Molecular Diagnostics in Coagulation

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1. INTRODUCTION

Coagulation testing has evolved from the use of the manual tilt-tube clotting time in the early 1900’s to the use of molecular diagnostics today. Over the years, the measurement of a clot endpoint has been the basis of testing. Automated instruments have replaced the manual visual methods and allowed for precise measurements of the clot endpoint. In addition, immunologic and chromogenic methodology has also been adapted into the coagulation laboratory, therefore providing an additional antigenic and enzymatic perspective. Today, most modern automated coagulation instruments offer a single test platform that incorporates optic, immunologic, and chromogenic methods. The arrival of molecular diagnostics now adds another dimension for the evaluation of hemostatic defects.

Historically, coagulation testing was confined mostly to the screening or work-up of patients with bleeding disorders. The availability of routine laboratory tests for patients with thrombosis has slowly increased over the years and the demand for this testing continues to grow. Molecular diagnostics has found an important role in this area of coagulation disorders. DNA-based tests are available for detection of the Factor V Leiden mutation, the Prothrombin 20120A mutation, and the methylenetetrahydrofolate reductase mutation.

At the same time, the significance of molecular testing for coagulation defects associated with thrombosis and the impact on patient care continues to be controversial. Physicians from different specialties are struggling with the questions surrounding the availability of a larger test “menu”: whom, when, and what to test? (1). Given the tendency to practice predictive medicine and the resulting increased screening of specific subgroups of patients for specific genetic information, it is anticipated that the role of molecular diagnostics for coagulation disorders will continue to increase with time (2).

In this chapter, a brief review of the procoagulant and anticoagulant systems will be followed by a general description of the molecular defects underlying various coagulation disorders. This, in turn, will provide a background for a review on the molecular genetic testing available in coagulation, particularly for the hereditary hypercoagulable states. Finally, the clinical significance of testing for Factor V Leiden, Prothrombin G20210A, and the methylenetetrahydrofolate reductase mutations will be discussed.

2. OVERVIEW OF COAGULATION

2.1. GENERAL CONCEPTS

The components of coagulation include the blood vessels, platelets, coagulation factors and cofactors, and the fibrinolytic proteins. As a result, hemostasis is a delicate balance between surface proteins on vessels/subendothelium, surface glycoproteins on platelets, procoagulant and anticoagulant proteins, and fibrinolytic proteins (3). The interaction among these components results in both fibrin clot formation and dissolution at the site of injury. Hereditary or acquired defects in any of these components can result in bleeding or thrombosis.

Hemostasis is generally viewed as two linked processes: primary and secondary hemostasis (4). Primary hemostasis, through platelet adhesion, activation, and aggregation, represents the platelet response to damaged endothelium and, at the same time, provides a template for the coagulation cascade. The coagulation cascade of proenzymes and cofactors is, in turn, referred to as secondary hemostasis. Together, these two processes form the basis of clot formation.

2.2. PRIMARY HEMOSTASIS

With damage to a blood vessel, the exposed collagen binds circulating Von Willebrand factor (VWF), which, in turn, functions as a “glue” between the subendothelium and the circulating platelets (5). Platelets adhere to the damaged vessel through the platelet receptor for VWF, glycoprotein Ib (GpIb), followed by platelet activation with release of ADP and serotonin, among others, from their storage granules. Platelet activation also results in expression of the surface receptor glycoprotein I Ib/IIa (GpIb/IIa), which, together with fibrinogen and other proteins, allows for platelet aggregation. Meanwhile, the presence of phosphatidylinerine on the surface of the platelet plug provides a negatively charged phospholipid surface, necessary for the subsequent formation of the fibrin clot (4).

2.3. SECONDARY HEMOSTASIS

Secondary hemostasis is characterized by the formation of a fibrin meshwork, which serves to reinforce the platelet plug. The system is comprised of a coagulation cascade of proenzymes and it’s activated forms,
along with cofactors and calcium (4). The procoagulants include the contact system proteins (factors XII and XI, high-molecular-weight kallikrein and kininogen), the vitamin K-dependent proteins (factors II, VII, IX, X), fibrinogen, the cofactors V and VIII, and factor XIII.

In vitro observations led to the traditional concept of two separate pathways involved in the generation of thrombin and the subsequent fibrin clot: the intrinsic and extrinsic pathways (3). In the intrinsic pathway, the components are found "inside the blood," best demonstrated when whole blood in a glass tube is left to clot by itself. In contrast, in the extrinsic pathway, clotting is initiated by tissue factor, which might originate from one of several sources located "outside the blood." The extrinsic pathway is generally considered the physiologic pathway in vivo while the intrinsic pathway provides a reinforcing mechanism (6).

The coagulation cascade is initiated when tissue factor binds to the small amount of circulating activated factor VII and this complex, in turn, activates both factors IX and X (3). Of note, the in vivo activation of factor IX by factor VII has replaced the traditional view of distinct intrinsic and extrinsic pathways. In the presence of cofactor VIII, activated factor IX will further activate factor X. Likewise, activated factor X, in the presence of cofactor Va, will subsequently convert prothrombin to thrombin, followed by the conversion of fibrinogen to fibrin.

Thrombin plays a central role in procoagulation: activating platelets, converting fibrinogen to fibrin, and providing positive feedback for further activation of cofactors V and VIII and factor XI. At the same time, thrombin also acts as an anticoagulant by activating the protein C system. Thrombin loses its procoagulant activity by binding to thrombomodulin present in most endothelial cell surfaces, followed by activation of the natural anticoagulant, protein C (6).

2.4. THE NATURAL ANTICOAGULANT SYSTEMS

Physiologic anticoagulant system(s) help keep thrombin formation in check, thus avoiding abnormal clot propagation. Tissue factor pathway inhibitor (TFPI), serine protease inhibitors (so-called serpins), the protein C system, and the fibrinolytic system are all necessary for the regulation of thrombin and fibrin formation (4).

The protein C system, through protein C, protein S, and thrombomodulin, plays a major anticoagulant role by inactiva-

The fibrinolytic system includes plasminogen, plasminogen activators and activator inhibitors, plasmin, and α2-antiplasmin (8). Plasminogen is activated mainly by tissue plasminogen activator (tPA) to form plasmin, which then degrades the clot. Plasminogen activation is confined to the fibrin clot, limiting the overall effect of fibrinolysis to the clot itself.

In sum, the physiologic balance of procoagulation and anti-
coagulation results in controlled thrombin formation limited to the site of vascular injury. A number of mutations and polymorphisms in the genes coding for these various proteins might result in increase risk of bleeding or thrombosis. A few of these genetic defects are now evaluable through molecular testing.

3. MOLECULAR DEFECTS UNDERLYING COAGULATION DISORDERS

3.1. POLYMORPHISMS AND MUTATIONS

Homologous areas of the genomic DNA contain variations within their nucleotide sequence. When this sequence variation has a greater than 1% frequency in the population, it is referred to as a polymorphism (9). These polymorphisms can occur in genes and gene-related sequences or noncoding extragenic DNA. Alleles represent polymorphisms within a single gene (10). When the sequence variation has a population frequency of less than 1%, this might be referred to as a mutation; however, some mutations might be present with >1% frequency (i.e., Factor V Leiden). Many different polymorphisms/mutations form the molecular basis of risk factors associated with coagulation disorders.

Different types of genetic polymorphisms, including single-nucleotide polymorphisms (SNPs), deletions, duplications, and insertions, have been variably associated with different coagulation disorders (11–15). With several genes of coagulation proteins now completely sequenced, a number of SNPs have been described, including point mutations in exons, introns, or regulatory regions (11). These mutations can result in loss of function (e.g., protein C deficiency) or gain of function (e.g., Prothrombin mutation 20210A) (11,14).

Not surprisingly, given their level of genetic complexity, coagulation disorders might be phenotypically heterogeneous. Many different mutations and polymorphisms might affect the same gene; there might be silent mutations or frameshift muta-
tions, the latter typically associated with a severe phenotypic abnormality (11). Levels of coagulation factors might vary depending on the type of polymorphism or mutation (14,16). This degree of genetic complexity remains a challenge for the molecular diagnostics laboratory.

The genetic complexity in coagulation disorders is further magnified by the interaction between different genes (17) and between genes and the environment (18). Although mutations in several different genes might be associated with hereditary thrombophilia, the age of onset, severity of the disease, and penetration are highly variable. Factor V Leiden has a high prevalence in selected ethnic groups; approx 3–7% of the white population of northern European ancestry are heterozygous carriers, but the disease has a relatively low penetration (19). In contrast, a condition with a low prevalence such as the homozygous state for protein C deficiency might be associated with a severe clinical syndrome, purpura fulminans, in the newborn (20). In the vast majority of adult patients, venous thrombosis most likely represents the inter-
action of environmental and genetic factors (18). The inter-
action with acquired risk factors compounds the risk of venous thromboembolism; a single gene defect alone might have a limited role in thrombosis, but in combination with known acquired risk factors, the two circumstances together might significantly increase the risk for disease (18).

3.2. MOLECULAR DEFECTS IN BLEEDING DISORDERS

The clinical use of molecular diagnostics apply mainly to thrombophilic disorders, however, the molecular defects under-
lying both bleeding and thrombotic disorders are similar.