Intermediate Physiology (PHGY 313) Term Paper Assignment

Case Study #6: X-linked Sideroblastic Anemia

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Abstract

X-linked sideroblastic anemia (XLSA) is a hereditary and congenital heme synthesis disorder, characterized by ringed sideroblasts. There are many clinical features that can indicate an XLSA diagnosis, including many of the blood parameters like hemoglobin concentration and hematocrit generally used to diagnose anemias. XLSA, however, is especially characterized by its ringed sideroblasts, where there is perinuclear accumulation of iron in erythrocytes. Its underlying cause is centered around the erythroid-specific 5-aminolevulinate synthase (ALAS2) gene mutation. ALAS2 is the enzyme for the first and rate determining step in heme biosynthesis, requiring the cofactor PLP to produce aminolevulinic acid (ALA). Without heme, there is inadequate amounts of protoporphyrin IX to react with the excess iron, resulting in the iron overload seen in XLSA patients. There are two types of XLSA: pyridoxine-responsive and pyridoxine-refractory. In pyridoxine-responsive XLSA, mutations in the ALAS2 enzyme lead to inefficient binding of PLP, thereby hindering heme synthesis. In pyridoxine-refractory XLSA, mutations in ALAS2 hinder its import into the mitochondria. The two types of XLSA can be treated with pyridoxine therapy or gene therapy respectively. Additionally, a recent study has shown that not all forms of XLSA mutations are directly related to ALAS2 but can in fact simply disrupt mitochondrial function while still causing XLSA in patients.

Clinical Features

XLSA[1] is diagnosed by measuring the values for various clinical features. Blood parameters are measured, and a peripheral blood smear is done. Among the blood indices, hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), serum ferritin, serum iron, and total iron binding capacity (TIBC) [1]. A decreased MCV[2] and MCH[3], usually indicates XLSA.

In the case study provided, Walter, a 21-year-old man admitted to the Jewish General Hospital, presented with these blood parameters (with the normal physiological ranges provided in parentheses): hemoglobin, 4.7 g/dL (115-155 g/L); red blood cell count, 3.4 x 10^9/L (4.4-5.7 x 10^{12}/L for males); hematocrit, 16% (42-52% for males); MCV, 45 fL (80-90 fL); MCH, 14.4 pg (27 to 34 pg); white blood cell count, 6.8 x 10^6/L (4.0-10.0 x 10^9/L); platelets, 600 x 10^6/L (130-400 x 10^9/L) [1]. Walter's iron status was also measured: serum iron level, 262 μg/dL (39-157 μg/dL); TIBC[4], 278 μg/dL (240-

1 XLSA: X-linked sideroblastic anemia
2 MCV: mean corpuscular volume
3 MCH: mean corpuscular hemoglobin
4 TIBC: total iron binding capacity

450 μg/dL); ferritin levels, 702 ng/mL (24-336 ng/mL). As demonstrated, Walter's count on all blood parameters were below the normal physiological range, strongly indicating a type of anemia, while his iron statuses, except for TIBC, were elevated, indicating iron overload. His TIBC, on the lower end of the spectrum, indicates that there is high iron in his blood, also signifying iron overload [2]. Walter's blood smear also revealed hypochromic and microcytic red blood cells with 60% ringed sideroblasts, supporting the results of decreased MCH and MCV respectively, furthering the diagnosis of XLSA.

Patients like Walter, who have been diagnosed with XLSA, also often have symptoms such as extreme fatigue and weakness, shortness of breath, headaches, and splenomegaly due to iron overload. Iron overload, a sign of XLSA, in itself, can also cause rheumatoid arthritis, growth disturbances, liver cirrhosis and heart failure [3].

Underlying Causes

To confirm Walter's diagnosis, his DNA was extracted and his ALAS2[5] gene was sequenced to find that he had point mutation, where a thymine was substituted for a guanine in exon 9 of the ALAS2 gene. This is one of many point mutations that can cause XLSA. There are two types of XLSA, pyridoxine-responsive and pyridoxine-refractory, and the two are caused by different mutations of the ALAS2 gene.

The pyridoxine-responsive XLSA occurs when the point mutation affects the binding capabilities of ALAS2 to pyridoxal 5'-phosphate (PLP), a cofactor required in the rate determining step of heme biosynthesis. These mutations are single base substitutions within exons 5 to 11 of the ALAS2 gene. This single base substitution causes a change in amino acid, leading to reduced ALAS2 activity or an early stop codon, which truncates the protein, also resulting in reduced or no ALAS2 activity. The reduced ALAS2 activity is usually due to decreased ability to bind to PLP[6]. Cotter et al. conducted a study showing that in exon 9, a T1424A transversion, resulted in a I471N substitution. This amino acid substitution resulted a change in the secondary structure of the ALAS2 gene, where a region of hydrophobic residues was disrupted. Instead of forming a β-sheet, an α-coil is formed. This mutation occurs at the PLP binding site of the ALAS2 catalytic region, reducing its ability to bind to PLP and catalyze the formation of ALA[7], the first step in heme biosynthesis, thereby inhibiting the production of heme. Since this study was done, many other point mutations caused by nucleotide changes have been discovered, followed by their amino acid substitutions: A547C, F165L in exon 5 [4]; G871A, G291S in

[5] ALAS2: erythroid-specific 5-aminolevulinate synthase
[6] PLP: pyridoxal 5'-phosphate
[7] ALA: aminolevulinic acid

exon 7 [5]; T647C, Y199H in exon 5; C1283T, R411C in exon 9; G1395A, R448Q in exon 9; C1406T, R452C in exon 9 [3]. The majority of point mutations that cause pyridoxine-responsive XLSA are located at lysine 391, the PLP binding site in exon 9, as potentially demonstrated by Walter's case study [6]. All these point mutations, although different from one another, result in the same consequence; the reduced ability of ALAS2 to bind to PLP results in decreased enzymatic activity, causing a buildup of iron and a deficiency of heme. Due to this iron overload, people with the human hemochromatosis (HFE) gene are predisposed to developing XLSA. Fortunately, pyridoxine-responsive XLSA can be treated with pyridoxine supplementation after iron chelation.

On the other hand, the pyridoxine-refractory XLSA occurs when the point mutation does not affect the binding of ALAS2 to PLP. There is less known about this type of XLSA and its treatment. In a study done by Furuyama et al., it was found that the base transversion A621T resulted in an amino acid substitution D190V. This point mutation affects the proteolytic processing of ALAS-E, the ALAS2 precursor, during or after its transport into the mitochondria [7]. This results in the enzyme's decreased stability and reduced activity, but is unrelated to PLP binding capabilities. Another study by Campagna et al. found that a mutation in transcriptional enhancer element in intron 1 of the ALAS2 gene could also be responsible for pyridoxine-refractory XLSA. This mutation disrupts the binding site of GATA, but is not actually in the coding region of the ALAS2 gene. Instead, this mutation is in a regulatory or noncoding region, rather affecting the expression of the gene itself as opposed to disrupting the translated protein [8]. It is suspected that other cases of pyridoxine-refractory XLSA are also caused by similarly placed mutations. However, another PLP-unrelated mutation was discovered by Bishop et al. In this case, the ALAS2 enzyme has completely normal enzymatic activity, kinetics and thermostability, unlike the other mutations. In fact, this mutation disrupts the binding between ALAS2 and the β subunit of succinyl-CoA synthetase (SUCLA2) [9]. The function of SUCLA2[8] is to bring succinyl-CoA, the substrate, to ALAS2 the enzyme. This mutation causes deletions of the carboxyl-terminal amino acids, truncating the protein and therefore disrupting the first step of heme biosynthesis. Because pyridoxine-refractory XLSA mutations are unrelated to PLP, patients are often unresponsive to pyridoxine treatments, so the only other option is gene therapy.

Pathological Physiology

The ALAS2 gene is transcribed and translated into the ALAS2 enzyme. This enzyme is responsible for catalyzing the first and rate determining step of heme biosynthesis, and by extension, effective erythropoiesis. The first step of heme biosynthesis is the condensation of succinyl-CoA and

[8] SUCLA2: succinyl-CoA synthetase

glycine, with required cofactor PLP, to form ALA, occurring in the mitochondria [4]. Under normal circumstances, this occurs without any problems. However, under pathological conditions, ALAS2 cannot bind PLP efficiently. Compared to the wildtype ALAS2 enzyme, the mutant requires 100X more PLP to reach its respective maximal enzymatic activity. This affects heme synthesis greatly. When heme synthesis is inhibited, the formation of protoporphyrin IX, a precursor of heme, is also inhibited. This causes unused iron to accumulate [5]. There is not enough protoporphyrin IX to use up the excess iron, resulting in the iron overload symptom of XLSA [3]. This leads to ineffective erythropoiesis, which can be observed in hypochromic, microcytic red blood cells with perinuclear accumulation of iron.

The regulation of heme synthesis also plays a role in the pathological condition. Under normal physiological conditions, erythropoietin activates the transcription of ALAS2 gene, as well as any other genes required for erythropoiesis such as globin genes. Excess heme also stimulates a negative feedback loop, that inhibits the import of ALAS2 enzyme into the mitochondria [6]. Similarly, there is a negative feedback loop with excess iron. These negative feedback loops exacerbate the pathological condition. Excess iron in the mitochondria downregulates the transport of mutant ALAS2 enzyme into the mitochondria, while low heme levels upregulate the import of mutant ALAS2 enzyme, disrupting its enzymatic activity even further. Due to the pathological physiology of XLSA, there is always a deficiency of heme and a surplus of iron.

Clinical Treatment

Pyridoxine-responsive XLSA can be treated with pyridoxine therapy, after iron accumulation is dissipated through chelation therapy or phlebotomy [1]. Iron overload must be treated before pyridoxine therapy because the excess iron disrupts mitochondrial function and by extension hinders heme biosynthesis [4]. XLSA cannot be declared unresponsive to pyridoxine therapy until iron levels are improved. Pyridoxine therapy works by administering 300 mg of subcutaneous pyridoxine [2] or 5-500 mg of oral pyridoxine daily [4]. This is effective because pyridoxine is a precursor to PLP, which pyridoxine-responsive XLSA patients require more of to maximize ALAS2 enzymatic activity and heme synthesis. This treatment usually results in an increase in hemoglobin values and a reduction in ferritin levels [1]. These values almost never reach normal physiological levels, but are still an improvement. Pyridoxine administration has been proven to correct hemoglobin levels in at least one-third of cases [6]. Folic acid and Vitamin B6, an inactive form of PLP, are also often administered. Upon determining which type of XLSA Walter has, he can be treated accordingly.

Recent Research Advances

In 2016, Lichtenstein et al. discovered that a mutation occurring in the respiratory complex I (RC-I) NDUFB11 is responsible for a new form of XLSA [10]. NDUFB11 is a mitochondrial complex I protein that is required for mitochondrial oxidative phosphorylation. Whole Exome Sequencing (WES) was used to find the mutation. The mutation in NDUFB11 is an in-frame deletion of 3 nucleotides, resulting in the loss of phenylalanine 93 (F93). This deletion occurs within a single transmembrane helical domain, making the protein hydrophobic, while shortening the domain and causing rotational rearrangement of following helices. This affects both the localization of NDUFB11 within the mitochondrial membrane and its interactions with other mitochondrial membrane proteins. However, a blood smear of a patient with this condition would show normocytic red blood cells because the mutation inhibits only proliferation, and not differentiation of the red blood cells, as shown via CRISPR/Cas9. NDUFB11 is part of the RC-I[9], so its mutation causes a deficiency of fully assembled and functioning RC-I, determined by standard assays. This loss of functioning RC-Is is associated with a 25% decrease in basal and maximum oxygen consumption rates, resulting in lactic acidosis and XLSA. This decrease was measured by assaying primary patient fibroblasts on a Seahorse XF96 Extracellular Flux Analyzer.

New techniques such as WES[10] and CRISPR/Cas9, combined with new technology, allow deeper understanding of the molecular genetics behind XLSA. WES, done using the SureSelect Target Enrichment kit by Agilent on a HiSeq 2500 sequencer by Illumina at a mean depth of approximately 50X, combined with custom-built, rule-based algorithm next-generation sequencing programs, allowed for the identification of the mutation and its location in the genome. The use of CRISPR/Cas9 showed that the mutation causes proliferation abnormalities as opposed to differentiation ones, supporting the evidence of normocytic red blood cells. Furthermore, the Seahorse XF96 Extracellular Flux Analyzer and Mitochondrial Stress Test kit used in measuring basal respiration, ATP production and respiratory capacity of the fibroblasts allowed for a quantitative observation to support the qualitative ones. Not only has technology facilitated sequencing, it has also opened up other possibilities. The advance of technology has allowed for researchers to determine that there are other causes of XLSA not directly related to the ALAS2 gene, but instead affect the function of the mitochondria, in this case, mitochondrial respiration.

[9] RC-I: respiratory complex I
[10] WES: Whole-Exome Sequencing

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