EVALUATION OF THROMBOGENIC EFFECTS OF DRUGS

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GENERAL CONCEPTS

More than 100 years ago the German pathologist Rudolf Virchow recognized that pathological changes of blood vessel walls, thrombotic diathesis, and altered blood flow were the major causes of thrombosis (1). It is remarkable that no fundamentally new pathogenetic factors have since been added to what is now known as *Virchow's triad.* In recent years biochemical and morphological studies have unraveled many of the processes taking place during hemostasis. As a result, we have also gained a better understanding of the mechanisms contributing to thrombus formation.

The notion that drugs play an important role in the development of thrombosis has gained widespread attention. Extensive investigations undertaken in an effort to explain the apparent thrombogenic effect of steroidal oral contraceptives (OC) have revealed a number of unusual toxicological problems:

1. Few chemical substances are known to cause outright thrombosis. However, many agents seem to increase the risk of thrombosis, that is, they create a state of impending thrombosis or thrombophilia, an ill-defined pharmacological endpoint.
2. Drugs may influence several factors involved in thrombus formation.
3. There are at least three fundamentally different forms of thrombosis, arterial and venous thrombosis, and disseminated microcirculatory thrombosis. Drugs may promote one or more of these forms to a variable degree.
4. Most laboratory methods were originally designed to measure defective blood clotting and platelet function. They are not well suited for the demonstration of impending thrombosis.
5. In man, the recognition of a thrombogenic drug effect often rests on epidemiological data. Serious problems of diagnosis, and the existence of disease-related and environmental factors that influence the incidence of thrombosis, render such studies difficult.

Mechanisms of Thrombogenic Drug Actions

It is generally acknowledged that all processes involved in normal hemostasis are also operative in thrombus formation. The physiological and pathological events most likely to be set in motion by thrombogenic substances are listed in Table 1. Remember, however, that the hemostatic equilibrium is maintained by many checks and balances. In some cases a drug-induced shift in a direction favoring coagulation invariably results in thrombosis. In other instances it may merely indicate that the statistical risk of developing thrombosis, however small, is higher than in the absence of the drug. In order to emphasize these differences, a somewhat arbitrary classification that recognizes *thrombogenic substances of the first, second, and third order* was proposed (2): 1. The principal biological action of a thrombogenic substance of the first order is a distinct activation of the hemostatic system, which is usually followed by thrombus formation. Such substances are not used as drugs, but serve as experimental tools to study mechanisms of thrombosis. 2. Thrombogenic substances of the second order also activate hemostatic mechanisms, but thrombosis is only caused at excessively high doses. 3. Thrombogenic substances of the third order include all compounds that shift the hemostatic equilibrium in the direction of thrombus formation without causing outright thrombosis, even at very high doses.

Table 1 Possible mechanisms of thrombogenic substances

Vessel wall	Platelets	Coagulation
Cytotoxic and irritant effect	Symptomatic thrombocythemia	Release of tissue thromboplastin
Generalized deendothelialization	Aggregation	Increased concentration of clotting factors
	Sensitization against ADP and serotonin	Activation of clotting factors
	Increased adhesiveness	Decreased antithrombin III
Blood flow	Reduction of surface charge	Inhibition of fibrinolysis
Vasoconstriction	Reduction of cAMP	
Stasis and hypotension	Induction of release reaction	
Turbulence		
Distension of vessels		

THROMBOGENIC SUBSTANCES ACTING ON THE VESSEL WALL

Cytotoxic and Irritant Effects

Perhaps the most frequent form of iatrogenic thrombosis is that occurring at injection sites. Prerequisites are localized vascular lesions where blood can interact with

subendothelial extracellular structures (3), and inflammatory reactions stemming from perivascular deposition of cytotoxic agents.

In order to test for thrombus induction, drugs are injected into a blood vessel that is temporarily occluded to permit the chemical to interact with the endothelium. Blood flow is then restored, and thrombus formation is assessed by visual inspection and histological evaluation. Chemicals may also be applied on the outer layers of a blood vessel. The rate of thrombus formation may then be measured by direct microscopic observation. York, Rogers & Kensler (4) have used this method on blood vessels of the hamster cheek pouch for an evaluation of new anticancer drugs of the phthalanilide series. The thrombogenic potency of three derivatives correlated well with incidence of thrombophlebitis observed in patients.

Generalized Endothelial Damage

That chemical substances may be responsible for widespread endothelial defects was learned from the study of homocystinuria, an inborn error of metabolism. These patients experience frequent episodes of arterial thromboembolism that result from parietal thromboses at the site of patchy deendothelializations. The same lesions were produced in baboons by intravenous (iv) infusion of homocystine (5). This could be diagnosed intravitam by demonstration of circulating endothelial cells. Such cells were also found after injection of endotoxin in rabbits (6), indicating that the potent thrombogenic effect of this agent is, at least in part, a result of endothelial damage.

Another method was used for the demonstration of endothelial lesions induced by polyanethol sulfonate (Liquoid ®, Roche). Rats were treated with this potent thrombogen; the aortas were then incubated in a medium containing tritiated thymidine. Areas of intensive endothelial repair could be demonstrated by autoradiography (7). An uncommon form of endothelial damage was found with sodium acetriozate, a radiocontrast agent. This compound caused disseminated thrombosis in veins and capillaries, even in the absence of platelets and all coagulation factors except fibrinogen. This was explained by the ability of acetriozate to extract a glycoprotein from the endothelium and to form an insoluble fibrinogen derivative (8).

THROMBOGENIC SUBSTANCES ACTING ON THE PLATELETS

Increased Platelet Count

Frequent thrombosis and bleeding is a well-known syndrome in patients whose platelet count exceeds 10^6 per mm^2. Testosterone, progesterone, somatotropic hormone, serotonin, and pantetine (a combination of pantothenic acid and cysteamine) caused a marked increase in platelet count in experimental animals (9). This indicates that drugs can induce symptomatic thrombocythemia. The effect was also seen in man, quite unexpectedly, with vinblastine and vincristine (10, 11).

Platelet Aggregation

Aggregation of platelets is a key step in the process of hemostasis and thrombus formation. It is readily induced by several naturally occurring substances, thrombin,

adenosine diphosphate (ADP), collagen, epinephrine, serotonin, fatty acids, and a cyclic endoperoxide formed from arachidonic acid during prostaglandin biosynthesis (12–14). The crucial alterations of platelets that make them aggregable are not known, but the kinetics of the process and the morphological changes of the cells are well studied (15).

Other chemical substances act less specifically, perhaps by direct binding to cell membranes (16), cell damage, or change in surface charge. Some compounds cause loose, reversible aggregates without impairment of platelet function. With others, aggregates are irreversible and associated with loss of function or platelet destruction (17). This qualitative difference is often overlooked, although it appears to be of considerable importance. Irreversible platelet aggregates are retained in the capillaries of the lungs as microemboli, leading rapidly to thrombocytopenia, often also pulmonary hypertension and death (18–20). Induction of reversible aggregates is usually not associated with a drop in platelet count. This indicates that most of the platelets are not sequestered in the capillary bed.

Many methods are available for in vitro testing of platelet aggregation, including turbidimetric measurements (21), microscopic observation of platelets in suspension (18, 22) or on plastic slides (23), and counting of unaggregated platelets in platelet-rich plasma (PRP) or blood before and after contact with the test substance (24, 25).

A drug found to aggregate platelets in vitro must be tested in vivo. Circulating aggregates may be seen as white bodies in exposed and illuminated blood vessels (26, 27) or in a glass tube bypass (28). The blood may also be passed through a filter where aggregates are retained and lead to an increase in filter pressure (29). Determinations of platelet survival give useful indirect information about the rate of platelet destruction following administration of aggregating agents.

A simple method consists of counting aggregates in blood samples suspended in buffered EDTA solutions (18, 30). It was used to demonstrate circulating platelet aggregates developing after iv injection of 2 azo dyes, Evans blue and Congo red, into guinea pigs.

The blood concentration necessary to cause formation of aggregates was determined and compared with blood levels reached during use of the agents in man. From these determinations a satisfactory "safety factor" was calculated for Evans blue, and a marginal one for Congo red (18, 31). It is noteworthy that several cases of shock and disseminated thrombosis have occurred after iv use of Congo red in man (32). These reactions were most likely due to drug-induced microembolization.

The antibiotic ristocetin also aggregates human platelets in vitro and causes dose-dependent thrombocytopenia in rabbits and man (33). It is interesting that this compound does not aggregate platelets of many von Willebrand patients. It thus needs a plasma factor, a property not shared by other aggregating agents (34–36).

As a further mechanism of thrombogenic action the possibility that drugs may sensitize platelets against endogenous aggregators must be considered. For example, platelets of women taking OC exhibit an enhanced response to ADP (37). A significantly increased aggregation response with serotonin was observed in psychiatric patients treated chronically with chlorpromazine (38). In vitro this drug reduces rather than promotes platelet aggregation. From these findings it is concluded that

potential drug-induced enhancement of platelet aggregation must not only be investigated in vitro but also during chronic administration.

Increased Platelet Adhesiveness

The major factor responsible for adhesion of platelets to vessel walls is endothelial damage with exposure of collagen fibers and other subendothelial structures (3). The belief that increased stickiness of platelets also promotes this process is based on the finding that platelets of certain thrombosis-prone patient populations have a higher tendency to adhere to foreign surfaces. (39–42). Many test methods have been proposed, using glass slides (39), or beads (43), latex particles (44), traumatized rat omentum (45), and tannic acid–treated human red cells (46) as structures for the platelets to stick on. Strong platelet aggregators, ADP, epinephrine, thrombin, and Evans blue, promote platelet stickiness. One could thus conclude that aggregation and adhesion are manifestations of the same process, so that there would be no need to use the cumbersome adhesiveness tests. There is, however, evidence that the two reactions are not identical: platelets of patients with Glanzmann's thrombasthenia fail to aggregate but adhere normally to subendothelial structures (47). Moreover, serotonin, a potent aggregating agent, did not enhance platelet adhesiveness to tanned red cells (46).

In order to test platelet adhesiveness in vivo, discrete vascular lesions must be induced, for example, with electric current (48), biolaser (49), or iontophoresis of ADP (50). Thrombus formation and embolization are assessed by direct observation. A drug that increases platelet adhesiveness is expected to produce larger thrombi earlier and to prolong microembolization. In a rat experiment in which venous thrombus formation was initiated by a mechanical lesion, urethane, in anesthetic concentrations, was indeed shown to prolong production of microemboli significantly (51).

Changed Platelet Surface Charge

From studies of pH-mobility relationship of platelets in vitro we are well informed about their electrical surface charges and the chemical groups with which they are associated (17). Normally there is a net negative surface charge that keeps platelets separated from each other. Positively charged macromolecules such as polylysine, polyornithine, protamine sulfate (17) were shown to bind to platelets and to cause aggregation when the electrophoretic mobility reached zero. Polyquaternary, a long-chain detergent increased adhesiveness to electrically stimulated blood vessels (52).

Reduced Cyclic AMP

A new concept of platelet function is based on the observation that prostaglandins stimulate adenylcyclase and increase cAMP content of platelets. The aggregation response of these platelets with ADP, arachidonic and other agents is reduced, and this effect is further enhanced by inhibitors of phosphodiesterase, for example, theophylline (14, 53, 54). In vitro ADP and epinephrine cause a rapid drop in platelet cAMP content. This has led to the suggestion that the aggregation response

was directly related to the cyclic nucleotide level. If this were the case any influence leading to a reduction of platelet cAMP might be expected to facilitate aggregation.

Release of Platelet Content

The release reaction (55), a liberation of ADP, serotonin, histamine, and enzymes, is set in motion under the influence of several endogenous platelet-aggregating substances, for example, thrombin and epinephrine (56). Of particular importance is the release of ADP which is probably the common effector agent responsible for the aggregating effect of long-chain free fatty acids (57), the cephalin preparation Thrombofax® (58), immune complexes (57), heparin (16), and vasopressin (59). The release reaction is of considerable toxicological importance, since it promotes further growth of platelet aggregates lodged in small blood vessels or deposited on vascular lesions.

Induction of release reaction is conventionally studied in vitro: PRP and test drug are stirred, and the released substances are determined in the plasma fraction (60). Platelets may also be labeled with ^{14}C-serotonin, which they release promptly under the influence of aggregating agents (61). In vivo studies of the release reaction are more difficult, because of the rapid metabolic disposition of the released substances.

THROMBOGENIC SUBSTANCES ACTING ON THE COAGULATION SYSTEM

A description of the intricate biochemical reactions taking place during blood clotting is outside the scope of this review. In the following paragraphs experimental and clinical evidence is presented that demonstrates that chemical substances can create a state of hypercoagulability which may progress to thrombosis. It will also become evident from the examples cited that not every shift in the biological equilibrium of the clotting system represents a significant thrombosis hazard.

Release of Tissue Thromboplastin

Thromboplastic substances, also called *tissue damage factors,* are present in most tissues. They are important as activators of the extrinsic clotting system. It is conceivable that irritant drugs injected into adipose and muscle tissue may cause enough cell damage to make appreciable amounts of such thromboplastic substances available.

Increased Concentration of Clotting Factors

The plasma level of many clotting factors is increased in women taking OC (37), but the significance of this finding is not well understood (62, 63). For the toxicological evaluation it is permissible to assume that increased levels of one or more clotting factors must not be regarded as indicating a state of hypercoagulability. After all, even under normal circumstances these are substances present in excess. Still, a drug causing marked and consistent elevation of one or several clotting factors must be subjected to careful scrutiny.

Acceleration of Clotting Reactions

Epinephrine is a typical example of a thrombogenic substance of the second order, causing fibrin thrombi in rabbits, but only at excessively high doses (64). It accelerates blood clotting in vitro (65) and in vivo (66) and causes an increase in Factors VIII and IX levels (66). Since it promotes clotting also in patients with hemophilia A (67) and in Factor IX–deficient dogs (66) it was suggested that it acted by a generalized catalytic influence on enzymatic processes (66). Several drugs release epinephrine from its body stores. It is conceivable that they also accelerate the clotting process. In rabbits the two hypotensives guanethidine and debrisoquin, known to release epinephrine, caused a transient state of hypercoagulability. The same effect was seen with tyramine, a pressor substance that also acted through epinephrine release (68).

Activation of Contact Factors

The generation of intrinsic thromboplastin is set in motion by activated Hageman factor and Factor XI (PTA). The presence of activated contact factors in the blood is demonstrated by shortened clotting time in siliconized glass tubes, decrease in partial thromboplastin time, acceleration of the thromboplastin generation test, and shortened thrombus formation time as measured with the Chandler loop technique (2, 69). It represents a state of hypercoagulability, but does not necessarily lead to thrombosis (70, 71). An important reason thrombosis does not always develop is probably related to the ability of the reticuloendothelial system of the liver to eliminate thromboplastin from the circulation (72). If this protective mechanism is impaired, the risk for thrombosis through activation of contact factors is increased.

Prolonged and intensive activation of contact factors may produce thrombosis without a contribution of other factors. For example, iv infusion of lactic acid into rats caused complete activation of Hageman factor, arterial thrombosis, and microembolization in the lungs (73). Similarly, polyinosinic-polycytidylic acid, a potent activator of contact factors (2), caused severe thrombosis when injected at high doses into dogs and monkeys (H. Levy, personal communication). Long-chain FFA are potent activators of contact factors (74, 75). This may be one of the reasons they cause thrombosis after iv injection (19, 24, 76, 77). Elevated FFA levels may also be produced by drugs, for example, catecholamines, anorexigenic phenethylamines (78), thymoleptics (78), ACTH (79), and nicotine (80). Fortunately, released FFA are bound by serum albumin, so that only very high levels, exceeding the binding capacity of the available serum albumin, may become hazardous. That such high FFA levels can cause thrombosis was demonstrated in experiments with rabbits that were given large doses of ACTH and died from pulmonary thrombosis (79).

Decreased Antithrombin

In 1965 Egeberg (81) described the occurrence of low antithrombin III levels in members of a family. The patients attracted attention because of frequent venous thrombosis, thrombophlebitis, and pulmonary embolism. This was confirmed in other families so that no doubt exists at present that antithrombin III is a very

important protective factor against intravascular clotting. It is understandable, therefore, that the finding of low antithrombin III levels in women taking OC attracted great attention (82, 83). It was also reported that the effect seemed to be due to the estrogen component of these drugs (84). There is some indication in the literature that women with the lowest antithrombin III levels are most likely to develop thromboembolic complications (84). However, the available data are not sufficient to permit a final evaluation of this remarkable observation. But of all changes induced by the OC, the effect on antithrombin III appears to be one of the most logical explanations of their suspected thrombogenic effects.

Inhibition of Fibrinolysis

The fibrinolytic system is the ultimate weapon against the consequences of intravascular coagulation. There is much experimental and clinical evidence that drug-induced inhibition of fibrinolysis represents a considerable hazard: just about every experimentally induced form of thrombosis takes a much more serious course if the animal was pretreated with a plasminogen antiactivator, e.g. ϵ-amino caproic acid and tranexamic acid, or a proteinase inhibitor, e.g. aprotinine and iniprol (85). In man such compounds were occasionally associated with thrombosis, particularly in patients with kidney disease (86–88). Corticosteroids, long suspected to increase the incidence of thrombosis, also inhibit fibrinolysis. The detrimental consequences of prednisolone pretreatment were shown in rabbits with thrombin-induced intravascular coagulation (89). Another group of compounds inhibiting fibrinolysis comprises mercurial derivatives, e.g. mercuric chloride (90) and the diuretic Novurit® (91).

THROMBOGENIC SUBSTANCES INDUCING CHANGES IN BLOOD FLOW

Vasoconstriction

Arterial thrombosis as a consequence of prolonged vasoconstriction is found in patients with ergotamine tartrate overdosage (92). It is probable that a lesion of the vessel wall develops as a consequence of the compression of the vasa vasorum during spastic contraction of the artery (93). Another example of thrombosis in a spastic blood vessel is that reported to occur after infusion of pitressin into the superior mesenteric artery (94).

Stasis and Hypotension

The importance of venous stasis as a contributing factor in venous thrombosis is generally accepted, but more on circumstantial evidence than on hard, experimental facts. It is proven experimentally that stasis alone does not produce thrombosis (95, 96). But if a state of hypercoagulability exists, thrombosis will develop preferentially in blood vessels with slow blood circulation (95). Drugs that cause venous stasis must therefore be considered as accessories to such an event. As an example, the OCs must again be cited. They are known to cause venous stasis in the lower extremities (97), an effect that may contribute to their thrombogenic potential.

A sudden lowering of arterial blood pressure, a possible side effect of autonomic blocking drugs, also reduces the blood flow in the periphery. It is noteworthy that formation of platelet aggregates and intravascular coagulation are well recognized consequences of hypovolemic hypotension (98). Further experiments in dogs proved that circulatory failure due to hypercapnia was associated with thrombosis in various organs (99). It is thus quite clear that various disturbances of the peripheral circulation must be considered as potential triggers for a thrombotic process.

Turbulence and Distension of Blood Vessels

Experimental evidence shows that platelet adhesion develops preferentially in regions of disturbed arterial flow, and that the size of the platelet deposits is related to the intensity of the turbulence (100). This indicates that a sudden elevation in blood pressure, which might occur after the injection of a sympathomimetic drug, could create a hazardous situation by increasing turbulence in areas of disturbed arterial flow (bifurcations, atherosclerotic plaques). Moreover, acute hypertension leads to hyperextension of blood vessel. This may damage the endothelial layer of the arteries and facilitate platelet deposition (101).

CONCLUSIONS

The investigation of the pathogenesis of thrombosis was greatly facilitated by the availability of chemical substances with which various forms of intravascular coagulation and thrombosis could readily be induced. Using these *Thrombogenic Substances of the First Order* as experimental tools, it was possible to identify various mechanisms of chemical thrombosis and to develop in vitro and in vivo methods for detection and quantitative assessment of thrombogenic effects. The mechanisms found to be most important for induction of experimental thrombosis included endothelial lesions, platelet aggregation, changes in platelet surface charge, activation of certain clotting reactions, and inhibition of fibrinolysis. Several other factors such as increased platelet counts and platelet adhesiveness, reduction in cAMP content of platelets, induction of platelet release reaction, increased sensitivity of platelets for aggregating action of ADP and serotonin, release of tissue thromboplastin, decreased antithrombin levels, increased concentration of clotting factors, and various changes in blood flow were also identified as being involved in thrombus formation. Most of these processes were shown to be susceptible to changes induced by chemical substances.

A second group of chemical compounds, *Thrombogenic Substances of the Second Order,* caused changes of hemostatic mechanisms similar to those described above. In most instances the compounds only produced a prethrombotic state, a change in the hemostatic equilibrium sometimes also referred to as hypercoagulability, impending thrombosis, thrombotic diathesis, or thrombophilia. But the fact that administration of excessively high doses of these agents was often followed by thrombosis represents a good argument that the changes of hemostatic functions are indeed valid indicators for an increased tendency to develop thrombosis and thromboembolism.

Finally, a sizable group of chemicals including many drugs were recognized as having some influence on hemostatic mechanisms, shifting the balance in the direction of thrombosis. Even at excessively high doses these compounds do not by themselves induce thrombosis. But it is possible that they represent a risk factor whose clinical significance must be determined by epidemiological investigations.

The OC may be mentioned as a prototype of these *Thrombogenic Substances of the Third Order.* Their varied effects on vasculature, platelet function, and clotting mechanisms indicate a distinct tendency to shift the hemostatic equilibrium in favor of thrombosis (37). But the actual occurrence of thrombotic episodes under the influence of these drugs is still a rare event. The evaluation of drugs with methods described in this review thus serves the purpose of identifying potential, however weak, thrombogenic properties of new and commonly used drugs. This knowledge should not discourage the therapeutic use of these compounds. But it must caution the clinician against potential hazards and provide him with useful directions on the methods to be used for monitoring of patients.

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