it is endemic in some populations such as the Athabascan Indians, Navajo Indians, and Yakutsk natives of Siberia. In other ethnic and racial groups, the defect occurs sporadically. Homozygotes or compound heterozygotes have methemoglobin concentrations of 10% to 35% and appear cyanotic but are usually asymptomatic even with levels up to 40%. Life expectancy is not shortened, and pregnancies occur normally. Significant compensatory elevation of hemoglobin concentration (polycythemia) is sometimes observed. The b5R activity of the erythrocytes of heterozygotes is approximately 50% of normal. Although this activity level is sufficient to maintain normal methemoglobin levels during normal conditions, oxidant stress can overwhelm the erythrocyte's capacity to reduce methemoglobin and produce acute symptomatic methemoglobinemia.

Ten percent to 15% of cases of enzymopenic congenital methemoglobinemia are type II, which is caused by a general deficiency of b5R in all cell types. Type II b5R deficiency is found sporadically worldwide. The main symptoms are cyanosis, mental retardation, and severe developmental delay. Life expectancy is significantly shortened due to the neurological complications, the pathophysiological basis of which remains unexplained. The cyanosis can be effectively treated with methylene blue or ascorbic acid, as in type I b5R deficiency; however, treatment is not indicated except for cosmetic reasons because it has no effect on the neurologic abnormalities. Amniotic cells con-

tain easily measurable b5R activity; thus, prenatal diagnosis of homozygous b5R deficiency is feasible.

In summary, congenital methemoglobinemias, with the exception of type II congenital methemoglobinemias, are asymptomatic and only of cosmetic concern to some. In contrast, comparable level of methemoglobin in patients with acquired methemoglobinemia is symptomatic due to severe acute tissue hypoxia and may be life-threatening.

Non-Enzymatic Functions of Red Cell Enzymes

There is a growing recognition that glycolytic enzymes have diverse, non-enzymatic functions including mobilization of cell movement, control of apoptosis, and interaction and modulation of regulation of oncogenes (comprehensively reviewed elsewhere).[18] Some of these discoveries have stemmed from the seminal work of Otto Warburg on respiration and energy metabolism of cancer cells[19,20] and their different regulation by hypoxia, which is now partially explained by their constitutive up-regulation of the master regulator of hypoxic responses; i.e., hypoxia inducible factor (HIF).[21]

One of the newly discovered functions of glycolytic enzymes is their role in tumor metastasis and cell motility. Glucose-6-phosphate isomerase acts as an autocrine motility factor (AMF) in cell migration. This activity has been studied in melanoma and it appears to be important in cancer metastasis.[18,22] Other glycolytic enzymes have a role in the regulation of apoptosis. Mitochondrial hexokinase appears to inhibit apoptosis via interaction with the pro-apoptotic protein BAD,[18,23] while glyceraldehyde phosphate dehydrogenase (GAPD) appears to have a pro-apoptotic function.[18,24] In addition, some glycolytic enzymes, including hexokinase, GAPD, enolase, and lactic dehydrogenase, have important roles in the regulation of the transcription of an array of genes by multiple mechanisms that are beyond scope of this review (reviewed in [18]).

It remains to be determined whether the non-erythroid clinical phenotypes of mutations of some red cell enzyme genes can be explained by their newly discovered non-glycolytic roles. However, the recent discovery of apoptotic erythroid progenitors in the spleen of a pyruvate kinase-deficient individual[12] suggests that other gene mutations previously considered to be hemolysis-specific may have other functions that contribute to the disease phenotype.

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