Coagulation disorders have been traditionally worked up by their clinical phenotypes and coagulation factor assays which are dependent on APTT- and PT-based techniques. Development of chromogenic substrates in the late seventies and early eighties allowed coagulation factors to be measured like enzymes. There was still a major lacuna in the understanding of the biology of different coagulation disorders. Modern molecular biology - which developed as an unique synthesis of biochemistry, immunology, cell biology, and genetics - allowed us to have a more comprehensive understanding of the pathobiology of many of these coagulation disorders. This overview presents several examples which show how we have enriched our understanding about the varied clinical phenotypes of different coagulation disorders.

Key words: Molecular Biology, Coagulation disorders, phenotypes, genotypes

Coagulation disorders, particularly bleeding disorders, have been traditionally defined by a combination of clinical phenotypes along with laboratory assays using APTT- or PT-based techniques. This left one unsatisfied because there was a lack of complete understanding of the disease process. For example it was expected that severe deficiency of factor VIII or IX should produce severe clinical phenotype, but in reality it is not always so.

Similarly, it is understood from classical genetics that a disease like severe hemophilia A or hemophilia B may even compromise the reproductive fitness of an individual; in which case the disease should have been wiped out from the population but for the fact that an asymptomatic carrier status exists among first-degree female relatives of hemophiliacs. We still do not know whether the hemophilia gene per se provides any survival advantage to female carriers of the disease, but the fact that around 25% of hemophilia cases arose by new mutations (i.e., de novo mutations) was not explainable through our knowledge of classical genetics, but it could be explained through molecular biology. The techniques of molecular biology have enriched our understanding of the pathobiology of bleeding and clotting disorders. In this short overview, using several examples, an attempt will be made to demonstrate how we have become wiser by looking at the coagulation disorders through the windows of molecular biology.

Heterogeneity of Clinical Phenotypes in Hemophilia

It was broadly known that clinical phenotypes, i.e., the severity of bleeding manifestations in hemophilia, are dependent upon the amount of circulating plasma clotting factor levels. Thus, patients with severe hemophilia have less than 1% of normal circulating clotting factor, patients with moderately severe disease have clotting factor activity between 1-5% of normal, and patients with mild disease have clotting factor activity above 5% of normal.[1] It is also known from classical pharmacokinetic studies that the level of any protein in blood is the algebraic sum of its production rate and its metabolic clearance rate. Hence, to understand why certain patients of hemophilia have severe deficiency of factor VIII or IX and others have milder deficiency, one has to understand if there is a difference in the production rate or the metabolic clearance rate of these factors in different categories of patients that is responsible for the difference in the plasma levels. The metabolic clearance rate of purified plasma-derived clotting factors can easily be measured in patients with severe hemophilia by injecting a given dose of clotting factor and then following its levels at different time intervals. Studies have shown that the half-life of injected factor concentrates vary little from one patient to another, showing that it is the production
rate of the clotting factor that decides whether hemophilia patients have mild, moderate, or severe disease. Thus, attention turned to the detailed investigations of the mutations of factor VIII and IX genes that result in the disease phenotype. The database for factor VIII and IX mutations are now available and are updated from time to time. These databases allow us to make some broad generalizations:

1. Missense point mutations usually produce moderate or milder factor deficiency.
2. Critical missense point mutations at the active site may produce a severe phenotype, with the proteins present in the circulation but without coagulation factor activity.
3. Small deletions tend to produce variable effects on factor activity and levels.
4. Large deletions and inversions totally disorganize the gene and produce no molecule whatsoever; these produce severe deficiency.
5. Early chain-termination mutations produce no active coagulation factor and, hence, produces a severe phenotype.
6. Missense mutations in critical glycosylating or proteolytic cleavage sites may produce a severe phenotype.
7. A gene is usually activated by a transactivator called transcription factor; a mutation in the attachment site for the transcription factor, or in the transcription factor itself, can produce a hemophilia phenotype which may improve as the patient gets older (factor IX Leiden).

It was also found that certain moderately severe and mild mutations in a community are more common because of the existence of a founder effect. Haplotype studies show that these common mutations in apparently unrelated subjects are due to a founder effect and not due to independent origin. Detailed structural analysis of these genes also reveals the mechanism by which these different types of mutations arise in these genes. For example, homology studies have shown that factor VIII, factor V, ceruloplasmin, and fungal protein discoidin arose from the same ancestral gene, though they have quite different biological functions. Similarly the genetic similarity of vitamin K-dependent coagulation proteins and inhibitors like protein C, protein S, and protein Z are striking and suggests that the origin is from the same ancestral gene. It was also found that a proportion of patients with severe hemophilia who have the same level of deficiency of coagulation factor (<1% of normal) have a milder clinical presentation. Study of the epistatic interaction of other genes, as well as of the role of environmental factors, will probably provide an explanation.

Spontaneous Mutation Causing Hemophilia - The Explanation from Molecular Biology

More than 25% of the hemophilias occur due to new mutations and these are of various types, i.e., missense, nonsense, small deletions, large deletions, and inversions. Hemophilia apparently had no selective advantage; why then are such mutations so common? A look at both factor VIII and factor IX genes gives us some explanation.

Sommers and his group from Mayo Clinic studied the prevalence of hemophilia in different countries to assess whether environmental mutagens like chemicals, background radiation, or cosmic radiation are likely to have some impact. They argued that severe factor IX deficiency is a disease that is unlikely to be subclinical and, therefore, prevalence studies would be unlikely to miss any case. The expected prevalence/incidence of the disease was likely to be different if external mutagens are the cause of the mutations. Surprisingly, they found the same prevalence/incidence of severe hemophilia B across the world, i.e., 1:10,000 for factor VIII deficiency and 1:40,000 to 50,000 for factor IX deficiency. This data suggested that an intrinsic mutagen must be the cause of such mutations. This intrinsic mutagen was found to be none other than oxygen, the very basis of life. Both factor VIII and IX genes are GC-rich genes. GC-rich areas in a gene becomes the “hot spot” for mutations because the free radicals generated from oxygen can convert guanine into 7-oxoguanine, which structurally resembles adenine, and in its next cycle of gene replication, pairs with thymine rather than cytosine. Hence, G → A becomes one of the common mutational events. Moreover, if there are GC-rich areas in a gene, there is slippage or secondary loop formation, leading to small or large deletions or insertion of GC repeats during meiotic crossover. Thus, in intron 22 of the factor VIII gene there is an island (a gene
within a gene), with similar structure but oriented in the opposite direction. This pseudogene becomes a spot where, during meiosis, a large part of intron 22 becomes inverted, producing severe hemophilia. This event occurs in between 40-50% of severe hemophilia patients. In India, several groups including ours have found a similar prevalence of intron 22 inversions. Similar events, involving intron 1 of factor VIII gene or large and small deletions of factor IX gene, also occur in a small proportion of severe hemophilia patients. A similar prevalence for factor VIII gene deletions in hemophilia has also been reported from this country, using the southern blot analysis.

**Development of Inhibitors in Mild or Moderate Hemophilia A Patients**

Hay and his group have reported the development of inhibitor in moderate or mild hemophilia patients when they were infused with factor VIII concentrate. This was not expected, because these patients have sufficient amount of factor VIII in their blood, hence infused human factor VIII should act like an autoantigen rather than alloantigen. Some of these patients developed inhibitor following minimal infusions. Molecular biology studies of factor VIII gene in these patients gave us the answer. Most of these patients have missense mutations causing changes in the secondary and tertiary structure of their own factor VIII molecule. This difference from the normal factor VIII molecule was sufficient for the normal factor VIII molecule to be considered antigenic by these patients. This finding also led to a change in clinical practice, i.e., presently, factor VIII concentrates are generally avoided in mild or moderately severe hemophilia and alternative drugs, such as desmopression, are used. These patients can develop factor VIII antibodies even after blood transfusion.

**Thrombophilia Genes**

The first case of hereditary thrombophilia was described long ago, in 1965, as antithrombin III deficiency, and a family with this deficiency was described by our group way back in 1982. Other causes of thrombophilia, such as protein C, protein S deficiency, were described from across the world, as well as from India, much later. Thrombophilia research got its impetus from molecular biology studies, which led to the detection of factor V Leiden, factor V Cambridge, factor V HongKong, PT G20210A polymorphism, fibrinogen gene polymorphisms, EPCR-23 base pair insertion, etc.

A few studies from India have shown the prevalence of many of these mutations and polymorphisms in Indian thrombosis patients and its striking difference, in some respects, from that in other parts of the world, i.e., absence of PT gene polymorphism, low factor V Leiden mutation in patients, and strong association of PAI 4G/5G polymorphism in recurrent fetal loss. This led to the concept that geography also, in some way, decides the prevalence of the thrombophilic gene in the local population. Molecular research in Glanzmann’s thrombasthenia in parts of our country also revealed the existence of many unique mutations, suggesting that one should look in the same genes for gain of function mutations predisposing to arterial thrombosis. Though platelet glycoprotein polymorphism may also be an important contributor.

**Interaction of Coagulation Factor Defect and Thrombophilia Genes**

As described earlier, severe hemophilia patients (i.e., those with <1% deficiency in factor levels) occasionally show a surprisingly milder clinical phenotype. Initially it was thought that some of these probably could be explained by a variant factor VIII molecule which gives less then 1% value by APTT-based assays but much higher levels of factor VIII by two-stage TGT-based assay. While this was found to be true for a small group of patients, in a larger series, up to 60% were found to have co-inherited one or more thrombophilia genes. In Western literature, factor V Leiden was found to be strongly associated with a milder clinical phenotype than was seen with severe factor VIII or IX deficiency, but in our study we found this effect across all thrombophilia markers. This finding has important prognostic implications: in early childhood, if a patient with severe factor VIII or IX deficiency has co-inherited thrombophilia gene, the parents can be counseled that the baby may have a milder course and it is possible that many of these children may not require the costly, prophylactic treatment that is now regularly prescribed for hemophilia patients.
It has also been observed that this ameliorating effect of thrombophilia is not restricted to hemophilia alone, but also to severe Glanzmann’s thrombasthenia where co-inheritance of the more thrombophilic PLA-1 b gene ameliorates the bleeding tendency.[36]

Molecular Insights into the Pathogenesis of Multiple Factor Deficiencies

**Combined factor V and VIII deficiency**

It was not easy to explain why two factors, i.e., factor V and VIII gene products, should be deficient in the same patient. These genes are not linked genes and they are located on totally different chromosomes. Factor VIII deficiency occurs in 1:10^3 births and factor V deficiency is know to occur in 1:10^6 births; if it were to happen purely by accident, these two deficiencies would coexist only in 1:10^9 births, i.e., 1 per billion population. But these two deficiencies occur much more frequently and families having this condition have been reported from across the world. The initial hypothesis about combined factor V and VIII deficiency was that it was due to the inheritance of an inhibitor to activated protein C,[37] but such an inhibitor was never detected. Modern molecular biologic studies in gene mapping mapped the locus for the disease to chromosome 18[38] in an Israeli family and this locus was found to contain a gene which was responsible for post-translational processing of factor V and VIII in endoplasmic reticulum and the Golgi apparatus. The gene which was called ERGIC-53, and is now known as the MCFD2 gene, is defective in these patients and causes retention of factor V and VIII in the Golgi apparatus/endoplasmic reticulum. As the MCFD2 defect could not explain all the cases of factor V and VIII deficiency, another locus for this disease called LMAN gene was detected.[39] We in India detected the first mutation of MCFD2 gene and a novel mutation of the LMAN1 gene in our combined factor V and VIII deficient families.

**Multiple vitamin K-dependent factor deficiency [MCFD1]**

This is one of the rare multiple coagulation defects where vitamin K-dependent coagulation factors are deficient. All over the world hardly 40-45 such cases have been described. The phenotypes of these patients are very variable. Some of them also have deficiencies of protein C and S. The patient we described had the additional problem of dysmorphism and mental retardation.[40] Two different gene products were found to be involved in causing such a defect, e.g., (i) vitamin K-dependent carboxylase and (ii) vitamin K-dependent oxidoreductase (VKOR-1).[41] Depending on whether the deficiency is in the carboxylase gene or the VKOR-1 gene, patients may or may not respond to high doses of vitamin K. VKOR-1 gene mutations/polymorphisms have also been linked to extreme resistance to vitamin K-dependent oral anticoagulants.

**Combination of severe factor XI deficiency and Bernard-Soulier syndrome**

We reported an unique family where factor XI deficiency and Bernard-Soulier (BS) syndrome were co-inherited.[42] The index case had no chromosomal abnormality and all patients with low levels of factor XI also had giant platelets in their peripheral smear and the GPⅠbⅩⅢ defect characteristic of BS syndrome. Analysis of this family suggests that this could be due to a mutation in the transcription factor common to transcription of factor XI in the liver and GPⅠb or GPⅠⅩⅢ in the megakaryocytes. This elusive transcription factor is yet to be described.

**Dominant Hemophilia now Classified by Molecular Biology**

Graham et al. reported a few families where factor VIII deficiency was found to be inherited in a dominant manner[43] so that both males and females were involved; there was apparently no defect in the von Willebrand factor in the family as detected by the then available techniques. Later on the defect was found to be a variant of the von Willebrand gene, with the disease caused due to loss or severe reduction in the binding activity of von Willebrand factor to the factor VIII molecule. Several phenotypic assays were developed to detect this defect and approximately 5% of von Willebrand disease patients in the West were found to have this kind of von Willebrand disease, known as the Normandy-type VWD or 2N-type VWD.[44]

We reported the first series of 2N-type of VWD patents from India and developed a relatively easy assay for this condition.[44] The assay needs some more modification to make it sufficiently sensitive. In our series, we found
that approximately 10% of our severe VWD patients have 2N-type VWD.

Other Coagulation Factors

Using molecular biology tools, detailed investigation of coagulation factors have revealed a multitude of defects, such as factor VII deficiency,[45] fibrinogen deficiency, i.e., fibrinogen - Mumbai;[46] prothrombin deficiency i.e. Prothrombin Vellore - 1[47] to name a few. In some of these defects, it was found that the active center of the enzyme had been compromised and in some, the secretion of the protein was compromised due to loss of the glycosylation site.

Prenatal Diagnosis

Prenatal diagnosis is important in the prevention of severe factor VIII or IX deficiency. Molecular biology has made it possible to detect the gene early in pregnancy so that abortion is possible without much harm to the mother. Several centers in India including ours have taken lead in prenatal diagnosis in hemophilia.[48-51] Some of the centers have even evolved technologies to diagnose prenatally von Willebrand disease.[52] Glanzmann’s thrombasthenia,[53] and other factor deficiencies.[54] As molecular biology techniques evolve and we develop a mutation database for various bleeding disorders in India, it will be possible to directly detect the mutation very easily with a combination of approaches. This needs close cooperation between the coagulation research centers and the various Hemophilia Chapters.

Conclusion

In this short overview I have tried to project, through several concrete examples of coagulation disorders, the important message learnt through molecular biology. It has been clearly shown at the level of the gene that every genetic disorder is likely to be heterogenous. Even if the disease-defining mutation is same, the phenotype of the disease could vary extremely due to complex interactions of synergistic and antagonistic gene products, a process known to geneticists as epistasis. Powerful modern techniques like microarray and proteomic analysis are likely to provide us with a more complete picture and allow us to determine the phenotype of a disease. Finally, we should not forget that the gene is not something fossilized in the tomb of the cell; rather a gene continuously modifies itself according to the information from the environment and can switch its action “on” or “off.”

India has a rich heritage in research in blood coagulation. Stalwarts like Prof. J.B. Chatterjea, Prof. A.K. Saraya, Prof. K.C. Das, Prof. Dipika Mohanty, Prof. Manorama Bhargava, Prof. B. Dub, Prof. N.N. Sen, Dr. S. Parekh, Dr. A.J. Desai, and Dr. Jaya Kasturi have led the way to the foundation of modern coagulation research in India. Prof. S.K. Sood, Prof. V. Ray, Prof. J.J. Jolly, and Prof. D.K.Bhattacharya standardized and led the foundation for blood component therapy in India. The names given above are indicative and not all inclusive.

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