A calibrated automated tool to assess the thrombotic-haemostatic system.

by Dr P. Giesen

Thrombosis is the leading cause of death in the West; world-wide, someone dies from arterial thrombosis, venous thrombo-embolism, stroke or other manifestation of this disease every second. On the other hand, we all survive regular minor injuries due to the complicated but effective system that keeps the blood fluid inside the vessels but causes it to clot at the site of a damaged vessel. Hence the thrombotic-haemostatic system is a major cause of mortality as well as being essential for survival. Biochemically there is no difference between thrombosis and haemostasis. Both result in the appearance and consolidation of a clot at the site of an injury or, in case of thrombosis, on the inside of a vessel wall that has pathologically changed so that it appears to be injured. This injury stimulates the system to respond. Blood at a wounded vessel comes into contact with exposed Tissue Factor (TF), severed cells with negatively charged phospholipids, and collagen. A mixture of TF and phospholipid membrane, also called thromboplastin, was formerly purified from brain tissue and used to trigger the in vitro clotting of blood or plasma.

Testing for thrombosis-haemostasis

For many years a simple test has been used, in various formats, to get a picture of the thrombotic-haemostatic system. This test requires the addition of a trigger, such as thromboplastin, to make the blood clot, and the time it takes for the clot to appear is known as the clotting time. However, clotting times are generally not very accurate and do not give much information; indeed they do not give the complete picture. It is important to have a test that can do so; subjects who have blood with a weak response to thromboplastin may suffer from haemophilia, whereas subjects with an abnormally strong response may have an increased thrombotic tendency. During the process of evolution it was more important to survive injury and childbirth than thrombotic disease, which occurs mostly in older (post gestational) age groups. This has made the clotting system "trigger happy" with the result that thrombosis is a very common disorder.

If an obese 50-year old man presents to a doctor complaining of thirst, frequent urination and fatigue, the doctor would provisionally diagnose diabetes and request a test for blood glucose level. In this case the measurement of blood glucose is a 'function test' of the ability of the patient's system to maintain glucose concentration within narrow limits. Is there a 'function test' for thrombosis and haemostasis? A plethora of information has been published that gives an insight into elevated thrombotic risk; factors such as smoking, high blood pressure, obesity, high cholesterol and poor eating habits are known to increase risk. However, a doctor would not prescribe insulin based on symptoms - s/he would always wait for test results. Similarly a patient would not be treated with anticoagulant drugs merely on the basis of having a number of risk factors. Sophisticated tests are available, e.g. for the determination of genetic disorders (factor V Leiden mutations, hyperprothrombinaemia, homocysteine mutations), and for activity and epitope of all clotting factors and anti-clotting factors. These however are not 'function tests' and may give circumstantial evidence for thrombotic risk but not sufficient proof to start treatment. The only 'function tests' available are clotting tests e.g. activated partial thromboplastin time (aPTT), International Normalised Ratio of the Prothrombin Time (INR), Ecarin clotting time, thrombin time and thromboplastin time. These tests are not capable of measuring increased thrombotic risk and are mostly used to monitor treatment with antithrombotic drugs. In addition, these tests are not general; one test does not suffice to monitor all of the available treatments or detect every thrombotic or haemostatic disorder. This means that doctors have to wait until a thrombotic event or a haemorrhage occurs. If the patient survives, the appropriate treatment can be initiated, but this is too late for the many who do not survive.

It would be preferable if there were a test that could detect increased as well as decreased clotting ability. This test would need to be sensitive to any antithrombotic treatment, any known or unknown pathology of the platelets or plasma, and any drug treatment or combination of treatments. The result would be a general 'function test' providing a reliable and global picture of the thrombotic-haemostatic system.

The thrombotic-haemostatic system

Bleeding occurs when a vessel wall is damaged so that it cannot contain the stream of blood passing through it. Exposure to a wounded or pathologically changed vessel triggers the blood to clot. For a wound this occurs during repair, but in the case of thrombosis the inappropriate response causes an obstruction to an otherwise intact vessel. In both cases a common set of events occurs. Firstly platelets adhere and activate, which exposes an increased negatively charged membrane area to which vitamin K-dependent coagulation factors bind. Thrombin is the pivotal enzyme in coagulation. It enables the production of more thrombin but also initiates a chain reaction that gradually causes its production to cease. A review of all the reactions involved in the whole system of coagulation will not be covered here, but it is essential to be aware of the central role of thrombin in order to understand why a measurement of its concentration over time during the reaction gives all the information necessary to provide a full picture of the system.

The schematic diagram [Figure 1] shows the core of the coagulation system. Tissue Factor, present in the deeper layers of the vessel wall, becomes exposed to the blood upon injury. It binds to factor VII, which when activated becomes factor VIIa and forms the TF:VIIa complex that initiates production of the first traces of activated factor X. Activated factor X (factor Xa) then leads to the production of thrombin from prothrombin. Thrombin activates factor V to factor Va, but also activates platelets (as mentioned above) thus providing the phospholipid surface on which the vitamin K dependent coagulation factors bind. The newly formed factor Va binds to the existing factor Xa on the platelet surface and this complex is called the 'prothrombinase' complex.
As soon as prothrombinase is formed, the production of thrombin is increased by several orders of magnitude. This clearly cannot continue indefinitely, otherwise one small injury would result in complete stasis of blood. It should be confined to the site of injury and should produce just sufficient clot to repair the leak. Therefore thrombin terminates its own formation through an indirect pathway. Thrombin binds to thrombomodulin (TM) and the TM-thrombin complex activates protein C. This activated protein C (APC) inhibits factor Va which forms factor V. Because this inactivation is indirect, it offers the system a window of time to produce a burst of thrombin, which converts fibrinogen into fibrin (the actual clot) and at the same time produces sufficient TM-thrombin complex to inhibit prothrombinase. This mechanism of rapid, direct activation and slower indirect inactivation is also seen with the inhibition of the VIIa-TF complex. Factor Xa is formed directly, whereas the inhibition of the VIIa-TF complex requires the formation of an Xa-TFPI (Tissue Factor Pathway Inhibitor) complex from factor Xa.

Monitoring treatment with antithrombotic drugs
When the coagulation system is too “trigger happy” such as when it responds too readily to vessel wall irregularities, it can be down-regulated through drug administration. These drugs have one common result, they all influence thrombin production. Three common drugs, i.e. heparin, aspirin and coumarin, all affect the system in different ways. Heparin is administered by injection (either subcutaneous or intravenous) and binds to anti-thrombin (AT, present in plasma). AT can directly inhibit thrombin; this inactivation is much more effective when combined with heparin. Aspirin inhibits platelet activation so that less pro-coagulant surface, to which the vitamin-K dependent coagulation factors bind, is available. The orally administered coumarin derivatives block carboxylation of these proteins so that they are unable to bind to activated platelets. Drug monitoring can ensure adequate treatment. However, these three different drugs require different tests: heparin is monitored by the aPTT, aspirin can only be monitored via its influence on platelet aggregation tests and coumarin must be monitored by the INR. The aPTT does not respond to aspirin or coumarin and the INR does not respond to aspirin or heparin. Apparently each test measures something different and does not measure the key parameter of the system. All three drugs however do influence thrombin generation: heparin treatment causes more rapid disappearance of thrombin, aspirin slows down and decreases prothrombinase formation, and coumarin produces “disabled” coagulation factors such as factor X, prothrombin and factor VII. The final result in all cases is less thrombin.

The measurement of thrombin generation
Monitoring the concentration of thrombin in clotting plasma following the addition of a trigger (diluted thromboplastin), better known as measuring the Thrombogram [Figure 2], used to be a laborious technique in which samples were taken manually every 20 seconds from a tube containing clotting plasma. The amount of thrombin in these samples was measured in a photometer using a chromogenic substrate. Later, in the early nineties, it was demonstrated that a chromogenic substrate with the right properties could be added to the plasma directly, and the velocity of conversion of the substrate by the increasing and decreasing thrombin concentration in time could be followed in a photometer. This technique was much easier to carry out but had the drawback that clot formation disturbed the measurement. This meant that either fibrin formation needed to be inhibited or fibrinogen needed to be removed prior to the experiment. This made the assay unsuitable for measurements using platelet-rich plasma and also overlooked the important role of the activity of thrombin bound to fibrin.

Fluorogenic substrate
The introduction of a fluorogenic substrate in 2002 was a great step forward because it can be used in turbid mixtures in which all components of the thrombotic-haemostatic system are present. The fluorogenic substrate is added to clotting plasma and converted by thrombin into the fluorophore Amino Methyl Coumadin (AMC). This is fluorescent and the increase in AMC concentration is followed in a fluorometer. However, it has been found that the fluorescent signal not only depends on the amount of thrombin but also depends on the colour of the plasma, which can vary greatly between donors, the instrument used and the age of the lamp and filters. In addition the signal is also influenced by two other effects. Firstly, when thrombin is measured together with fluorogenic substrate in buffer, it produces a curved graph. The reason for this curve is that the substrate is being consumed by thrombin. This lowers the concentration and velocity at which thrombin converts the substrate. Secondly, the relationship between the concentration of AMC and fluorescence is not linear, therefore at higher AMC concentrations the amount of fluorescence is relatively lower - this is called the ‘inner filter’ effect. The combination of these effects causes the system to “saturate”: more thrombin does not give more signal. When these effects are not taken into account, the system produces curves that perfectly resemble a Thrombogram, but which on closer inspection contain disturbing artifacts.

Normally when a signal resulting from an unknown amount of enzyme is measured, the experiment should be repeated with a known amount...
of enzyme to establish the relationship between signal and enzyme. However thrombin cannot be added to plasma because it would immediately start thrombin formation, form a clot and be inhibited by anti-thrombin. Nor can thrombin be added to a buffer or water because then the donor-to-donor differences in plasma colour would not be taken into account.

The calibrated automated thrombogram
Research by Professor Hemker’s group in Maastricht has lead to a modified thrombin with known activity that does not interact with any plasmatic substrate or inhibitor - the ‘Thrombin Calibrator’. With this new chemistry, the plasma sample is divided into two. Measurement of thrombin generation, i.e., the clotting reaction, is carried out with one sample, and ‘Thrombin Calibrator’ is added to the other. The fluorogenic substrate is added to both samples and the curved line of the Thrombin Calibrator is first converted into a straight line to eliminate the substrate consumption and inner filter effect. The parameters that are needed to correct for these effects are then applied to the fluorescence values derived from the thrombin generation wells [Figure 3]. The first derivative is then taken from this corrected signal. Thrombin disappears from plasma through two main pathways: binding to AT and binding to alpha2-Macroglobulin. The latter large molecule wraps around thrombin and prevents it from binding to any of its natural substrates. However, this complex is still able to convert the fluorogenic substrate, and its activity should be subtracted from the curve so that finally, nanomoles of thrombin over time can be presented.

The Calibrated Automated Thrombogram (CAT) assay, marketed by Thrombinscope BV (www.thrombinscope.com), is designed for running on a Thermo Electron (www.thermo.com) Fluoroskan Ascent connected to a personal computer. The Fluoroskan Ascent [Figure 4] is an advanced microplate fluorometer with high sensitivity for a wide variety of fluorometric applications. For the CAT it comes equipped with an on-board dispenser, an incubator and a shaker, and allows the measurement of a whole 96-well plate within 20 seconds, a requirement for the CAT method, making it the only fluorometer on the market suitable for measuring the Thrombogram. Data interpretation and presentation is processed by the Thrombinscope software package [Figure 5].

Conclusions
The chance of having a heart attack, a stroke or other disease related to thrombosis is largely determined by how well a person’s blood clots. The easier it is to trigger the coagulation system, the higher the thrombotic risk. For this reason the Thrombogram surpasses any existing test in providing an overall picture of blood coagulation.

The Calibrated Automated Thrombogram system provides a sensitive measurement of thrombin generation in plasma samples with all the natural components affecting blood coagulation present. The automated technique is based on fluorescence and, therefore, is not affected by the appearance of a clot or the presence of platelets. The system offers speed, automation and an increased throughput of up to 30 samples per hour. Additionally, the user-friendly software together with calibration of the system enables data standardisation and easy comparison between studies. The CAT system is an excellent solution for research laboratories monitoring any antithrombotic drug or drug combination.

Bibliography

The author
Peter Giesen, MD, Ph.D.,
Thrombinscope,
PO Box 616,
6200 MD Maastricht,
The Netherlands
Fax +31 43-356 7899