CHAPTER 14

HUMAN PATHOLOGIES AND ABERRANT SULFUR METABOLISM

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14.1 INTRODUCTION

Sulfur is found in relatively small quantities in biological molecules. It has numerous properties that make it valuable in facilitating protein structure–function relationships that are critical to life processes. Sulfur has the essential chemical properties to exist in a biologically reduced sulfhydryl state where the pKa of the thiol group is ~9.65, accounting for its nucleophilicity. Sulfur and thiol homeostasis are maintained in cells by a complex series of balanced pathways. Methionine, cysteine, and glutathione (GSH) sequester the majority of cellular sulfur. Within pathways that regulate these molecules there exist defects that translate into human disease states. In this chapter (and others within this volume) we will discuss some of the phenotypes that can result in aberrant physiologies in human populations.

14.2 BIOSYNTHESIS AND METABOLISM OF METHIONINE AND CYSTEINE

Methionine is required for protein synthesis, while its activated form, *S*-adenosylmethionine (SAM), serves as a methyl donor in numerous biological reactions. ATP is attached to the sulfur atom of methionine to form SAM, a reaction catalyzed by methionine adenosyl transferase (MAT) and SAM condenses with glycine, releasing a methyl group and sarcosine, to form *S*-adenosylhomocysteine (SAH). This reaction is catalyzed by the cytosolic enzyme glycine *N*-methyltransferase (GNMT) [1], a regulator of the SAM : SAH ratio. Where the methyl group supply is limiting, GNMT activity is reduced in order to provide a greater supply for other SAM-dependent methyltransferases. Conversion of SAH to homocysteine is

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the intersect between the transulfuration pathway and the folic acid cycle. Indeed, GNMT is a folate binding protein, but can also act as a sensor to maintain cellular thiol balance.

14.2.1 Folic Acid Metabolism

A functional link between sulfur amino acid metabolism and the metabolism of methyl groups is provided by the conversion of homocysteine to methionine, resulting in the formation of tetrahydrofolate (THF), the active form of the water-soluble vitamin folic acid. Folic acid is synthesized by two sequential reductions both requiring NADPH and catalyzed by dihydrofolate reductase (DHFR). 5,10-Methenyl-THF while also involved in the metabolism of glycine and serine can be thought of as a central compound with respect to the various folate derivatives. It can be generated from THF either through conversion of serine to glycine or through the decarboxlyation of glycine. Its reduction to 5-methyl-THF is essentially irreversible and with the possible exception of some central nervous system functions, 5-methyl-THF is of limited direct biological consequence.

This compound is the predominant extracellular form of folic acid and once transported into cells it must be demethylated to THF. Because of the irreversibility of the reaction catalyzed by DHFR it cannot be oxidized to 5,10-methylene-THF. Demethylation may be achieved where homocysteine accesses the methyl acceptor and is converted back to methionine. This is a reaction in which both folic acid and vitamin B12 participate. While this reaction is of central significance to folic acid homeostasis, it is relatively minor in terms of regeneration of methionine, one characteristic that makes methionine an essential amino acid.

Inhibition of GNMT is achieved by SAM-induced allosteric inhibition of 5,10-methylene tetrahydrofolate reductase (MTHFR) with concomitant decreased levels of 5-methyl-THF [2]. GNMT is a folate-binding protein and is subject to inhibition by 5-methyl-THF [3]. Thus, under conditions of low availability of methyl groups, low SAM levels prevail and this leads to an increase in 5-methyl-THF with subsequent inhibition of GNMT [4]. Conversely, with excess methyl groups, SAM levels increase, with subsequent decrease in MTHFR and 5-methyl-THF and enhancement of GNMT activity.

14.2.2 Transulfuration Pathway

Homocysteine is a sulfur-containing amino acid that plays a significant role in one-carbon metabolism and methylation reactions. In humans, the sole source of homocysteine is through dietary methionine intake and subsequent metabolism. The inability of certain individuals to metabolize homocysteine via methylation and/or transulfuration can result in its systemic build up in the circulation. In the initial step of the transulfuration pathway, serine is combined with homocysteine in a reaction catalyzed by the B6-dependent enzyme cystathionine β-synthase (CBS) to form cystathionine, which is converted to cysteine via c-cystathionine. Subsequent metabolism of cysteine may eventually lead to taurine, a nonstandard amino acid that is critical in fetal and

childhood development and is usually added as a supplement in many infant formulas. While high plasma concentrations of cysteine can prove to be toxic, the amino acid can be limiting in the synthesis and maintenance of cellular glutathione pools. Glutathione is a primary source of cellular nucleophiles and critical to the maintenance of a reduced environment by maintaining a balanced cellular redox potential. Of equal biological importance, sulfur amino acids contribute to critical regulatory pathways that involve one carbon metabolism.

14.2.3 One-Carbon Metabolism

One-carbon metabolism is the transfer of one-carbon groups from one compound to another in a complex array of interrelated biochemical reactions that in its most simple view serves two critical functions: methylation of nucleic acids and synthesis of nucleotides and thymidylate [5, 6]. THF can function as a coenzyme in the transfer of one-carbon units, critical in a number of important cellular synthetic reactions, perhaps the most important of which is the synthesis of thymidylate. The conversion of 5-methyl-THF to THF provides the one-carbon group that is used in the remethylation of homocysteine to produce methionine, a reaction catalyzed by the B12-dependent enzyme methionine synthase. It is also worth noting that an oxidized metabolite of choline, betaine (trimethylglycine), can also serve as a methyl donor. Its participation in the remethylation of homocysteine to methionine produces dimethylglycine, which is not a methyl donor. However, when the methyl groups are removed as formaldehyde, oxidation to formate can lead to the formation of 10-formyl-THF. Although the major significance of this pathway probably relates to the biosynthesis of phospholipids, a conversion of choline to glycine can be thought of as a salvage pathway for one-carbon units. One-carbon metabolism has been attributed to >80 reactions and frequently has folate and the B vitamins as coenzymes. Modest dietary deficiencies of these coenzymes are associated with important diseases, including neural tube defects (NTD), cardiovascular disease, and cancer. Although cysteine is not directly involved in one-carbon metabolism, it can be synthesized from serine and methionine and these two amino acids are directly involved. Thus, overall, serine and methionine together with glycine, homocysteine and various THF moieties are major contributors to one-carbon metabolism.

14.3 DEFECTS IN THE TRANSULFURATION PATHWAY

14.3.1 Homocysteinuria

Previous studies have suggested that homocysteine is a specific risk factor and/or a marker for human pathologies such as cardiovascular disease [7–9]. Whether homocysteine is a cause or effect of the increased incidence of vascular disease is not clear; however, there is a meaningful correlation between elevated plasma homocysteine and mortality from all causes [10].

Both methionine and homocysteine accumulate within cells and bodily fluids of individuals with homocysteinuria, with the result that cysteine biosynthesis is

impaired, reducing the availability of this amino acid. The build up of homocysteine has numerous pathological effects, including the alteration of normal collagen cross-linking [11]. Interference with normal collagen formation may contribute to ocular, skeletal, and vascular complications in patients. In the eye, changes in the ligaments of the optic lens can affect lens stability. In bones, the matrix may be compromised, leading to progressive osteoporosis. Interference with vascular wall formation and maintenance may contribute towards arterial and venous thrombotic disease. Increased homocysteine accumulation may also contribute towards enhanced platelet adhesiveness, thereby providing the baseline for thrombotic occlusive disease.

In addition to homocysteinuria, an elevated plasma concentration of homocysteine is common among patients with cardiovascular disease. While treatments to solve the underlying metabolic malfunction would be optimal, there is evidence that lowering homocysteine levels pharmacologically may provide some therapeutic benefit. Several studies have shown an inverse relationship between homocysteine levels and folic acid and/or the B vitamins [11]. Hence, dietary supplementation with the corresponding vitamin deficiency is beneficial. Numerous studies have shown that folic acid supplementation (dietary or supplements) can reduce plasma homocysteine levels [12, 13].

14.3.2 Homocystinuria

Homocystinuria is a metabolic disorder discovered in the 1960s independently in the United States and Ireland by observations of elevated homocystine (the disulfide of homocysteine) levels in the urine of mentally retarded individuals [14, 15]. While the worldwide incidence is 1 in 344,000, Celtic regions have a significantly higher incidence of 1 in 65,000. Homocystinuria affects the eyes, central nervous system, skeletal and vascular systems. The disorder is characterized by seven biochemically distinct alterations, resulting in increased concentrations of homocystine and methionine in body fluids. The most prevalent form of the disease is characterized by decreased activity of CBS, while other forms have resulted from impaired conversions of homocysteine to methionine [16]. Decreased CBS activity has been classified in three categories: (1) no residual activity, (2) decreased activity with normal affinity for the cofactor pyridoxal phosphate, and (3) decreased activity with low affinity for the cofactor [17]. CBS is a 63 kDa heme containing enzyme that forms a homotetramer and catalyzes the condensation of homocysteine with serine to form cystathionine. CBS requires SAM that can stimulate both pyridoxal phosphate and heme and its activity. The locus for the enzyme is the q21 region of chromosome 21 [18, 19]. The gene contains 23 exons and has an unusually high number of Alu repeats that may predispose it to deleterious rearrangement [20]. Nearly 92 disease-associated mutations in the CBS gene have been identified in laboratories around the world [21]. The most common mutation in the Celtic region is the G307S mutation [22]. Individuals homozygous for this allele have been shown not to respond to pyridoxine supplementation [21], whereas individuals with an I278T mutation respond favorably to dietary supplementation [21]. Moreover, $>80\%$ of homozygous individuals for complete synthase deficiency have optical defects. Mental retardation can occur in

approximately 50% of the patients and this is sometimes accompanied by behavioral symptoms. It is interesting to note the wide range of cognitive abilities in individuals with homocystinuria. Nearly one-third of individuals with this disorder have normal intelligence [23]. Of the individuals that show altered capabilities, two categories have been defined; those that respond to B6 supplementation (mean IQ of 79) and B6 nonresponsive (mean IQ of 57) [16]. Approximately one-quarter of the patients die from vascular occlusive disease before the age of 30. Heterozygote patients may also be at increased risk of premature peripheral and cerebral occlusive vascular disease. Heterozygocity shows a dominant negative effect as enzyme levels are ~25% to 30% of normal, rather than 50%. This may be attributed to the formation of the homodimer. Effective treatment is enhanced by early diagnosis. Newborn screening for decreased CBS activity began in Ireland in 1971. Infants diagnosed have been successfully treated with methionine-restricted cystine-supplemented diets. In addition, oral supplementation with pyridoxine can provide a reduction in urinary methionine and homocystine. This benefit reflects the capacity of residual enzyme activity to be enhanced by the presence of the cofactor.

14.3.3 Cystathioninuria

Cystathioninuria is an autosomal recessive disorder attributed to a defect in the cystathionase γ-lyase (CTH) gene that involves the cleavage of cystathione to cysteine. Because of the elevated plasma concentrations, there is an increase in urinary excretion of cystathione. Mutations in the CTH gene result in a decreased capacity for the enzyme to bind its cofactor, pyridoxal phosphate [24]. Cystathioninuria is considered to be a benign biochemical anomaly that has a low occurrence of 1 per 14,000 live births [25]. Cystathioninuria is not clinically associated with consistent or striking pathologic features; however, some individuals may have developmental defects, convulsions, thrombocytopenia, and mental retardation.

14.4 INHERITED DEFECTS IN MEMBRANE TRANSPORT

14.4.1 Methionine Malabsorption

At least 10 disorders of amino acid transport have been described. Both cysteine and methionine are represented in these disease states of methionine malabsorption, folate malabsorption, and cystinuria. Frequently, these conditions affect transport in the kidney and the gastrointestinal tract, or both. Only rarely is there an impact on other tissues. For example, the autosomal recessive methionine malabsorption trait has direct effects on jejunal mucosa, with clinical manifestations that include mental retardation, convulsions, hyperpneic attacks, white hair, and α-hydroxybutyricaciduria [26]. The disorder is diagnosable primarily as a consequence of urinary excretion of α-hydroxybutyric acid, a by-product of the breakdown of unabsorbed methionine by intestinal flora and adds an odor reminiscent of malt to the urine. Treatment of such individuals with a methionine-restricted diet can produce improvement in the symptoms.

14.4.2 Folate Malabsorption

Folate malabsorption is a hereditary defect in transport of folic acid in the intestine as well as the blood–brain barrier [27]. Individuals with this disorder experience megaloblastic anemia, mental retardation, convulsions, and movement disorders. Some studies show that treatment of such individuals with folic acid reduces seizures while others show the condition to be aggravated [28, 29].

14.4.3 Cystinuria

Cystinuria, one of the most common inborn errors of amino acid transport, is inherited as an autosomal recessive trait resulting from mutations in membrane transport proteins for structurally related amino acids. In the early 1800s,Wollaston [30] identified yellow stones in the urine of some patients that he proposed were composed of sulfur-containing amino acids, and termed the substance cystic oxide, and referred to the syndrome as cystinuria. The disease is characterized by excessive urinary excretion of lysine, arginine, ornithine, and cystine and is a consequence of restricted tubular reabsorption of these amino acids. Although a similarly impaired absorption also occurs in the intestinal tract, clinical symptoms of cystinuria manifest as a build up of cystine stones in renal, ureteral, and bladder calculi. These calculi occur primarily because cystine is one of the least water-soluble amino acids and is more likely to precipitate in target organs. Characteristic of the disease is the fact that at physiological pH, the solubility of cysteine is approximately 300 mg per liter. Individuals who suffer from the disease frequently excrete 600 to 1800 mg of cysteine per day, producing an environment conducive to the formation of stones. While these stones are characteristically found in the second or third decade of life, in some individuals they can occur in the first year.

Type I, II, and III cystinuria have been described. These designations are based on the excretion of cystine and dibasic amino acids in the urine of heterozygous individuals. Specifically, type I refers to heterozygotes who excrete normal amounts of cystine and dibasic amino acids; in type II, heterozygotes excrete 9 to 15 times more; in type III, heterozygotes excrete twice the normal range. Type III patients respond to cystine supplementation whereas type I and type II do not. The underlying mechanism leading to this disorder has been attributed to mutations in at least two amino acid transporters. Type I cystinuria has been attributed to the SLC3A1 amino acid transport gene localized to chromosome 2 [31, 32]. Mutations in a second amino acid transporter that contains a heavy and light chain and is encoded by two genes (SLC7A9 and SLC7A3) were identified in type II and type III patients [32]. Mutations in SCL7A9 are characteristic of mild (A182T allele) to severe (G105R, V170M, and R33W alleles) cystinuria [33]. Medical treatment of the disease includes a high fluid ingestion, usually in excess of 4 : l per day. Ideal urinary cysteine excretion should be less than 250 to 300 mg per liter. These high levels of water intake can serve either to prevent crystal formation, or to dissolve existing crystals. While alkalinization of urine can also positively impact on stone formation, such a treatment modality must be balanced with the possibility of inducing calcium-based stones and

other nephrology complications. A further treatment modality involves the use of penicillamine, which can redox exchange with cysteine to form mixed disulfides of penicillamine and cysteine. This disulfide is significantly more soluble than cystine and can therefore help to reduce the physiological concentrations of amino acids.

14.5 PATHOLOGIES ASSOCIATED WITH FOLIC ACID METABOLIZING ENZYMES

14.5.1 Methionine Synthase Reductase Deficiency

Methylation of homocysteine via methionine synthase results in the formation of methionine. Methionine synthase requires the cofactor cob(I)alamin, which becomes oxidized to cob(II)alamin, resulting in the inactivation of the enzyme. Hence, a second enzyme, methionine synthase reductase (MTRR) is required to maintain methionine synthase in a functional state [34]. MTRR is a 77.7 kDa protein containing 698 amino acids [34]. RT-PCR analyses have identified a variety of mutations in the MTRR gene that are associated with homocystinuria–megaloblastic anemia and spina bifida [34–37].

14.5.2 Methylenetetrahydrofolate Reductase Deficiency

5,10-Methylenetetrahydrofolate reductase (MTHFR) is the enzyme that catalyzes the conversion of 5,10-methylene-THF to 5-methyl-THF, the cosubstrate for remethylation of homocysteine to methionine. A 7.2 kb transcript of MTHFR was identified in all tissues; however, a 9 kb transcript was identified in brain, muscle, placenta, and stomach [38]. The tissue-specific transcript has been shown to be a product of an alternate transcriptional start site and polyadenylation signal.

A number of polymorphisms exist within the MTHFR gene and some are associated with a decrease in enzyme activity that leads to MTHFR deficiency, a process that alters folate metabolism and is associated with a variety of disorders, including homocystinuria, homocysteinemia, NTD, and coronary heart disease. While 24 point mutations have so far been identified that alter enzyme activity [39], a high degree of MTHFR deficiency has been causally associated with nine of these [40]. The C559T mutation which gives rise to a termination codon was identified in Native Americans who lack MTHFR activity and have severe homocystinuria [41]. A second mutation (G482A) also decreased enzyme capacity [41] and this allele was associated with a milder disorder.

Perhaps one of the most widely studied allelic variants of MTHFR is the thermolabile C667T allele that has decreased enzyme activity [41]. This allele has been identified in all populations studied and ranges in frequency of 0.1 to 0.38 [42–44] Chromatographic studies showed that the distribution of red blood cell (RBC) folates is altered in individuals carrying the C667T allele [45]. High pressure liquid chromatography and mass spectrum analysis of DNA from the RBC of individuals homozygous for the C667T allele showed a positive correlation of DNA methylation and an

inverse correlation with plasma homocysteine levels [46]. Folate can stabilize the thermolabile enzyme [42]. Subsequent studies have shown that increasing serum folate levels >15.4 nM appear to neutralize the clinical manifestations due to the thermolabile allele [47].

Homozygous individuals carrying the C667T allele have a threefold increased risk of developing premature cardiovascular disease [48]. The risk factor of the thermolabile allele with coronary disease was confirmed in a meta-analysis of dozens of independent studies [49]. The risk was particularly enhanced when serum folate levels were decreased.

14.5.3 Neural Tube Defects (NTD) and MTHFR Deficiency

NTD are developmental abnormalities of the spinal cord that are recessively inherited. NTD include a wide variety of disorders, such as spina bifida occulta, diastematomyelia, and intradural or extradural lipoma. The C667T allele of MTHFR was characterized as the first genetic risk factor for NTD at the molecular level [50]. Ou et al. [51] showed that C667T homozygosity was associated with a 7.2-fold increased risk for neural tube disorders. Conflicting results with the thermolabile allele and NTD exist [51–53]. These studies provide evidence that a number of factors contribute to the development of NTD.

Folic acid can serve to lower homocysteine levels by providing the factors necessary for the remethylation of homocysteine to cysteine. Evidence from the Centers for Disease Control suggest that administration of folic acid prior to conception and during the first 4 weeks of pregnancy can prevent ~50% of NTD [54]. In 1998, the United States mandated that grain products were to be fortified with folic acid (140 μg/100 g) to prevent NTD in pregnant women. It is important to note that NTD do not arise from a nutritional deficiency of folate but from metabolic defects that can be corrected by large doses of folic acid at developmentally critical times.

14.5.4 Polymorphisms in MTHFR and MTRR as Risk Factors for Spina Bifida

Spina bifida is one of the disorders associated with NTD and is prevalent in the general population (0.14%). Polymorphisms of the MTHFR (C677T), MTRR (A66G), and methionine synthase (A2756G) genes have been studied in families who have members with spina bifida and compared to individuals from unaffected families [34, 55]. Determining the significance of these enzymes to the disease is complicated by consideration of other maternal and embryonic risk factors. Pietrzyk et al. [55] concluded that maternal homozygosity for polymorphisms in MTHFR and methionine synthase genes confers a high risk. In prior studies, [56] this same causal relationship was shown and it was suggested that the risk of having a child with spina bifida increased with the number of maternal alleles. From these and other studies, enzymes involved in the homocysteine-folate metabolic axis should be considered as independent risk factors for spina bifida.

14.5.5 Down Syndrome

Down syndrome, trisomy 21, is one of the most common human disorders associated with chromosomal imbalance. Clearly the location of the CBS gene on chromosome 21 has a significant impact on affected individuals who have an additional chromosome. The CBS protein is overexpressed in individuals with Down syndrome and alters homocysteine metabolism, resulting in a metabolic imbalance such that folate-dependent resynthesis of methionine is compromised [57].

In a manner similar to potential risk factors for spina bifida, polymorphisms in the MTHFR (C667T) and MTRR (A66G) genes were examined and have been linked to the etiology of Down syndrome [58]. Wisniewski et al. [59] reported the presence of senile plaques and neurofibrillary tangles in the brains of patients with Down syndrome. These findings are neuropathologic hallmarks of Alzheimer's disease; however, the presence of plaques appears at an earlier age. Polymorphisms in the MTHFR gene, as well as increased plasma homocysteine levels, have been associated with Alzheimer's disease in some populations [60]. In general, MTHFR polymorphisms have been linked to a wide variety of disorders that are associated with impaired mental dysfunction, including Alzheimer's disease [60], Down syndrome [59], and spina bifida [55, 56]. Studies are ongoing to clarify the impact of allelic variation for folate metabolizing enzymes in individuals who have Down syndrome.

14.5.6 Cancer

Some cancer cell primary cultures and cell lines express an unusual dependence on methionine for growth [61]. Conversely, most nontransformed cells are methionine independent [62]. Initially, a defect in the enzyme methionine synthase was considered a viable explanation for the methionine dependence. Indeed, decreased activity of this enzyme has been demonstrated in some tumor cell lines [63], but not all [64]. Furthermore, some methionine-dependent cells have defects in cobalamin metabolism [65], while others are associated with defective expression of methylthioadenosine phosphorylase [66]. Thus, methionine dependence is multifactorial. Matsuo et al. [67] examined polymorphisms of methionine synthase reductase (MTRR), methionine synthase, and MTHFR in rectal cancer patients and compared the incidence to noncancer patients. Individuals homozygous for the A66G polymorphism in MTRR had a significantly higher risk of colorectal cancer than other genotypes in Japanese populations [68]. These same three genes were analyzed in Caucasians with non-Hodgkin's lymphoma, multiple myeloma, and noncancer patients. The methionine synthaseA2756G polymorphism was shown to confer a 2.4-fold lower risk in lymphoma patients. Alterations in one-carbon metabolism have also been associated with the pathology of cancer. Indeed, aberrant DNA methylation is a common phenotype in human neoplasias. Decreased SAM levels could contribute to altered DNA methylation in tumor cells. The underlying cause could be a consequence of nutritional imbalance or allelic variation in genes governing SAM metabolism. Paz et al. [68] analyzed a wide range of cancer types for the methylation status of three genes involved in methyl group metabolism. A positive correlation

was found among the C677T allele of MTHFR and the 2756G allele of methionine synthase [68]. Efforts continue to unravel what role, if any, these enzymes may have in tumor progression. Whether therapeutic targeting of these pathways could be used to achieve anticancer effects remains to be established. 5,10-Methylene-THF and its 5,10 precursors play critical roles in purine and pyrimidine biosynthesis. The pool of 5,10-methylene-THF increases with diminished activity of MTHFR. Skibola et al. [69] hypothesized that the enhanced 5,10-methylene-THF pools could result in decreased misincorporation of uracil into DNA, thereby resulting in fewer double strand breaks. Hence, folate status would be critical in the development of rapidly proliferating cancers (colorectal and leukemias) that have high DNA synthesis rates. The 667TT genotype was shown to confer a 4.3-fold decreased risk among patients with acute lymphocytic leukemia (ALL) [69]. Further studies in pediatric leukemia patients showed a significant association with the C667T genotype when compared to healthy newborns [70]. 5-Formyl-THF (leucovorin) is used pharmaceutically as a rescue agent in combination with cancer drugs of the antimetabolite class. The role that folate-metabolizing enzymes play in the etiology of cancer is not clearly understood. However, a better understanding of the intricate relationship between cancer risk and thiol status may unravel new initiatives that could incorporate folate supplementation as a means of chemoprevention in high-risk individuals.

14.6 HETEROGENEITY OF GSH METABOLIZING ENZYMES AND ASSOCIATED HUMAN PATHOLOGIES

Alterations in GSH levels are associated with a wide variety of pathologies, including cancer, HIV, lung disease, and Parkinson's disease. Hence, polymorphisms in the genes governing GSH levels are considered contributory to the etiology of these disorders. Polymorphisms within the γ-GCS gene have been identified within the heavy subunit [71]. The gene, located on chromosome 1, encompasses 22 kb and contains seven exons and six introns. Three alleles of the γ-GCS-HS subunit have been identified that differ by the number of GAG trinucleotide repeats in the 5 coding and noncoding region. The contribution of these alleles toward GSH homeostasis pathologies remains unclear. There are examples of altered γ-GCS activity associated with a variety of diseases. The essential role of γ-GCS in GSH synthesis has been demonstrated in knockout mice that were incompetent to form the heavy chain of γ-GCS [72]. The mutation was shown to be embryonic lethal; however, cell lines were derived from the mutants when supplemented with either GSH or *N*-acetyl cysteine (NAC). In humans, γ-GCS activity is diminished in patients with hemolytic anemia [73]. These patients were shown to carry an A → T mutation at nucleotide 1109 that results in a histidine to leucine transition at amino acid 370. Two additional alterations were identified in an intron (+206) and a CGC repeat in the 3-untranslated region [73]. Some patients with malignant mesothelioma have a deletion on chromosome 1 in the region that encompasses the γ-GCS gene [74]. This deletion can lead to γ-GCS deficiency and it was proposed that this could contribute to the malignant phenotype of this disease.

14.6.1 Diseases Associated with Altered Glutathione Metabolism

14.6.1.1 Defects in Enzymes of the γ-Glutamyl Cycle To date, hereditary defects have been described in four of the major enzymes that mediate glutathione metabolism through the γ-glutamyl cycle [75]. Polymorphic variants of γ-GCS have been linked to syndromes that include hemolytic anemia, either with or without hepatosplenomegaly. Hereditary defects in glutathione synthetase are autosomal recessive and can lead to mental retardation and neuropsychiatric dysfunction in approximately 50% of patients, while this deficiency is routinely accompanied by metabolic acidosis and hemolytic anemia. The cycle intermediate 5-oxoproline is found in excess in the bloodstream of these patients, presumably because of the lack of feedback inhibition by GSH on γ-GCS. Where glutathione synthetase deficiency is restricted to erythrocytes, the hemolytic anemia is not accompanied by a generalized oxoprolinuria [76]. Glutathionemia (excess GSH in the blood) occurs with aberrant expression of γ-glutamyl-transpeptidase. This can lead to an imbalance in glutamic acid homeostasis, classifying the disease as an inherited disorder of dicarboxylic acid catabolism. Symptoms can include mental retardation, but it is not clear whether there is a straightforward inheritance pattern for this genetic abnormality.

14.6.1.2 Parkinson's Disease While GSH is found in millimolar concentrations in the brain [77] this organ is more susceptible to oxidative damage than other tissues [78]. Hence an alteration in GSH homeostasis that may lead to oxidative stress has been associated with neurodegenerative diseases, such as Parkinson's disease (PD). Parkinson's disease, affecting nearly 1% of individuals over the age of 65, is a progressive neurodegenerative disorder that results in impaired motor and cognitive functions [79]. The underlying cause of the disease stems from the destruction of dopaminergic neurons in the substantia nigra pars compacta (SNpc) region of the midbrain [80]. These cells are involved in the metabolism of dopamine and PD is characterized by a dopamine deficiency. During normal endogenous dopamine metabolism, ROS are generated and their removal by GSH serves to protect the SNpc. The progression of PD is associated with a depletion of GSH levels and an increase in ROS within the SNpc [81, 82]. Using a murine model, it was shown that BSO treatment depleted GSH and resulted in selective damage to the neurons within the SNpc [83]. In a clinical study, improvements in patients with PD were observed following administration of reduced GSH [83]. Whether prolonged, systemic treatments with reductive agents that cross the blood–brain barrier would prove to be an effective preventive treatment for patients at high risk remains to be established.

14.6.1.3 HIV Nearly 21.8 million deaths associated with HIV/AIDS were reported worldwide between 1981 and 2000. Prior to clinical manifestations of the disease, the immune system is compromised. GSH levels have an impact on many immune functions, including activation of lymphocytes. Consequently, it was postulated that GSH deficiency could lead to the progression of immune dysfunction, a hallmark of AIDS. Glutathione levels are depleted in plasma, epithelial lining fluid (ELF), peripheral blood mononuclear cells, and monocytes in asymptomatic

HIV-infected individuals and in AIDS patients [84]. Systemic glutathione deficiency has also been reported in symptom-free HIV seropositive individuals. [84]. Clinical studies have shown that GSH deficiency is correlated with morbidity [85]. It seems reasonable to conclude that a generally impaired antioxidant system is an obvious contributory factor that may contribute to these clinical findings. However, the precise importance of GSH deficiency is a more complex scenario. Decreased GSH levels have been shown to activate NF-κB, leading to a series of downstream signal transduction events that allow HIV expression [86]. The long terminal repeat of HIV (HIV LTR) contains an NF-κB site. In vitro studies have shown that NF-κB binds to and activates genes controlled by the HIV LTR [87]. *N*-acetyl-cysteine (NAC) supplementation blocked HIV LTR gene expression, thereby confirming the importance of thiol status in HIV-positive cells [86]. Depletion of the CD4+ T cell lymphocytes accompanies the etiology of HIV progression. Decreased GSH levels are known to be one contributory factor in the induction of apoptosis in CD4+ T lymphocytes [88]. Oxidative stress indices were measured in blood samples from HIV/AIDS patients and compared to healthy individuals [89]. These studies showed that reduced GSH levels were accompanied by an increase of DNA fragmentation in lymphocytes, indicative of apoptosis [89].

These factors suggest that maintenance or restoration of GSH levels is a potential therapeutic approach in HIV patients. Several studies have shown that NAC restores GSH levels, prevents the activation of NF-κB and replication of HIV [90]. Treatment with NAC has provided beneficial effects for HIV-infected individuals, even though GSH levels in lymphocytes are not altered [90]. Plasma GSH levels in HIV-infected individuals were increased to 89% of the uninfected controls following an 8-week treatment with oral NAC [91]. Supplementation with cysteine-rich whey proteins achieves short-term increases in plasma GSH levels [92]. In concordance with other dietary protocols, GSH precursor amino acid supplements seem, at least on the surface, to be a beneficial additive.

14.6.1.4 Liver Disease High intracellular content of GSH in liver are congruous with the detoxification functions of this organ. Inherited disorders in glutathione synthesis and metabolism can significantly disrupt liver function and in some instances can be conditionally lethal. In humans, regular dietary intake of precursor sulfur containing amino acids will maintain hepatic intracellular GSH levels in the 5 to 10 mM range. Alterations in liver GSH are either the cause or effect of a number of pathologies. For example, in alcoholics, pools of mitochondrial GSH are depleted, with the concomitant result that ROS damage can be exacerbated producing cell death and contributing to cirrhosis. The defect that leads to reduced levels of mitochondrial GSH involves a partial inactivation of a specific mitochondrial membrane transport protein [93]. Thus, while a build up of cytosolic GSH occurs, inability to transport this into mitochondria is caused by physicochemical alterations to the inner mitochondrial membrane caused by long-term alcohol exposure. The selective depletion of GSH in this organelle can sensitize hepatocytes to the oxidative effects of cytokines such as tumor necrosis factor (TNF) [94]. In patients with chronic hepatitis C infections, GSH levels are severely depleted in hepatic and plasma fractions and

also in peripheral blood mononuclear cells. These conditions were more pronounced in patients who had a concomitant HIV infection [95].

14.6.1.5 Cystic Fibrosis Cystic fibrosis (CF) is a genetic disorder afflicting nearly 250,000 children worldwide per year. The lung dysfunction that characterizes the disease is due to an alteration in an ion transport protein, cystic fibrosis transmembrane conductance regulator (CFTR). The recessive mutation renders the channel dysfunctional or absent. CFTR is an organic anion efflux channel with functional properties that are redundant to MRP, a related class of ATP binding cassette transporters. CFTR maintains a cellular homeostatic balance of ions, including sodium, chloride, and GSH. Normal levels of GSH in the ELF of the lung are 150 times higher than other tissues [96] where it serves as an essential antioxidant that protects the tissue from inhaled toxins. However, the presence of GSH in the ELF also provides a sensor system for maintaining surfactant production, as well as a trigger for inflammation. CF is characterized by systemic GSH deficiency that progresses over time [97]. Cellular GSH deficiency has been associated with an increase in transcription of NF-κB, which participates in the regulation of the inflammatory cytokines [96]. Low levels of GSH lead to inflammation, a hallmark of CF, and oxidative stress that can lead to damage to cell membranes, cellular proteins, and DNA. In support of the causative influence of ROS in CF, these patients frequently have higher levels of lipid peroxidation by-products [98, 99]. Adding to the complexity of disease progression is the expression pattern of GSTM in CF patients. GSTM1 plays a role in the detoxification of hydroperoxides. Additionally, GSTM1 is a negative regulator of ASK1, a kinase involved in the regulation of apoptotic pathways [100]. CF patients with the null phenotype for GSTM1 (GSTM*0) have a poorer prognosis than individuals with other GSTM1 alleles [101]. At this time, the impact of allelic variation on the regulation of kinase signaling is unknown. We do know, however, that CF patients have a diminished immune response that is attributed to decreased GSH levels leading to premature apoptosis in macrophages and neutrophils recruited to the lung [96].

Some therapeutic strategies for CF are aimed at restoring GSH levels, particularly in the ELF. Cysteine supplementation has been undertaken in other disorders associated with GSH depletion, including PD. Because GSH deficiency is due to an aberration in efflux rather than synthesis, augmentation with GSH is desirable. Three clinical strategies to supplement GSH have included intravenous, oral, or inhalation of GSH or *N*-acetyl cysteine [102]. Due to stability and uptake, inhalation methods appear more promising. Recent studies in seven CF patients treated with 600 mg of a GSH aerosol administered twice daily for 3 days showed an increase in ELF GSH levels and should provide a platform for future clinical trials [103].

14.6.1.6 Aging Much has been written concerning the plausible inverse correlation between the generation of free radicals and subsequent ROS and the longevity of an organism. Intuitively, one might assume that natural selection would provide an adaptive force to secure cellular protective mechanisms that permit more efficient protection against such stresses. However, a number of issues cloud this principle and

influence the selective advantage of efficient ROS detoxification systems. Specifically, protection of organisms early in life to permit the attainment of reproduction will serve to propagate the species and provide selective advantage. Thus, when diseases of prepuberty result in production of ROS through, for example, a protective inflammatory response, the consequence would be survival to reach a reproductive age. To achieve this, unavoidable cumulative collateral damage may adversely influence the organism in the later stages of life. This outcome would have little consequence to the success of the individual in terms of selective advantage. In other words, the biological advantage of protecting somatic cells in old age will not have a major impact on survival of the species, whereas energy expended at younger ages will be a strong selective advantage. Therefore, there is significant advantage to counteracting childhood infections with macrophage-mediated ROS defenses. Notwithstanding the age-related relevance of the cellular defense mechanisms, the pleiotropic and redundant array of protective enzyme systems that counteract ROS include many that utilize GSH directly. The type and complement of these protective systems can vary significantly, both between tissues and between organisms. In the blood and tissues of numerous organisms any or all of the following can contribute to protection against ROS: water soluble radical scavengers, including GSH, ascorbate, or urate; lipid soluble scavengers, α-tocopherol, γ-tocopherol, flavonoids, carotenoids, ubiquinol; enzymatic scavengers such as superoxide dismutase (SOD), catalase and glutathione peroxidase and some glutathione *S*-transferases; small molecule thiol-rich antioxidants such as thioredoxin and metallothionein; the enzymes that maintain small molecule antioxidants in a reduced state, thioredoxin reductase, glutathione reductase, dehydroascorbate reductase, the glyoxalase sytem; the complement of enzymes that maintain a reduced cellular environment, including glucose-6-phosphate dehydrogenase, in part responsible for maintaining levels of NADPH. The functional redundancy and cooperative interactions between these defense pathways illustrates just how critical protection against ROS is to survival.

Numerous reports have correlated age-related induction of oxidized or glycated proteins with a decreased ratio of GSH to GSSG in both invertebrates and vertebrates. A number of important metabolic enzymes, including aconitase III, can be inactivated by oxidation [104]. This enzyme participates in the citric acid cycle and possesses an active site iron-sulfur cluster which is sensitive to inactivation by superoxide ($O_2^{\bullet -}$) [105]. Carbonic anhydrase III has two reactive sulfhydryls that are subject to conversion to cysteine sulfinic acid or cysteic acid in the presence of H_2O_2, peroxy radicals, or hypochlorous acid (HOCl). These reactions are competitively inhibited by GSH, perhaps as a consequence of *S*-glutathionylation of the affected cysteine residues [106]. These authors reported that in menadione-treated rats, the extent of cysteine sulfinic acid damage to aconitase III was higher in older animals compared to young ones. In light of the potential widespread occurrence of protein *S*-glutathionylation, it seems reasonable to propose that this mechanism may prove to be functionally protective for a number of critical cellular proteins.

Some credence has been given to the relevance of ROS-induced cell membrane damage as a causative event in cell aging and senescence. In this context, oxygen toxicity can be promoted by metals (such as iron and copper) that catalyze the

cleavage of ROOH groups. This Fenton reaction generates hydroxyl radicals ($OH^{\bullet}$) which can abstract protons to initiate lipid peroxidation reactions. In healthy individuals, the potential catalytic activity of these metals is negatively regulated through binding to proteins such as ferritin and transferrin [78]. In aging humans the total body content of iron increases with age (in women after menopause). It was proposed that this increase would promote the occurrence of oxidative damage during the aging process [107] and that perhaps lipid peroxide-induced membrane damage could be crucial to the onset of geriatric disease. A further indication that lipid peroxidation may be linked to aging is provided by studies in *Drosophila* [108]. By disrupting a microsomal glutathione-*S*-transferase (mGST)-like gene these authors showed that the mutant flies had a significantly reduced lifespan compared to controls. One of the characteristic properties of mGSTs is their efficacy in detoxifying products of lipid peroxidation, particularly in the membrane compartments of cells.

A recent small scale study in healthy humans aged 19 to 85 measured the ratios of cysteine to cystine and GSH to GSSG in plasma [109]. For the former, a linear oxidation rate was observed throughout life. For GSH : GSSG ratios, there was no alteration in the redox balance until age 45, after which there was an enhanced oxidation at a nearly linear rate. While the correlation between low GSH levels or low GSH : GSSG ratios and aging seems clear, the precise reasons for the age-related change in the content of GSH is less well characterized. One recent report has shown that a downregulation of the regulatory subunit of γ-GCS occurs in the rat brain during aging [110]. As the combination of catalytic and regulatory subunits of GCS contributes to the de novo synthesis of GSH, this age-related alteration in expression of this enzyme subunit may be a contributory factor in leading to the lower GSH levels.

Support for the free radical theory of aging is also provided from clinical studies in accelerated aging diseases such as Down syndrome, progeria—both adult (Werner syndrome) and childhood (Hutchison–Gilford). In each case a significantly shortened lifespan is accompanied by evidence of increased oxidative stress and disturbance in the redox balance of host cells [111].

The immune response with respect to balance of Th1 and Th2 production can also be influenced by redox conditions. For example, thioredoxin transgenic mice had a longer life expectancy than their wild-type counterparts. In these mice, peritoneal resident macrophages showed a higher ratio of GSH to GSSG compared to age-matched wild-type animals [112]. These so-called reductive macrophages predominated and were associated with sustained maintenance of Th1 prevalence during aging until 2 years in the transgenic mice, while wild-type littermates showed a rapid polarization to Th2 at 8 months. Cytokine production was different in these animals, suggesting that altered redox balance, particularly as a consequence of GSH changes, could influence immune response.

Dietary supplements frequently claim to be enriched in antioxidants and free radical scavengers. Are such claims of value to prevention of aging and the diseases associated with age? In the absence of carefully controlled (and possibly long-term) clinical studies, this is a difficult question to answer. Inclusion of GSH in over-the-counter supplements is of limited value, since the reduced state will not be maintained when exposed to normal atmospheric conditions and room temperature. Perhaps the

oxidized product (GSSG) will provide a supply of the constituent amino acids, where, in particular, cysteine may be useful in stimulating gastrointestinal synthesis of GSH. There is sufficient evidence that thiol-containing compounds can rescue patients from acute exposure to oxidative or electrophilic stress (for example, *N*-acetyl cysteine in acetaminophen overdose). It is also a general principle that cells prefer (and thrive in) a mildly reduced environment and that mild oxidative stress can stimulate growth in a manner not conducive to benign cellular homeostasis. As such, there would seem to be no harm in supplementing a diet with reducing equivalents. However, whether this will prove to be the elixir of longevity remains to be seen.

14.6.2 Diseases Associated with Glutathione *S*-Transferase Polymorphisms

Glutathione *S*-transferases (GSTs) are a family of phase II detoxification enzymes that have co-evolved with GSH and are abundant throughout most phyla. GSTs catalyze the conjugation of GSH to a wide variety of endogenous and exogenous electrophilic compounds. Human GSTs are divided into the membrane-bound microsomal and cytosolic family members. Microsomal GSTs play a key role in the endogenous metabolism of leukotrienes and prostaglandins [113]. Cytosolic GSTs are divided into 6 classes: α, μ, ω, π, θ, and ζ. GSTs can be induced by structurally unrelated compounds known to result in chemical stress and carcinogenesis, including phenobarbital, planar aromatic compounds, ethoxyquin, butylated hydroxyanisole (BHA), and trans-stilbene oxide [114]. Some of the compounds known to induce GSTs are themselves substrates of the enzyme, suggesting that induction is an adaptive response. Many clinically useful drugs are also potential substrates for GST and development of drug resistance can frequently be a key element in cancer treatment failure. GSTs have been linked with the development of resistance toward chemotherapy agents, insecticides, herbicides, and microbial antibiotics [115].

In addition to their catalytic function, GSTs have been shown to form protein-protein interactions with members of the Mitogen Activated Protein (MAP) kinase pathway, thereby serving a regulatory role in the balance between cell survival and apoptosis. By interacting directly with MAP kinases, including c-Jun N-terminal kinase 1 (JNK1) and ASK1 (apoptosis signal-regulating kinase), GSTs function to sequester the ligand in a complex, preventing interactions with their downstream targets [100]. Many anticancer agents induce apoptosis via activation of MAP kinase pathways, in particular those involving JNK and p38 [116]. This novel, non-enzymatic role for GSTs has direct relevance to the GST overexpressing phenotypes of many drug-resistant tumors. As an endogenous switch for the control of signaling cascade pathways, elevated expression of GST alters the balance of regulation of kinase pathways during drug treatment, thereby conferring a potential selective advantage. This process can also provide a plausible explanation for the numerous examples of drug resistance linking GST overexpression with agents that are not substrates for these enzymes.

While polymorphisms have been identified within each class of GSTs, only a limited number have been shown to contribute to human pathologies or clinical drug

response. The μ and τ class of GST have a null phenotype (GSTM*0 and GSTT*0) where individuals do not express catalytically active protein. The presumed inability to detoxify carcinogens is associated with an increased risk toward a variety of cancers. The GSTM1*0 allele is observed in ~50% of the Caucasian population [117] and is associated with an increased risk of lung, colon, and bladder cancer and is a risk factor for pulmonary asbestosis [118, 119]. The GSTT*0 phenotype varies between ethnic groups and is found to be highest in Chinese (65%) and lowest in Mexican American (9%) populations [120]. The GSTT*0 phenotype is associated with an increased risk of tumors of the head and neck, oral cavity, pharynx, and larynx [121, 122].

The μ class of GSTs has five genes (GSTM1-5) [123] that are found in a gene cluster on chromosome 1 [124]. The GSTM1 gene contains four alleles and has been the most widely studied. GSTM1*A has been associated with a decreased risk of bladder cancer and has an allele frequency of 20% [117]. Neurodegenerative diseases such as Parkinson's disease and schizophrenia are characterized by the degeneration of dopaminergic neurons. GSTM2-2 has been shown to catalyze the conjugation of GSH to aminochrome, a reactive oxygen species generated in the redox cycling of orthoquinones within dopaminergic neurons [125]. Hence, GSTM2-2 has been proposed to play a protective role against neurodegenerative diseases.

A single gene spanning ~3 kb located on chromosome 11 encodes for proteins designated in the π class of GSTs [126]. Polymorphisms at the GSTP1 locus result in four alleles, GSTP1*A-D, that differ structurally and functionally [127]. The promoter region contains a TATA box, two SP1 sites, an insulin response element, and an antioxidant response element within an AP-1 site [127]. GSTP1*A plays a role in the acquisition of resistance to cisplatin by enhancing the capacity of the cell to form platinum-glutathione conjugates [128]. GSTP1*B is an allele in which a single nucleotide (A → G) substitution at position 313 substantially diminishes catalytic activity [129]. Homozygocity for GSTP1*B is favorable in the treatment of cancer patients because they have a diminished capacity to detoxify platinum-based anticancer agents [130]. GSTP1*C or an allelic variant that is more predominant in malignant glioma cells differs from other GSTP1 variants by two transitions resulting in Ile104Val and Ala113Val [127c]. No major functional property has yet been assigned to this polymorphism.

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