Hemoglobin research and the origins of molecular medicine

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Much of our understanding of human physiology, and of many aspects of pathology, has its antecedents in laboratory and clinical studies of hemoglobin. Over the last century, knowledge of the genetics, functions, and diseases of the hemoglobin proteins has been refined to the molecular level by analyses of their crystallographic structures and by cloning and sequencing of their genes and surrounding DNA. In the last few decades, research has opened up new paradigms for hemoglobin related to processes such as its role in the transport of nitric oxide and the complex developmental control of the α-like and β-like globin gene clusters. It is noteworthy that this recent work has had implications for understanding and treating the prevalent diseases of hemoglobin, especially the use of hydroxyurea to elevate fetal hemoglobin in sickle cell disease. It is likely that current research will also have significant clinical implications, as well as lessons for other aspects of molecular medicine, the origin of which can be largely traced to this research tradition. (Blood. 2008;112: 3927-3938)

Introduction

During the past 60 years, the study of human hemoglobin, probably more than any other molecule, has allowed the birth and maturation of molecular medicine. Laboratory research, using physical, chemical, physiological, and genetic methods, has greatly contributed to, but also built upon, clinical research devoted to studying patients with a large variety of hemoglobin disorders. During this period, the pioneering work of Linus Pauling, Max Perutz, Vernon Ingram, Karl Singer, Herman Lehmann, William Castle, Ruth and Reinhold Benesch, Titus Huisman, Ernst Jaffé, Ernest Beutler, and many others still active has been instrumental in these studies. Our understanding of the molecular basis of hemoglobin developmental and genetic control, structure-function relations, and its diseases and their treatment is probably unparalleled in medicine. Indeed, this field, especially during the first 25 years of the existence of the American Society of Hematology, provided the model for developments in many other areas of research in hematology and other subspecialities. This review attempts to highlight some recent developments in hemoglobin research most relevant to the hematologist in the context of the current understanding of the functions of these proteins and their genes. I am occasionally asked, "What's new in hemoglobin?" I believe that this review will show that we are still learning much that is very relevant to our understanding of human physiology and disease.

Hemoglobin structure

The human hemoglobin molecules are a set of very closely related proteins formed by symmetric pairing of a dimer of polypeptide chains, the α- and β-globins, into a tetrameric structural and functional unit. The $\alpha_2\beta_2$ molecule forms the major adult hemoglobin. Their main function in mammals is to transport oxygen (O_2) from the lungs to tissues, but they also specifically interact with the 3 other gases, carbon dioxide (CO_2), carbon monoxide (CO), and nitric oxide (NO), that have important biological roles.

The functional properties of hemoglobin molecules are primarily determined by the characteristic folds of the amino acid chains of the globin proteins, including 7 stretches of the peptide α-helix in the α-chains and 8 in the β-chains (Figure 1).[1,2] These helices are in turn folded into a compact globule that heterodimerizes and then forms the tetramer structure.[3] These 4 polypeptides of the hemoglobin tetramer each have a large central space into which a heme prosthetic group, an iron-protoporphyrin IX molecule, is bound by noncovalent forces, and thus the iron atom is protected from access of the surrounding aqueous solution. The iron atoms in this environment are primarily in the physiologic ferrous (FeII) chemical valence state, coordinated to 4 pyrrole nitrogen atoms in one plane, to an imidazole nitrogen atom of the invariant histidine amino acid at position 8 of the "F"-helix, and to a gas atom on the side opposite (with respect to the porphyrin plane) the histidine residue. The reversible binding of gases to these 4 ferrous iron atoms in the tetramer of globin polypeptides allows hemoglobin to transport O_2, CO, and NO.[4] CO_2 is transported in the blood in solution and by interactions with the amino-terminal residues of hemoglobin as a weak carbamino complex and not by binding to the iron atoms.

In recent years, knowledge of the properties of the characteristic folds of each of the globin polypeptides and their ability to bind heme prosthetic groups has led to the development of a detailed evolutionary tree to describe the ontogeny of this family of genes from bacteria to vertebrates.[5,6] In bacteria, they are known as flavohemoglobins and appear to be primarily NO dioxygenases for detoxifying NO; in the protist and plant taxa, these single-chain globin proteins are largely involved with electron transfer and O_2 storage and scavenging. In invertebrates, the O_2 transport function of the globins develops as do several other biochemical functions. It is in the vertebrate taxa that the characteristic pattern of highly expressed intracellular globins, frequently functioning as multimers, for oxygen transport over relatively long distances evolved (Figure 2). These several globin proteins also include, however, the single-chain myoglobin, in high concentrations in many muscle tissues, as well as the homologous (to myoglobin and to each other) α- and

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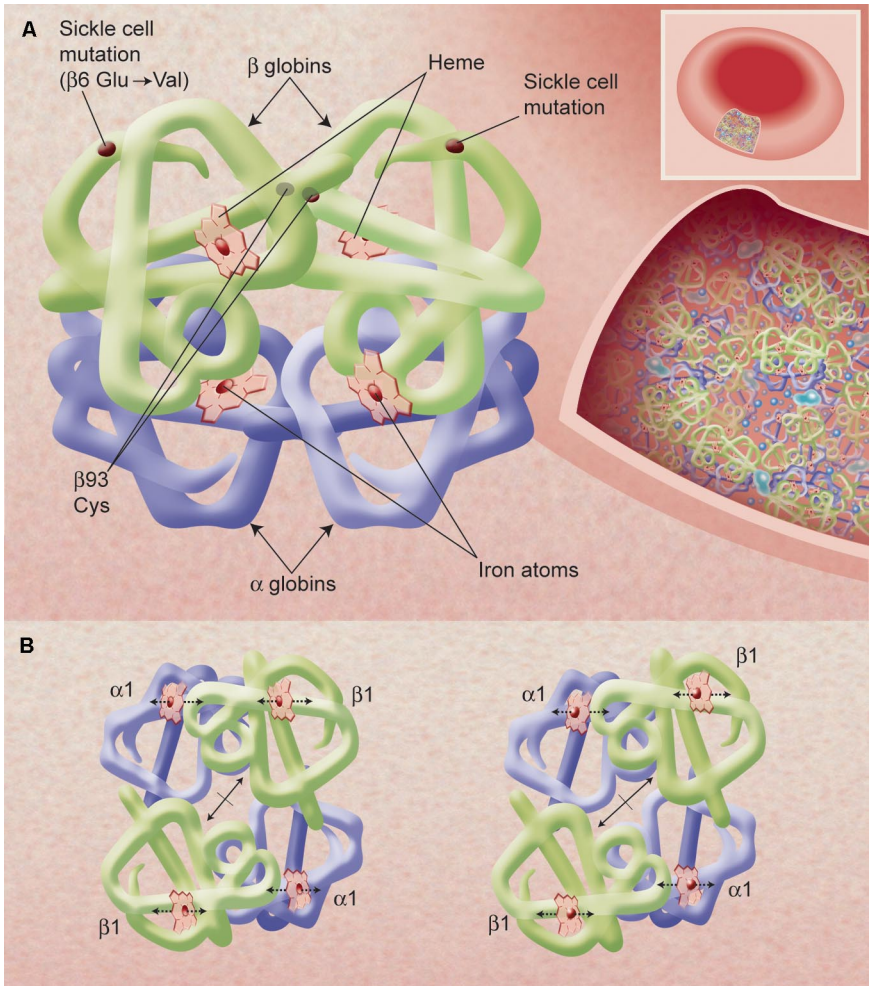

Figure 1. The X-ray determined structure of the hemoglobin molecule and a representation of its very high concentration in the erythrocyte. (A) The arrangement of the α-helices (shown as tubes) in each αβ unit—one on the left and one, 180° rotated, on the right—is shown, as are the 4 heme groups with their iron atoms where gas molecules bind. The site of the sickle mutations on mutant β-chains as well as the β93 conserved cysteine residues is also shown. Hemoglobin molecules in the red blood cell, shown in an inset on the right, are very tightly packed (at a concentration of approximately 34 g/dL) and have little access to solvent; this allows efficient oxygen transport by each cell but also affects the chemical behavior of the molecules, such as promoting sickle cell hemoglobin polymerization upon slight deoxygenation. (B) A representation of the quaternary structural changes in the hemoglobin tetramer, in a top-down view, in the transition from the oxy conformation (left) to the deoxy conformation (right). The iron atoms shift relative to the planes of the heme groups and a central cavity between the β-chains opens, facilitating 2,3 BPG binding. These diagrams are based on drawings of Irving M. Geis. Illustration by Alice Y. Chen.

β-globins and their very stable α/β dimers that pair to form hemoglobin. In the highly specialized mammalian enucleated cell, the erythrocyte, these molecules are expressed at very high concentrations (Figure 1), resulting in a tremendously efficient transport mechanism. The genes of myoglobin (and other globins) separated from the α- and β-globin genes during vertebrate evolution, and these 2 genes themselves evolved into complex genetic loci on separate chromosomes. The numbers of

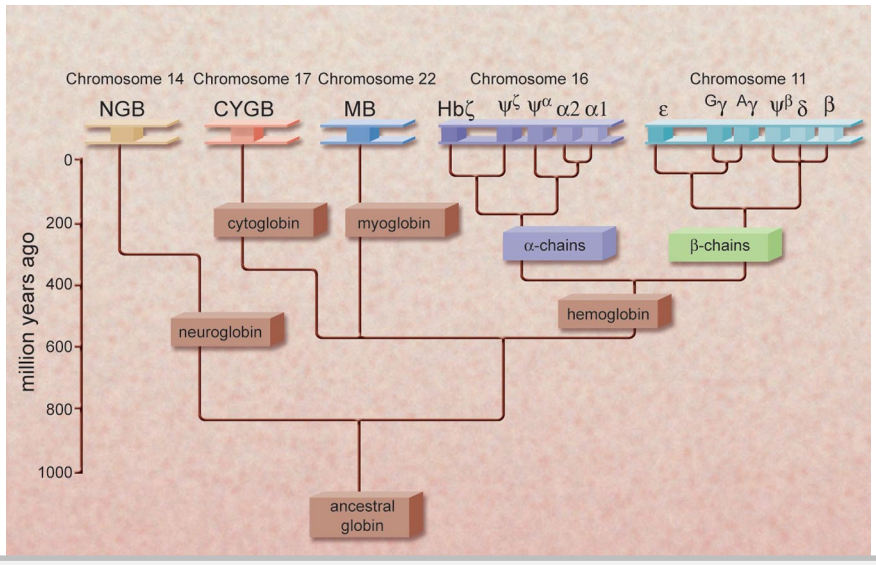

Figure 2. A diagram of the proposed evolutionary relationships of the human globin proteins as inferred from sequence analyses. NGB, neuroglobin;

these genes, their chromosomal locations, and their developmental control vary greatly among species; however, the basic globin gene structure and protein folds are conserved in evolution among all mammals.

Myoglobin has a very high affinity for O_2 compared with hemoglobin, but a detailed understanding of its function has still not been achieved. Mice with knockout of the myoglobin gene have almost normal physiology. Myoglobin is thought to serve more to facilitate oxygen diffusion in muscle, especially to mitochondria, than to act as a storage site, as previously thought.[7] Myoglobin also appears to act as an NO dioxygenase and a nitrite reductase. In the last decade, several other homologous proteins—neuroglobin and cytoglobin—have been detected in low amounts in certain tissues and appear to protect against hypoxia; again, however, there is much controversy about their functions.[8,9]

Hemoglobin function

The role of erythrocyte-encapsulated hemoglobin in transporting oxygen has been the focus of many of the greats of physiology, including Christian Bohr, August Krogh, J. B. Haldane, F. J. W. Roughton, and others in the last century and has been reviewed in detail.[10,11] More recently elucidated was how this finely tuned system is regulated via heterotropic interactions with other molecules, such as protons, anions, and bisphosphophoglyceric acid (2,3 BPG or, in the older convention, 2,3 DPG),[12] and by intramolecular, or homotropic, interactions for optimal normal respiratory function.[13] Cooperative oxygen binding can be explained very precisely in terms of the allosteric model[14] of protein regulation of Monod, Wyman, and Changeux, but alternative models are still being developed.[15] Understanding the physiologic fine-tuning of this function by proton binding (the Bohr effect) or 2,3 BPG binding has been a triumph of basic protein chemistry and applied physiology during the last 50 years.[10,11]

In the 1950s, the methods of protein sequence determination and X-ray crystallography allowed the determination of the amino acid sequences of various hemoglobins and the spatial arrangements of their atoms. This work, marked in particular by the high-resolution structural analysis—among the first for any protein—by Nobelist Max Perutz (Figure 3) and his colleagues in the late 1960s,[2,16] soon resulted in a detailed explanation of the relationship of hemoglobin function as an oxygen transporter to its molecular structure. Furthermore, this information allowed for the explanation of the clinical phenotypes of most of the many hundreds of characterized mutations in the globin genes and proteins, which cause changes in function and include the many "hemoglobinopathy" diseases, in terms of this molecular structure. These correlations, initiated by Perutz and Lehmann[17] and pioneered in the United States by Ranney, Beutler, Nathan, Bunn, Forget, and others, remain among the landmark accomplishments of the then-new field of molecular medicine. Although much of this information is now securely rooted in the textbooks, studies of hemoglobin function have recently become quite active again.

In the last decade, there has been considerable attention to understanding the interactions of normal hemoglobin with CO, in recognition of the fact that as well as being a toxic hazard, CO is produced in the body from free heme by heme oxygenase and can itself activate soluble guanylyl cyclase.[18] For this and other reasons, it has potential pharmacological applications. This attenimportant realization in the mid-1980s that NO is a ubiquitously produced cell signaling molecule, acting via both soluble guanylyl cyclase production of cyclic GMP and other mechanisms, throughout almost all life forms. It is especially important in mammals in the regulation of vascular tone, cell interactions, and neural function.[19]

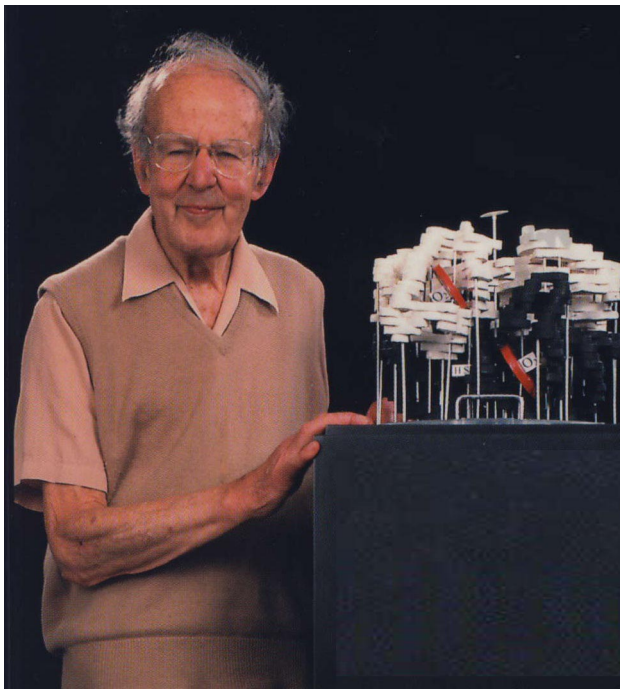

Figure 3. A photograph of Max F. Perutz (1914-2002) demonstrating an early model of the structure of hemoglobin. He devoted more than a half-century to the study of the detailed molecular structure of hemoglobin but was always directly concerned with the relevance of his work to understanding its function and its role in human disease. Courtesy of the Medical Research Council (London, United Kingdom).

It has been known since before World War I that NO reacts with oxyhemoglobin to produce methemoglobin, with ferric (FeIII) iron and nitrate ions. Recent work suggests that most of the methemoglobin circulating in red blood cells is derived from this oxidation process,[20] which is normally reversed by the erythrocytic methemoglobin reductase system. In the past 40 years, a second reaction of NO with deoxyhemoglobin to form nitrosyl(heme)hemoglobin (NO-hemoglobin), with the NO liganded to the ferrous iron atom, has also been studied intensively. Like the reaction with oxyhemoglobin, this reaction had generally been assumed to be irreversible. However, there is now evidence that NO-hemoglobin in the circulating red blood cell may be capable of releasing NO molecules—thus potentially allowing a mechanism for hemoglobin-based, endocrine-like transport of NO from one tissue to another within the body.[21]

Ten years ago, a third reaction of NO with oxyhemoglobin was postulated to be physiologically important: the binding of NO to the strongly conserved β-chain cysteine amino acid at position 93 (Figure 1) to form *S*-nitrosylhemoglobin (SNO-hemoglobin).[22] It was suggested that SNO-hemoglobin can physiologically dissociate to release NO at low oxygen concentrations. Thus, this could be a mechanism for homeostatic control of blood flow to tissues, because the NO released would promote vascular dilatation and increase blood flow and oxygen delivery. This hypothesis, although teleologically attractive, has been very controversial, with many studies negating it, and very recent work with transgenic mice lacking the β93 cysteine residues appears to disprove it.[23] More

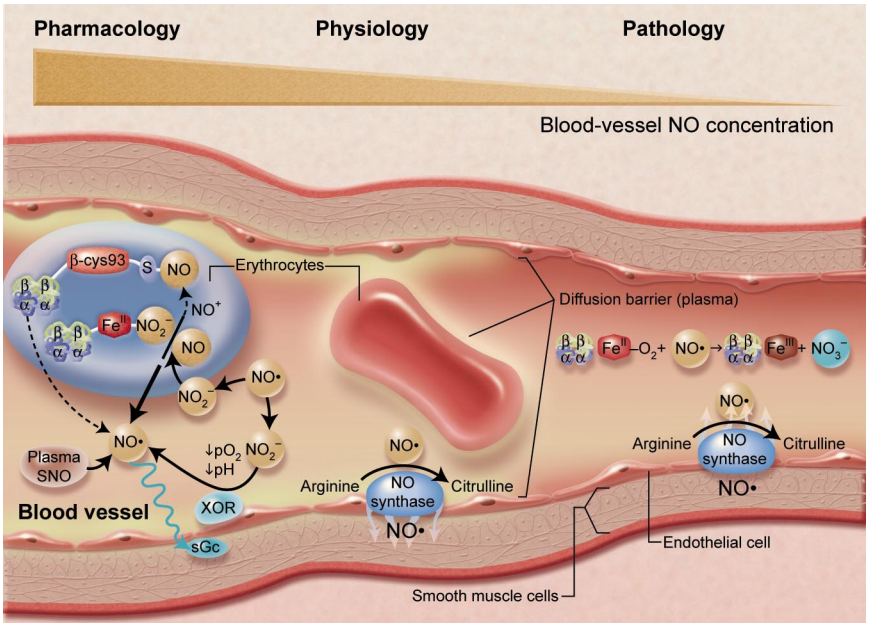

Figure 4. A representation of nitric oxide (NO)/hemoglobin reactions in the arterial microcirculation. Reactions that appear to predominate under physiologic conditions (center), as well as pathologic lesions due to hemolysis (right) and results of high or pharmacologic levels of NO (left) are indicated. Under basal conditions, NO (a short-lived free radical) produced by endothelial NO synthase enzymes largely diffuses into surrounding smooth muscle to activate soluble guanylyl cyclase (sGC) to produce cyclic GMP and regulate vascular tone. The interactions of NO with red cells under these conditions seem to be limited by several barriers to diffusion, at the red cell membrane and streaming of plasma near the endothelium. With hemolysis (or with administration of hemoglobin-based blood substitutes), cell-free oxyhemoglobin acts as an efficient scavenger of NO, causing vasoconstriction and perhaps pathological organ conditions. When endogenous NO levels become very high, or when it is administered by inhalation or by infusion of nitrite ions or other NO donors, reactions in the plasma and within erythrocytes become very important. Reactions with oxygen will tend to oxidize NO to nitrite and nitrate. Reactions with plasma molecules will form thiol (SNO) compounds and other species; plasma nitrite can also be reduced by endothelial xanthine oxidoreductase (XOR) to NO. Small amounts of SNO-Hb form, but its function is not at all clear. Nitrite from the plasma may enter the red cell or be formed in the cell itself, where reactions with hemoglobin and the ascorbate cycle can reduce it to NO. Although the prevalent reactions with oxyhemoglobin to form methemoglobin and nitrate tend to destroy NO bioactivity, these other reactions may allow its preservation and modulation for physiologic functions. Adapted from Schechter and Gladwin (N Engl J Med. 2003;348:1483-1485), with permission. Illustration by Alice Y. Chen.

nitrite ions within erythrocytes can be reduced to NO by deoxyhemoglobin—with reaction kinetics maximal at approximately 50% oxygen saturation—so that NO is increasingly generated as red blood cells enter regions of relative hypoxia.[24] Thus, there are now several potential explanations for a likely central function of NO in controlling blood flow via hypoxic vasodilation (Figure 4).

These recent studies of NO interactions with hemoglobin point to the increasing realization in the last few years that hemoglobin has evolved with functional properties important for the physiology of several gases, especially NO, as well as that of the paradigmatic delivery of O_2. There is also some indication that abnormalities in hemoglobin levels or localization (for example, the increases in total intracellular hemoglobin that occur in polycythemia or of cell-free hemoglobin in chronic and acute anemias [Figure 4]) may result in clinical abnormalities because of their overall tendency to deplete available NO. The major toxicities of all hemoglobin-based blood substitutes seem to be similar and are likely to be due largely to enhanced destruction of NO by the cell-free hemoglobin[25] but could possibly be overcome by replacement of the NO.[26]

The hemoglobin phenotype

In erythrocytes of normal human adults, hemoglobin A ($\alpha_2\beta_2$) accounts for approximately 97% of the protein molecules, hemoglobin A_2 ($\alpha_2\delta_2$) for 2%, and hemoglobin F or fetal hemoglobin ($\alpha_2\gamma_2$) for 1% (Figure 5). This distribution reflects the patterns of expression of the α-globin gene locus on human chromosome 16 has undergone complex changes that resulted in the presence of multiple genes and nonexpressed pseudogenes in the human genome. The pattern of expression of these genes shifts from the more 5′ genes on the DNA to more 3′ genes during fetal, then neonatal, and then adult development stages (Figure 6).[27] In the fetus, the ζ and ε genes are initially expressed primarily in the yolk sac, para-aortic region, and then the liver, resulting in the formation of hemoglobins Gower 1, Gower 2, and Portland. Their down-regulation in early embryonic life is followed by the expression of the 2 α-genes and the 2 γ-genes ($^{G}\gamma$ and $^{A}\gamma$); they are functionally identical but are different in that there is either a glycine or an alanine at position 136. This causes the accumulation of hemoglobin F, which predominates in the last 2 trimesters of gestation and has a slightly higher oxygen affinity than the adult hemoglobins because it binds 2,3 BPG less strongly. At birth, although the α genes remain fully active, the γ genes are effectively down-regulated and the β-like (δ and β) genes are up-regulated so that, normally, by the end of the first year of life, the "adult" hemoglobin phenotype, hemoglobins A and A_2, is predominant. In some cases, expression of the γ-globin persists in adult erythroid cells; this largely asymptomatic state is known as hereditary persistence of fetal hemoglobin (HPFH).[28]

The covalent modification of the major adult hemoglobin by nonenzymatic glycation of the β-chain amino-terminal residue by glucose forms hemoglobin A_{1c}.[29] This was observed in electrophoretic studies of hemoglobin phenotypes and has opened a vast area of diabetes-related research. There has also been much progress in understanding the diverse causes, manifestations, and

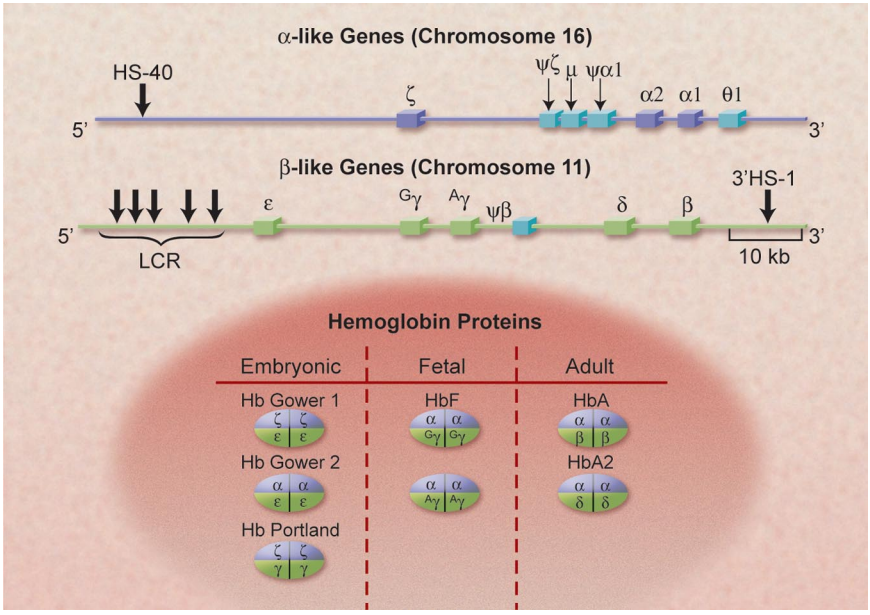

Figure 5. The genomic structure of the clusters of α-like and β-like globin genes, on chromosomes 16 and 11, in human beings. The functional α-like genes are shown in dark blue and the pseudogenes are in light blue; 2 of these (μ and θ-1) code for small amounts of RNA. The functional β-like genes are shown in light green. The important control elements, HS-40 and the LCR, discussed in the text, are also shown at their approximate locations. The α-gene cluster is approximately two thirds of the length of the β-gene cluster; it is transcribed from telomere toward centromere, the opposite of the β cluster. The various hemoglobin species that are formed from these genes, with their prime developmental stages, are shown in the lower part of the figure. Illustration by Alice Y. Chen.

as a cause of familial methemoglobinemia may be considered the first description of an enzyme defect in a hereditary disorder.[32] There have also been significant advances in understanding the complex physiologic adaptive responses to acute and chronic hypoxia, especially of populations at high altitudes.[33]

During the last 30 years, an enormous amount of effort has been devoted to understanding the molecular and cellular mechanisms that underlie these changes (called hemoglobin "switching") in expression of the α- and β-globin gene clusters.[34] This has been because of the intrinsic interest of this system as one of developmental gene control but also because of the potential relevance of this information to developing therapies for the 2 most common groups of genetic diseases of hemoglobin, the sickle cell syndromes and the thalassemia syndromes. Before reviewing these studies of globin developmental control, I note some of the relevant work—especially recent findings—on the pathophysiology of these 2 groups of diseases and how altering the hemoglobin phenotype might be clinically beneficial.

Sickle cell disease

The discovery by Linus Pauling and his associates in 1949[35] that the molecular basis of sickle cell anemia is due to an abnormal hemoglobin virtually created the field of molecular medicine and moved research hematology to its forefront. It is sometimes forgotten that this molecular medicine paradigm also required understanding of the inheritance pattern of this disease, which was supplied in the same year by J. V. Neel,[36] whose publication is also one of the founding articles of the field of medical genetics. We now have a detailed understanding of how a single nucleotide change (A to T) in the β-globin gene leads to the valine for glutamic acid substitution[37] in the β-globin protein. This in turn allows the formation of stable intermolecular interactions (linear polymers of the tetramers) in the concentrated intracellular solutions of deoxyhemoglobin S ($\alpha_2\beta_2^S$ or sickle hemoglobin).[38] This process is the basis for our understanding of the pathophysiology of this disease[39,40] at the genetic, molecular and cellular levels. Sickle cell anemia pathophysiology is a consequence of this reduced solubility, causing polymerization of hemoglobin S tetramers in red blood cells upon partial deoxygenation and the impaired flow of these cells in the microcirculation.[38] Other mechanisms secondary to intracellular polymerization have been extensively studied, especially in animal models, but their relative importance to human pathophysiology remains unclear.

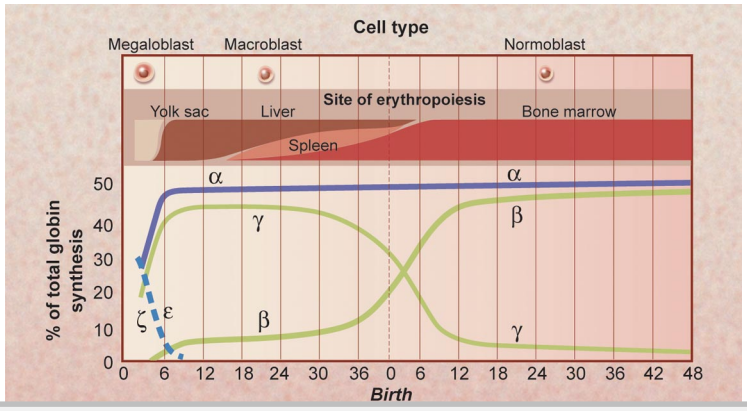

Figure 6. The timeline of the expression of the human globin genes from early stages of fetal development to the changes that occur at birth and in the first year of life. Also shown are the major sites of erythropoiesis and the types of hemoglobin-containing cells during these periods. These analyses are largely based on observations of clinical

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