G6PD - An Old Bottle with New Wine

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The major role of glucose-6-phosphate dehydrogenase (G6PD) is to generate reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is indispensable to reductive metabolism and maintenance of cellular redox homeostasis. Most advances in this field have been made in the pathophysiology of G6PD-deficient erythrocytes and the molecular characterization of different G6PD variants. Recently, numerous studies have shown the importance of G6PD in cell growth, development and disease progression. *(Chang Gung Med J 2005;28:606-12)*

Glucose-6-phosphate dehydrogenase (G6PD), the key regulatory enzyme in the hexose monophosphate shunt, catalyzes the oxidation of glucose-6-phosphate (G6P) to 6-phosphogluconolactone and the production of reducing equivalents in the form of NADPH to meet cellular needs for reductive biosynthesis and maintenance of the cellular redox status.[1] Clinically, deficiency of this enzyme affects as many as 400 million individuals worldwide.[1] This deficiency predisposes affected individuals to neonatal jaundice, drug or infection-mediated hemolytic crisis, favism, and less commonly, to chronic non-spherocytic hemolytic anemia. Over 400 biochemical variants have been reported.[2]

G6PD deficiency has been determined to be an X-linked genetic disorder.[3] The human G6PD gene, which contains 13 exons and is about 21 kb long, has been mapped to the Xq28 region.[4,5] To date, about 140 mutations or combinations of these mutations have been found.[6] Nearly all G6PD mutations lie in the coding sequence of the gene. The majority are point mutations causing amino acid substitutions. The diversity of point mutations and possible interactions with other genes account for the phenotypic heterogeneity of G6PD deficiency.

G6PD deficiency was discovered half a century ago and is still the most common inherited enzyme defect. Many excellent reviews on G6PD deficiency have been published. We will focus on certain issues of G6PD deficiency that are of special interest in Taiwan and nearby regions. Neonatal screening and health education have reduced the incidence of induced hemolysis. However, the possible involvement of G6PD deficiency in the pathogenesis of other diseases remains elusive. In light of recent developments in G6PD research, we will discuss some novel findings that have implications in the pathologic roles of G6PD mutation other than hemolysis.

G6PD deficiency in Taiwan and Southern China

It has been the known for a long time that favism occurs in Chinese population.[7,8] Kernicterus due to erythrocytic enzyme deficiency has been reported in Chinese neonates.[9] Reports show that favism, kernicterus, and neonatal jaundice are consequences of G6PD deficiency.[9,10] In 1961, an outbreak of hemolytic anemia which claimed several lives in Hsin-Chu, Taiwan, was linked to ingestion of fava

beans.[11]

G6PD deficiency is a common genetic disorder in southeast China and Taiwan. The incidence is as high as 5.5% in Guangdong;[10] it is even higher in certain Chinese national minority groups, such as the Li (6.74%) and the Miao (16.67%).[8]

Taiwan has conducted widespread neonatal screening for G6PD deficiency since 1985. Based on data from more than 400000 neonates, the incidence of this deficiency in Taiwan is estimated to be about 3% in male infants and 0.9% in female infants.[12] The incidence in Taiwanese adults is similar to that of infants.[13]

Genetics and Classification of G6PD Variants

1. Biochemical Variants

Over 400 biochemical variants have been characterized so far. Nearly three quarters of these were identified by methods recommended by the World Health Organization Scientific Group. Among the selection criteria are G6PD activity; electrophoretic mobility; Michaelis constant (Km) values of glucose-6-phosphate and NADP; utilization rates of substrate analogs deoxyglucose-6-phosphate and deamino-NADP; pH optimum; and heat stability.[2] Advances in polymerase chain reaction technology have expedited progress in molecular characterization of these biochemical variants. Many variants that were considered distinct on the basis of their biochemical properties have been found to be identical at the molecular level. For instance, G6PD Canton, G6PD Taiwan-Hakka, G6PD Gifu-like, and G6PD Agrigento-like are caused by a 1376 G→T mutation at the DNA level.[6] Ambiguity has arisen because some variants have been given different names in different parts of the world. In particular, G6PD deficiency associated with chronic non-spherocytic anemia is sporadic. Although the corresponding variants were formerly considered unique, some of these cases have been found to be caused by the same mutations.

2. World Health Organization (WHO) Classification

All biochemical G6PD variants are categorized into five classes according to the level of enzyme activity in erythrocytes and clinical manifestations in affected individuals. Class I includes severely deficient variants associated with chronic non-spherocytic hemolytic anemia. Class II variants have enzyme activity of less than 10%. Class III is characterized by moderate to mild deficiency in enzyme activity. Class IV variants have nearly normal activity. Class V includes variants with increased activity.

Most biochemical variants in the Chinese population, such as G6PD Taipei, G6PD Chinese-1, G6PD Fushan, and G6PD Taiwan-Hakka, belong to Class II.[14] The frequent occurrence of Class II variants may account for the high incidence of favism, drug- and infection-induced hemolytic anemia in this ethnic group.

3. The G6PD Gene and Genetic Variants

In 1986, the *G6PD* gene was cloned independently by Persico *et al.* and by Takizawa *et al.*[4,5] The structural organization of gene was compiled from the sequences available and is now known. The gene consists of 13 exons, and spans nearly 21 kb. The first exon has no coding sequence, and the intron intervening exon 2 and 3 is over 9 kb. A GC-rich promoter typical of housekeeping genes is situated at the 5' end. Selective demethylation of the GC-rich islands is associated with gene expression on the active X chromosome.[15] The G6PD gene is tightly packed with other genes within the Xq28 region and is closely apposed to the gene encoding NF-kappa-B essential modulator (NFMO) in head-to-head orientation.

Almost all G6PD mutations affect the coding region. Only one splicing mutation, G6PD Varnsdorf, has been identified and no mutation has been found in the promoter region. The majority of mutations affecting the coding sequence are single base missense mutations. A number of variants with small, in-frame deletions can also be found. G6PD Sunderland represents deletion of nucleotide 105-107 (of cDNA), whereas G6PD Stonybrook is caused by deletion of nucleotide 724-729 (of cDNA).[14] So far, no large deletion or frameshift mutation has been documented, suggesting that complete lack of G6PD expression is not tolerated during mammalian development. The apparent exception is G6PD Georgia caused by nonsense mutation at tyrosine 428.[16] It is not clear whether the truncated protein is partially active or not. The fact that this mutation was only detected in a heterozygous individual implies its lethality in the hemizygous state and the essentiality of G6PD for survival.

Mutations are distributed non-randomly

throughout the coding region of the gene. A few of mutations associated with class I variants are mapped to the first 500 bp (of cDNA); none of those that cause class II and III deficiencies lie in the last 500 bp. Interestingly, there is a cluster of class I mutations in exon 10. Recent crystallographic study suggests that these mutations lie close to the dimer interface and interfere with G6PD dimerization, which is essential to enzyme activity.[17]

4. Genetic Variants and Clinical Severity

The clinical presentation of G6PD deficiency is rather heterogeneous, ranging from no symptoms to chronic non-spherocytic hemolytic anemia. Whether diverse point mutations in the G6PD gene can lead to phenotypic and clinical heterogeneity is speculative. There seems to be no correlation between G6PD activity and clinical severity. It is probable that additional factors come into the play. In drug-induced favism, inherited differences in drug metabolism play a significant role in response to particular hemolytic drugs. If an individual can efficiently catabolize these drugs, hemolysis will not be apparent. Conversely, these drugs may cause hemolysis in people with inefficient drug metabolizing activity. In neonatal jaundice and kernicterus, UDP-gluconosyl-transferase activity may affect the clinical outcome. An interaction between G6PD deficiency and Gilbert Syndrome resulting in hyperbilirubinemia has been reported.[18] Additional factors such as nutritional status and environment may also play a role in the pathophysiology of G6PD deficiency. How different factors interact to influence the clinical manisfestations of G6PD deficiency remains to be elucidated.

Roles of G6PD in Cells Other Than RBCs

Most studies of G6PD have focused on the molecular characterization of different G6PD variants, the pathophysiology of G6PD-deficient erythrocytes, and the hemolytic aspect of G6PD deficiency. The roles of G6PD in nucleated cells have been largely overlooked. Recently, a number of studies have shone light on how G6PD status affects life.

1. G6PD as regulator of life and death?

A number of studies suggest that G6PD is essential to cell growth. Using a putative inhibitor of G6PD dihydroepiandrosterone (DHEA), Tian *et al*. showed that suppression of G6PD activity led to

diminished proliferation of several cell lines.[19] It is controversial if DHEA inhibits G6PD activity in cultured cells. Previous studies had demonstrated that DHEA could inhibit the activity of a purified G6PD preparation.[20] However, it appears that DHEA and analogs do not exert a long-term inhibitory effect on G6PD activity in cultured cells: G6PD activity dropped transiently after DHEA treatment and returned to normal several hours later. Similar kinetics of G6PD inhibition was observed in erythrocytes. It is plausible that the effects of DHEA are not caused by inhibition of G6PD activity.

With this question in mind, we proceeded to study the growth-regulatory role of G6PD using foreskin fibroblasts derived from a neonate carrying the G6PD Taiwan-Hakka variant.[21] Compared with normal foreskin fibroblasts, these G6PD-deficient cells showed retarded cell growth and reduced replicative potential upon serial cultivation. The slowdown in growth preceded an early entry of these cells into a non-dividing state reminiscent of cellular senescence. These cells exhibited signs of aging as indicated by large, flattened morphology and senescence-associated ß-galactosidase staining. The levels of the cell cycle inhibitors p16 (INK400) and p21 (CIP1) and the tumour suppressor p53 increased during the process. Meanwhile, an opposite trend was observed in the level of the molecular chaperones HSP27 and HSP70. These molecular changes are characteristic of senescent cells. The crucial role of G6PD activity in cell growth was further demonstrated by the ability of exogenous G6PD to rescue these deficient cells from growth retardation and early onset of senescence. As to the mechanism involved, we demonstrated that increased oxidative stress rather than accelerated telomere shortening is responsible for the early onset of senescence. Our findings highlight the importance of G6PD in cellular proliferation and senescence.

The involvement of reactive oxygen species in cellular senescence is not unprecedented. Reactive oxygen species (ROS) produced during metabolism cause cumulative damage, resulting in senescence. Owing to the imperfect nature of respiration, roughly 1-2% of electron flow contributes to chemical reduction of O_2 to $O_2^{\bullet-}$, which is sequentially converted to other ROS, such as hydrogen peroxide and hydroxyl radical. These ROS are known to damage proteins, lipids, mitochondrial DNA and genomic DNA in a

relatively indiscriminate manner. As this damage accumulates, the ability of cells to grow is ultimately impaired, provoking senescence. This view is supported by studies with *Drosophila* strains overexpressing Cu/Zn superoxide dismutase and/or catalase, where significantly extended mean and maximum lifespans were observed,[22] and studies in which exogenous SOD1 expression in the motorneurons of *Drosophila* increased the organism's normal lifespan by up to 40%.[23] As G6PD is indispensable to maintenance of the redox balance and detoxification of ROS, it is likely that G6PD deficiency cripples the antioxidant defense, resulting in the buildup of oxidative damage and thus cellular senescence. Consistent with this notion G6PD-deficient cells had lower intracellular G6PD activity and NADPH/NADP$^+$ ratio but higher level of 8-hydroxydeoxyguanosine (8-OHdG) compared with normal counterparts.[24] The redox status is increasingly tilted towards the oxidizing end during their serial passage. This correlates well with their tendency to undergo senescence. Moreover, G6PD-deficient cells display increased propensity for H_2O_2-induced senescence. Our findings suggest the involvement of ROS in G6PD deficiency-induced cellular senescence.

Apart from its role in growth and senescence, G6PD may play an important role in death signaling. Human fibroblasts deficient in G6PD activity showed an altered biological response to nitric oxide (NO).[25] Deficient cells underwent apoptosis after treatment with NO donor. This is in contrast to normal cells in which proliferation was enhanced by the same treatment. Very likely, the cellular G6PD status modifies the signaling pathway in such a way that switches the outcome of cells from life to death.

2. G6PD and Viral Infection

Our G6PD-deficient cells provide a good model for studying how an altered redox balance affects cellular physiology. One area that has aroused much interest is the interaction between oxidative stress and viruses. It is well-documented that the redox environment affects the outcome of viral infection. For example, replication of Coxsackievirus, rhinovirus and influenza virus is modulated by redox milieu;[26,27] Coxsackieviruses replicated to a higher titer in C3H/JHe mice fed with diets deficient in selenium (Se), vitamin E or both than in mice given a normal diet;[27] glutathione administration has an

antiviral effect on influenza virus.[26] All these findings suggest that redox imbalance is conducive to viral replication and virulence. Our preliminary findings have shown that the cellular G6PD status determines the outcome of enteroviral and coronaviral infection. It appears that G6PD deficiency enhances both the cytopathic effect and the number of progeny viruses produced.

3. G6PD in Development

As mentioned earlier, G6PD deficiency seems to be incompatible with mammalian development. This issue has been studied by creation of G6PD knockout mice. The earliest attempt by Pandolfi *et al.* resulted in production of mouse embryonic stem (ES) cells with an inactivated G6PD gene.[28] These cells were viable, but they showed a very high sensitivity to oxidative stress, suggesting that G6PD is dispensable for pentose synthesis but essential for antioxidant defense. In a similar fashion, Filosa et al. used a cre-lox approach to generate a G6PD-nullizygous ES line, which was unable to cope with exogenous oxidants.[29] Their study is particularly interesting in the following aspect: expression of the bacterial form of G6PD could substitute for the endogenous enzyme in protection against oxidative stress. Under conditions that encouraged in vitro differentiation, deficient ES cells were able to give rise to mesodermal cells, cardiomyocytes, hepatocytes, and primitive erythroid cells in embryoid bodies. However, definitive erythocytes underwent apoptosis after hemoglobin switching.[30] It is reasoned that adult hemoglobin with a lower oxygen affinity readily releases oxygen, leading to excessive oxidative damage and death in G6PD-deficient erythroid cells. These studies emphasized that G6PD serves primarily in maintenance of the proper redox balance.

A murine model of a "nearly-complete" G6PD deficiency was generated.[31] ES cells used in this study still retained about 2-30% of normal G6PD activity as a result of an artificial splicing. The mouse generated from this ES line displayed an intriguing inheritance pattern: after crossing the chimera with a normal female, the first generation (F1) G6PD (+/-) heterozygotes born were phenotypically normal. However, when F1 female heterozygotes were bred to normal males, only normal mice were born. Hemizygous G6PD (-) males as well as heterozygous G6PD (+/-) females died *in utero*. The

latter were supposedly devoid of G6PD activity as a consequence of inactivation of the paternal X chromosome. It was found that the placental development of these embryos was severely impaired. Longo *et al.* hypothesized that these embryos may die from oxidative stress and damage upon the establishment of circulation and impairment of placental function.

Riddles which Remain to be Solved

With the advancements in the field of G6PD research in the past few years, it has become certain that G6PD is an indispensable component of antioxidant defense. Still, whether G6PD deficiency plays pathogenic roles in diseases other than hemolytic disorders remains to be clearly defined.

It appears that G6PD deficiency is associated with an increased susceptibility to certain diseases. For instance, G6PD-deficient individuals suffer from an increased risk of diabetes and cataract.[32,33] It is plausible that the reduced proliferative capacity of G6PD-deficient cells impairs the turnover of damaged parenchyma cells. This, together with increased oxidative damage, can undermine the normal physiological functions of various tissues.

The correlation between G6PD and cancer is ambiguous. Previous studies revealed elevated G6PD activities in malignant tissues in various cancers.[34] Consistent with this, NIH3T3 cells overexpressing G6PD gave rise to tumors in nude mice, implying that G6PD is a promoter of tumorigenesis.[35] On the other hand, several epidemiologic studies did not reveal any difference between G6PD-deficient and healthy patients.[36,37] A more recent mortality follow-up study in Sardinia showed an association between G6PD deficiency and non-Hodgkin's lymphomas.[38] More epidemiological studies are needed to make definite conclusions. Perhaps, G6PD-deficient individuals may be less prone to the risk of cancers because of diminished proliferative potential and premature senescence of their cells. Additional mutations in key regulatory genes like p53 may be needed for these cells to evade senescence. This also means that the role of G6PD deficiency will be overshadowed by mutations of these genes. Moreover, G6PD deficiency may affect tumorigenesis indirectly. Indeed, we have found an association between G6PD activity and the relapse rate in nasopharyngeal cancer.[39] The mechanism behind this is currently unknown.

G6PD may play subtle roles in other aspects of health. For example, G6PD deficiency predisposes affected subjects to a higher risk of hypertension. This may be due in part to impaired production of nitric oxide.[40] Moreover, as mentioned above, G6PD deficiency may affect the outcome of viral infections. It is of interest to see if G6PD-deficient individuals are more susceptible to certain diseases such as viral infections and degenerative disorders.

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