The William Allan Memorial Award Address:
Thalassemia: Molecular Mechanism and Detection

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INTRODUCTION

It is indeed a great honor to be chosen as the twenty-second recipient of the William Allan Memorial Award, especially since I began my career as a hematologist. I became interested in thalassemia in the late 1960s, and my work on these diseases led me into the field of genetics. With the advent of the DNA technology, like many others in the field, I began to use molecular approaches to study these disorders. These studies have provided many new insights into the molecular mechanisms of genetic diseases. I will briefly cover several topics relating to my particular fields of interest, outlining first the different types of molecular lesions that may give rise to thalassemia, then reviewing how the new knowledge has provided us with a molecular approach to prenatal diagnosis of thalassemia and sickle-cell anemia, as well as insights into the evolution of the globin gene. Finally, I will discuss the possibilities of gene therapy for thalassemia and sickle-cell anemia. Many laboratories have contributed to the explosion of knowledge in this field, and, in this short summary, I can highlight only some of the major advances, sometimes illustrating them with work from our laboratory.

MOLECULAR MECHANISM OF THALASSEMIA

As this topic has been the subject of several recent reviews [1–4], I will not discuss it in detail. Recent findings show that mistakes can affect numerous steps during human hemoglobin synthesis, resulting in defective globin production. Defects can occur at virtually any point along the pathway of protein synthesis. For example, globin structural gene deletion has been shown to result in both α- and β-thalassemia. Deletions may affect a portion of the globin structural genes, or can encompass the entire α- or β-globin gene cluster. To date, more than 10 different deletions have been described for the β-globin gene cluster and another 10 in the α-globin gene cluster. Deletions in the β-globin

gene cluster result in β-thalassemia, δβ-thalassemia, hereditary persistence of fetal hemoglobin, or γδβ-thalassemia syndromes. However, while deletion is the most common cause of α-thalassemia, the clinically important β-thalassemias result from point mutations.

α-Thalassemia represents the first genetic disorder found to be caused by gene deletion [5, 6]. Two main types of deletions affecting the duplicated α-globin structural genes are associated with this disease. In α-thalassemia-2 or α^+-thalassemia, the deletion results in the loss of one of the duplicated α-globin gene loci, and one functional α-globin remains. The single deletion arose by unequal crossover as a result of malalignment in homologous regions of the two chromosomes [7]. Depending on the location of the malalignment along the α-globin gene cluster, a leftward or rightward crossover occurs [8]. Several different crossover events have occurred as multiple rightward crossovers have now been defined [9]. The chromosome containing the single α-globin locus is extremely common and has an extensive worldwide distribution that closely parallels that of malaria. Although it remains to be proved that individuals who carry this deletion are somehow more resistant to malaria, this hypothesis offers an explanation for the very high frequency of this gene in some populations. If a single α locus offers a selective advantage and yet homozygosity for this gene is benign, the frequency of the gene will increase greatly because homozygotes are healthy and will continue to reproduce. In contrast, whereas the β-thalassemia and sickle genes also protect against malaria, homozygosity for these conditions results in a disease state, the affected individuals do not usually reproduce, and their genes are lost from the gene pool. Hence, given the same selective pressure, the frequency of α^+-thalassemia will exceed β-thalassemia and sickle-cell anemia.

In the α-thalassemia-1 (or α^0-thalassemia) genotype, the function of both α-globin genes is abolished by the deletion. The deletion can be confined to the α-globin genes, or may involve the entire α-globin gene cluster, including the ζ-globin gene [4]. Homozygosity for this deletion results in the lethal form of α-thalassemia associated with hydrops fetalis, a condition that occurs frequently in Southeast Asia, rarely in the Mediterranean region, and has never been described in people of African origin [10]. The pattern of distribution directly correlates with the frequency of the α^0-thalassemia genotype in these populations.

Since the first point mutation was defined in β-thalassemia [11], more than 40 such mutations have been found, involving every step in β-globin gene expression [1–3]. These mutations can completely or partially abolish β-globin chain synthesis from the affected chromosome, causing the β^0- and β^+-thalassemia phenotypes, respectively. One type of mutation results in decreased mRNA transcription from the affected β-globin locus and produces a β^+-thalassemia chromosome. The lesions affect the promoter region of the β-globin gene and arise from point mutations either in a C-rich region located 88 and 87 nucleotides upstream from the β-globin gene or in the ATA region about 25 nucleotides upstream [12, 13].

Point mutations can also affect the splicing of the globin mRNA, resulting in

β^0- or β^+-thalassemia (for review, see [3]). The mutations can abolish normal splice sites, generate alternate splice sites, or activate usually dormant cryptic sites. When the mutation affects the invariant dinucleotide GT at the donor splice junction, or the AT dinucleotide at the acceptor splice junction, it completely abolishes normal splicing of the intervening sequence. All the mRNA from the affected locus is improperly spliced, and no functional β-globin mRNA is produced from this locus, resulting in β^0-thalassemia. Splicing abnormalities produce β^+-thalassemia when the function of the normal splice site is not completely abolished, although a variable amount of abnormally spliced mRNA is produced. The severity of the β-thalassemia depends on the relative amounts of normally and abnormally spliced mRNA produced. Thus, in the most common type of β^+-thalassemia in the Mediterranean region which is due to a point mutation at position 110 of the first intervening sequence, the ratio of normal to abnormally spliced mRNA is about 1 to 10, causing a severe disease. In contrast, in the so-called Portuguese mutation, where the mutation occurs at the sixth nucleotide of the first intervening sequence, a large amount of normally spliced mRNA remains and a mild form of β-thalassemia ensues.

When point mutations affect the polyadenylation signals, mRNA processing is disrupted. Normally, mRNA transcription extends some distance beyond the end of the mature mRNA. The RNA is processed back to about 25 nucleotides 3′ from the polyadenylation signal AAUAAA, and the polyA sequences are added. Point mutations in this sequence cause abnormal termination of mRNA processing, resulting in unstable mRNA and the α^+- or β^+-thalassemia phenotypes [3, 14].

A large number of mutations interfere with mRNA translation. These include mutations affecting the initiation and termination codons, and premature termination of translation due to nonsense or frame shifts. To date, initiation and termination codon mutations have been described only in the α-thalassemias. Initiation codon mutations cause one type of nondeletion α-thalassemia in Italians [15]. In the most common termination codon mutation, Hb Constant Spring, a U-to-C mutation extends the α-globin chain by 31 amino acids. This disorder is often associated with Hb H disease in southeast Asia [16].

Globin chain translation can terminate prematurely because of nonsense mutations resulting from a single nucleotide substitution in an amino acid codon [11]. Frameshift mutations in which one, two, or four nucleotides are deleted, or one nucleotide is added, have been described. These mutations shift the reading frames and produce an early termination codon downstream from the frame shift [3]. Both nonsense and frameshift mutations result in β^0-thalassemia. The last group of thalassemias is due to abnormal polypeptide chains. If the amino acid substitution produces an extremely unstable chain, the thalassemia phenotype results rather than an unstable hemoglobin syndrome. Examples include Hb Indianapolis which produces a β-thalassemia [17] and Hb Quong Sze which gives rise to an α-thalassemia [18].

Thus, virtually every step of protein synthesis and gene expression can be affected in the thalassemia syndromes. Perhaps the only lesion that has not been described is an enhancing sequence mutation. In many mammalian genes,

certain sequences enhance expression, often in a tissue-specific manner. These sequences have been best described in the immunoglobulin genes in which an enhancer directs their expression in the lymphocyte. It is not known whether such enhancers exist in the globin system, and if so, where they lie. Perhaps a mutation in such a region will be found for the globin genes and will help delineate tissue-specific enhancers in this system.

PRENATAL DIAGNOSIS BY DNA ANALYSIS

The introduction of DNA technology has also provided us with new methods for diagnosis of genetic disorders. Previously, diagnosis of hemoglobin disorders was accomplished by analysis of fetal blood obtained by fetoscopy or placental puncture [19]. The blood would then be analyzed for the presence of the β^s chain in sickle-cell anemia, or quantitated for β-globin chains in β-thalassemia. This method has two drawbacks. Fetal blood sampling, a complicated technique not readily available in many medical centers, carries a 3%–6% risk to the fetus. Second, in β-thalassemia, especially β^+-thalassemia, the levels of β-chain synthesis in the fetal blood often overlap in heterozygous and homozygous fetuses, clouding the distinction between the two genotypes. The advent of DNA technology resolved both these problems. Since the DNA for analysis can be obtained from amniotic fluid cells, the diagnosis relies on amniocentesis, which is a safe procedure. Second, as the most common approach to DNA analysis is direct detection of the mutation, homozygosity can clearly be differentiated from heterozygosity. Recently, chorionic villus sampling was introduced as a method of obtaining DNA for analysis [20]. This procedure can be performed before the 10th week of gestation and appears to be associated with low risk.

Once gene deletion was found to cause α-thalassemia, diagnosis by DNA analysis became possible. Initially, solution hybridization was used, but this method required at least 25 μg of DNA, which could be obtained only by culturing the amniotic fluid cells for 3–4 weeks [21]. With the introduction of Southern blot analysis, cell culture is not required in most instances because less DNA is needed [22]. The assay has now been further simplified by the dot-blot technique, in which undigested DNA is directly applied to a filter, eliminating the need for restriction enzyme digestion and electrophoresis [23]. These improvements should make the test practical in countries where thousands of pregnancies are at risk annually for α-thalassemia.

Point mutations are detectable by restriction endonuclease analysis if the mutation involves a restriction enzyme recognition site. The best example is sickle-cell anemia in which the mutation at the sixth amino acid of the β-globin chain happens to occur at the a cleavage site for several restriction enzymes (MnlI, DdeI, and MstII). MstII or the isoschizomer SalI have become the enzymes of choice for prenatal diagnosis of sickle-cell anemia [24–26]. They cleave the normal β-globin gene at the position corresponding to amino acid $\beta^{5,6,7}$. This site is abolished by the GAG-to-GTG mutation in the sickle gene. Digestion of genomic DNA with MstII generates a longer DNA fragment in the sickle gene as compared to the normal.

Since so many different lesions can cause β-thalassemia, the diagnosis of these disorders by DNA analysis is more complex. In a few cases, the mutation involves restriction sites and can be detected by digestion with the appropriate enzymes (as in sickle-cell anemia). However, most β-thalassemia lesions do not involve restriction sites. DNA analysis of these types of β-thalassemia was initially accomplished by linkage analysis with polymorphic restriction sites—a method first used for diagnosis of sickle-cell anemia before the MstII test was available [27]. When normal DNA is digested with the enzyme HpaI, the β-globin gene is contained in a 7.6-kb fragment. In DNA from sickle-cell anemia, the β-globin gene often resides in a fragment of 13 kb. This 13-kb mutation originated in West Africa, whereas the sickle genes in East Africa and Asia are usually not associated with the 13-kb fragment. Hence, in families that display linkage between the sickle gene and the polymorphic HpaI site, prenatal diagnosis could be performed once the linkage is established.

At least 15 different polymorphic sites have now been detected along the β-globin gene cluster. Kazazian, Orkin, and coworkers have systematically analyzed various thalassemic populations with restriction enzymes and classified the β-globin chromosome into different haplotypes according to these polymorphic restriction sites [28]. These haplotypes are normal chromosome backgrounds from which β-globin mutations arose. Although specific mutations are associated predominantly with one haplotype, exceptions have been described in which a single mutation resides in more than one haplotype or several mutations occur in the same haplotype. By performing family studies, it is possible in many cases to use these polymorphic sites as chromosome markers for tracing the inheritance of the paternal and maternal chromosomes containing the β-thalassemia lesion [29]. Thus, the method can be used for diagnosis of β-thalassemia, although in cases where no previous offspring with the homozygous genotype are available for study, it may be difficult to establish the linkage.

Wallace et al. devised another technique for detecting point mutations [30]. In this approach, a pair of oligonucleotide probes, usually 19 nucleotides long, are synthesized. One is homologous to the normal sequence, and the other, which differs by a single nucleotide, is homologous to the mutated sequence. These oligonucleotides are used as hybridization probes for genomic DNA. Under strictly controlled hybridization and washing conditions, the normal probe will hybridize only to the normal chromosome, while β-thalassemia probe hybridizes only to the β-thalassemia chromosome. This assay will clearly differentiate among the normal, heterozygous, and homozygous β-thalassemia genotypes and has been successfully used for prenatal diagnosis of point mutations in sickle-cell anemia and β-thalassemia.

Determining which of the many lesions that cause β-thalassemia may be a difficult task in families at risk for this disorder. This has recently been simplified by mapping the common lesions present in a given area. Once the predominant mutations in a given area have been determined, the majority of lesions are detectable using a few sets of enzymes or oligonucleotide probes. For example, in Sardinia, the β^{39} nonsense mutation accounts for 95% of the β-

thalassemia mutations on the island, and prenatal diagnosis is routinely performed by DNA analysis with oligonucleotide probes [31].

In summary, prenatal diagnosis of hemoglobinopathies is evolving from linkage analysis with DNA polymorphisms to direct detection of the mutation. These techniques are also being applied to other genetic disorders as probes for defective genes become available.

GENE EVOLUTION

DNA polymorphism provides a useful approach for studying the evolution of genes. The *Hpa*I polymorphism associated with sickle-cell anemia was used to trace the origins of the sickle gene to at least two parts of the world [32]. It is now known that more than five chromosomes with different haplotypes harbor the sickle mutation [33], although it is not clear how many of these haplotypes actually represent separate mutational events. As human DNA is much more unstable than was originally thought, some of these haplotypes could have been generated by crossing over and gene conversion.

The findings from Sardinia aptly illustrate the frequency of crossing over in human chromosomes [34]. Hybridizing with oligonucleotide probes specific for the β^{39} nonsense mutation, Pirastu et al. found nine different chromosome haplotypes containing this mutation. The restriction enzyme recognition sites near the β-globin gene were identical, while the more distant sites upstream from the gene differed. The multiple haplotypes probably resulted from the crossing over of the chromosome somewhere between the $\psi\beta$ and β loci, which generated new restriction patterns upstream from the β locus. Interestingly, in two cases, the affected chromosomes are associated with increased γ-globin expression. In one, the β^{39} mutation was associated with the nondeletion type of $\delta\beta$-thalassemia with increased $^A\gamma$ production [35]. In another, a chromosome containing three γ-globin genes in the order $^G\gamma$-$^G\gamma$-$^A\gamma$ was produced.

TREATMENT OF THALASSEMIA

Homozygous α-thalassemia is almost invariably fatal. Only one exception has been described in a Chinese infant who was born prematurely and transfused before the diagnosis was established [36]. At the time of this writing, the baby is over 1 year old and is still surviving, although he requires monthly transfusions. His future physical and mental development may indicate whether heroic treatment for α-thalassemia is warranted.

The treatment for homozygous β-thalassemia consists of monthly transfusions and iron chelation therapy. Recently, bone marrow transplantation has been advocated in cases where an HLA compatible donor can be found [37]. The transplantation must be performed early, before the child becomes overly isoimmunized by transfusions, but even under ideal circumstances bone marrow transplantation is currently associated with about 25% mortality. The drug 5-azacytidine has been successfully used to increase fetal hemoglobin synthesis in certain cases [38]; however, long-term administration of this drug carries risk of oncogenesis. Other nonmutagenic drugs such as hydroxyurea are now being tried but their effectiveness and safety have not yet been established.

Since all the normal globin genes are available in pure form, replacement of the defective gene by a normal one has been suggested. The two principal requirements for gene therapy are that the gene must be introduced into the target cells with high efficiency and that the inserted gene must be expressed at a high level. Recently, the first requirement appears to have been satisfied by using retrovirus vectors [39, 40]. A structural gene is cloned into a retrovirus vector and made infective by cotransformation with a helper virus which supplies essential viral function to the recombinant viral vector. This type of experiment has been performed in mice and could possibly be applied to human bone marrow.

The problem of adequate globin gene expression is harder to solve. While genes encoding for enzymes do not have to be expressed at high levels in order to correct enzyme-deficiency states, the globin gene must be expressed at a high level since hemoglobin ultimately forms over 90% of the protein of the red blood cell. This is where our present state of knowledge is most deficient, because we do not yet know what signal turns on globin gene expression in the bone marrow cell. Recent experiments with transgenic mice may provide a model as the introduction of exogenous globin genes into mouse germ line resulted in a high level of tissue-specific expression that appears to be developmentally regulated [41, 42]. If this type of experiment can localize the signal and control mechanisms that direct tissue-specific expression of globin genes, gene therapy could perhaps be contemplated.

An alternate approach to gene therapy would be to use suppressor tRNA genes in those types of β-thalassemia due to nonsense mutations [43]. The β^{39} Gln (CAG→UAG) mutation is a significant problem in the Mediterranean area and affects thousands of individuals. Suppressor tRNAs that insert Gln or Lys into the UAG codon have been constructed. The level of expression and effects of introducing these genes into bone marrow cells by retrovirus vectors are being tested.

CONCLUSION

The DNA technology has now given us a fairly complete picture of the mutations that give rise to the thalassemia syndromes and enabled us to devise methods of directly analyzing the DNA for prenatal diagnosis. These studies have shed light on the evolution of normal and mutant genes and, hopefully in the foreseeable future, will enable us to find ways of actually correcting the genetic defects.

ACKNOWLEDGMENTS

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