Chapter B
Pharmacological Assays in Thrombosis and Haemostasis[1]

[1] Contributed by Shaker A. Mousa based on contributions by M. Just and V. Laux.

B.1 General Introduction

Cardiovascular, cerebrovascular, and venous thromboembolic disorders continue to be the leading causes of death throughout the world. Over the past two decades, great advances have been made in the pharmacological treatment and prevention of arterial and venous thrombotic disorders (e. g., tissue plasminogen activators, platelet GPIIb/IIIa antagonists, and ADP receptor antagonists such as clopidogrel, low-molecular weight heparins, and direct thrombin inhibitors). New research is leading to the next generation of antithrombotic compounds such as direct coagulation FVIIa inhibitors, tissue factor pathway inhibitors, gene therapy, and orally active direct thrombin inhibitors and coagulation factor Xa (FXa) inhibitors. In vitro assays as well as animal models of thrombosis have played and will continue to play crucial roles in the discovery and validation of novel drug targets, the selection of new agents for clinical evaluation, and the provision of dosing and safety information for clinical trials. In addition, these models have provided valuable information regarding the mechanisms of these new agents and the interactions between antithrombotic agents that work by different mechanisms. This comprehensive manual presents the pivotal models that led to the development of drugs that have proven to be effective clinically. The major issues regarding the use of animal models of thrombosis—such as the use of positive controls, appropriate pharmacodynamic markers of activity, safety evaluation, species-specificity, and pharmacokinetics—are highlighted. Finally, the use of genetic models in thrombosis/hemostasis research and pharmacology is also presented.

B.2 In Vitro Tests

B.2.1 Blood Coagulation Tests

PURPOSE AND RATIONALE

The coagulation cascade consists of a complex network of interactions resulting in thrombin-mediated conversion of fibrinogen to fibrin, which is one major component of a thrombus. The coagulation cascade can be initiated either by the "exogenous pathway," the release of thromboplastin (tissue factor) leading to activation of factor VII to the tissue factor/factor VIIa complex, or by the "endogenous pathway," so-called contact activation leading via factors XII, XI and IX to the assembly of the tenase complex consisting of activated factors VIII and IX and Ca^{2+} on a phospholipid surface. Both complexes can activate factor X, which induces the formation of the prothrombinase complex consisting of factor X_a, factor Va and Ca^{2+} on a phospholipid surface. The latter leads to the activation of thrombin, which, in turn, cleaves fibrinogen to fibrin. The three coagulation tests (prothrombin time [PT], activated partial thromboplastin time [APTT], and thrombin time [TT]) allow one to differentiate between effects on the exogenous or endogenous pathway or on fibrin formation. The influence of compounds on the plasmatic blood coagulation is determined by measuring the coagulation parameters PT, APTT, and TT *ex vivo*.

PROCEDURE

Male Sprague-Dawley rats weighing 200–220 g receive the test compound or the vehicle (controls) by oral, intraperitoneal, intravenous, or other route of administration. After the end of the absorption time, they are anesthetized by intravenous injection of 60 mg/kg sodium pentobarbital. The caudal caval vein is exposed by a midline incision or by cardiac puncture and 1.8 ml blood is collected into a plastic syringe containing 0.2 ml 100 mM citrate buffer pH 4.5 (Behring Werke, Marburg). The sample is immediately agitated and centrifuged in a plastic tube at 1500 × *g* for 10 min. Plasma is transferred to another plastic tube and the coagulation tests for the determination of TT, PT, and APTT are performed within 3 h.

In general, citrated plasma is coagulated by the addition of the respective compounds (see below), and the time to clot formation is determined in the coagulometer (= coagulation time).

For detailed laboratory diagnosis of bleeding disorders and assessment of blood coagulation see Palmer (1984) and Nilsson (1987).

Prothrombin Time (PT). An aliquot of 0.1 ml of citrated plasma is incubated for 1 min at 37°C. Then 0.2 ml of human thromboplastin (Thromborel, Behring Werke, Marburg) is added and the coagulometer (Schnittger+Gross coagulometer, Amelung, Brake) is started. The time to clot formation is determined. The PT measures effects on the exogenous pathway of coagulation.

Activated Partial Thromboplastin Time (APTT). To 0.1 ml of citrated plasma 0.1 ml of human placenta lipid extract (Pathrombin, Behring Werke, Marburg) is added and the mixture is incubated for 2 min at 37°C.

The coagulation process is initiated by the addition of 0.1 ml 25 mM calcium chloride when the coagulometer is started and the time to clot formation is determined. The APTT measures effects on the endogenous pathway of coagulation.

Thrombin Time (TT). To 0.1 ml of citrated plasma 0.1 ml of diethylbarbiturate-citrate buffer, pH 7.6 (Behring Werke Marburg) is added and the mixture is incubated for 1 min at 37°C. Then 0.1 ml of bovine test-thrombin (30 IU/ml, Behring Werke Marburg) is added and the coagulometer is started. The time to clot formation is determined. The TT measures effects on fibrin formation.

EVALUATION

Mean values of TT, PT, and PTT are calculated in dosage groups and vehicle controls. Statistical evaluation is performed by means of the unpaired Student's *t*-test.

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B.2.2 Thrombelastography

PURPOSE AND RATIONALE

Thrombelastography (TEG) was developed first by Hartert (1948). The thrombelastograph (Haemoscope Corp, Skokie, Illinois, USA) is a device that provides a continuous recording of the process of blood coagulation and subsequent clot retraction. The blood samples are transferred to cuvettes and maintained at 37°C. The cuvettes are set in motion around their vertical axes. Originally, a torsion-wire suspended mirror in the plasma remains immobile as long as the plasma is fluid. The cuvette and the mirror become dynamically related as fibrin forms, resulting in transmission of cuvette motion to the mirror. The mirror then oscillates with an amplitude governed by the specific mechanical properties of the clot and reflects its light to a thermopaper. The modern thrombelastograph transfers the analogous recording to a digital signal that is evaluated by a computer program.

PROCEDURE

TEG can be performed in either whole blood or in citrated platelet-rich or platelet-poor plasma after recalcification. Blood samples are obtained from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.7–2.5 kg, from Wistar rats weighing 150–300 g, or from humans. The test subjects receive the compound by intravenous (i.v.), subcutaneous (s.c.), or oral administration. Ten or 20 min post dosing (i.v., s.c. administration) or 60, 90 or 180 min post dosing (oral administration) blood is collected. The blood samples are mixed with 3.8% trisodium citrate solution (1 part citrate solution to 9 parts blood) as anticoagulant. The citrated whole blood is recalcified by adding 0.4 ml isotonic calcium chloride solution. An aliquot of 0.36 ml of the recalcified whole blood is transferred to the prewarmed cup of the thrombelastograph. After the apparatus has been correctly adjusted and the samples sealed with liquid paraffin to prevent drying, the time for the whole procedure is noted. The thrombelastogram is recorded for 2 h.

EVALUATION

The following measurements are the standard variables of TEG:

1. Reaction time (*r*): the time from sample placement in the cup until onset of clotting (defined as amplitude of 1 mm). This represents the rate of initial fibrin formation.
2. Clot formation time (*k*): the difference from the 1 mm *r* to 20 mm amplitude. *k* represents the time taken for a fixed degree of viscoelasticity achieved by the forming clot, caused by fibrin build up and cross linking.
3. Alpha angle ($\alpha°$): angle formed by the slope of the TEG tracing from the *r* to *k* value. It denotes speed at which solid clot forms.
4. Maximum amplitude (*MA*): greatest amplitude on the TEG trace. *MA* represents the absolute strength of the fibrin clot and is a direct function of the maximum dynamic strength of fibrin and platelets.
5. Clot strength (*G* in dynes per square centimeter): defined by $G = (5000\,MA)/(96 - sMA)$. In a tissue factor-modified TEG (Khurana et al. 1997), clot strength is clearly a function of platelet concentration.
6. Lysis 30, Lysis 60 (*Ly30*, *Ly60*): Reduction of amplitude relative to maximum amplitude at 30 and 60 min after time of maximum amplitude. These parameters represent the influence of clot retraction and fibrinolysis.

TEG Parameters	TF (25 ng)	LPS (0.63 ug)	Xa (0.25 nM)	Thrombin (0.3 mU)
		Mean ± SEM		
R (minutes)	29.7 ± 2.3	23.4 ± 1.4	15.6 ± 2.9	3.4 ± 0.6
K (minutes)	5.8 ± 1.0	7.6 ± 0.9	4.8 ± 0.5	5.5 ± 0.8
α (angle)	45.0 ± 2.6	47.8 ± 3.2	61.5 ± 2.1	57.8 ± 2.9
MA (mm)	58.2 ± 1.7	50.0 ± 2.0	65.0 ± 0.8	50.1 ± 2.4

Citrated human whole blood plus 2 mM calcium. Data represent mean for n = 6 ± SEM.

Table 1 Effect of various stimulus on platelet/fibrin clot dynamics as shown by Mousa et al. (2000)

MODIFICATIONS OF THE METHOD

Bhargava et al. (1980) compared the anticoagulant effect of a new potent heparin preparation with a commercially available heparin by TEG *in vitro* using citrated dog and human blood. Barabas et al. (1993) used fibrin plate assay and TEG to assess the antifibrinolytic effects of synthetic thrombin inhibitors. Scherer et al. (1995) described an endotoxin-induced rabbit model of hyper-coagulability for the study of the coagulation cascade and the therapeutic effects of coagulation inhibitors using various parameters, including TEG.

Khurana et al. (1997) introduced tissue factor-modified TEG to study platelet glycoprotein IIb/IIIa function and to establish a quantitative assay of platelet function. With this modification, Mousa et al. (2000) found two classes of glycoprotein IIb/IIIa (GPIIb/IIIa) antagonists, one with high binding affinity for resting and activated platelets and slow platelet dissociation rates (class I) demonstrating potent inhibition of platelet function, in contrast to those with fast platelet dissociation rates (class II). Additionally, Mousa et al. (2005) utilized the TEG in phase II clinical trial in monitoring the efficacy of oral platelet GPIIb/IIIa antagonist on platelet/fibrin clot dynamics.

CRITICAL ASSESSMENT OF THE METHOD

Zuckerman et al. (1981) compared TEG with other common coagulation tests (fibrinogen, prothrombin time, activated thromboplastin time, platelet count, and fibrin split products) and found that there is a strong relationship between the thrombelastographic variables and these common laboratory tests. Mousa et al. (2005) and others expanded the use of TEG in differentiating among different antiplatelets, anticoagulants, and optimal combinations of both. Moreover, TEG has an increased sensitivity for detecting blood clotting anomalies; it contains additional information on the hemostatic process. This is due to the following: (1) the fact that most laboratory measurements end with the formation of the first fibrin strands while TEG measures the coagulation process on whole blood from initiation of clotting to the final stages of clot lysis and retraction, and (2) the ability of TEG to use whole non-anticoagulated blood without influence of citrate or other anticoagulants.

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B.2.3 Chandler Loop

PURPOSE AND RATIONALE

The Chandler loop technique allows the production of *in vitro* thrombi in a moving column of blood (Chandler 1958). The thrombi generated in the Chandler device show morphology very similar to that of human thrombi formed *in vivo* (Robbie et al. 1997), with platelet-rich upstream sections (“white heads”) that are

relatively resistant to t-PA-mediated thrombolysis in contrast to the red blood-cell-rich downstream parts ("red tails") (Stringer et al. 1994).

PROCEDURE

One millimeter of non-anticoagulated whole blood is drawn directly into a polyvinyl tube with a length of 25 cm and an internal diameter of 0.375 cm (1 mm = 9.9 cm tubing). The two ends of the tube are then brought together and closed by an outside plastic collar. The circular tube is placed and centered on a turntable, tilted to an angle of 23°, and rotated at 17 rpm. At the moment the developing thrombus inside the tube becomes large enough to occlude the lumen, the blood column becomes static and moves around in the direction of rotation of the tube.

EVALUATION

Time to occlusion of the tube by the thrombus establishes a definite end point in this system.

MODIFICATIONS OF THE METHOD

Stringer et al. (1994) used this method to determine the influence of an anti-PAI-1 antibody (CLB-2C8) on the t-PA-induced lysis of Chandler thrombi *in vitro*. They used citrated blood and supplemented it with 5.8 µM [^{125}I]-labeled fibrinogen prior to recalcification. After generation in the Chandler loop, the thrombi were washed with isotonic saline and then cut transversally into an upstream (head) and a downstream part (tail). The radioactivity of both parts was determined in a gamma counter (pre-value). The head and the tail were then subjected to thrombolysis by adding 300 µl phosphate-buffered saline containing plasminogen (2 µM) and t-PA (0.9 nM). During the observation time of 240 min, aliquots of 10 µl were taken at 30, 60, 120, 180 and 240 min, and the radioactivity was determined. The relation of the measured radioactivity to the pre-value was expressed as percentage of clot lysis.

Van Giezen et al. (1998) used this method to differentiate the effect of an anti-PAI-1 polyclonal antibody (PRAP-1) on human or rat thrombi.

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B.2.4 Platelet Aggregation and Deaggregation in Platelet-Rich Plasma or Washed Platelets (Born Method)

PURPOSE AND RATIONALE

Platelets play a crucial role in primary hemostasis by forming hemostatic plugs at sites of vascular injury. Moreover, they contribute to intravascular thrombus formation mostly upon rupture of an atherosclerotic plaque. The contact of unactivated platelets to exposed subendothelial tissue leads to adhesion via two main mechanisms: binding of subendothelial von Willebrand factor (vWF) to the platelet GPIb-IX-V-complex at high shear rates and binding of collagen to two receptors, integrin $\alpha 2\beta 1$ and GPVI. Platelet adhesion initiates the reactions of shape change, secretion, and activation of GPIIb-IIIa-ligand binding sites. These reactions result in the formation of platelet aggregates. Activation of GPIIb-IIIa is also achieved through signaling by a number of agonists that bind to G-protein-coupled receptors. Consequently, for the measurement of platelet aggregation, platelets are activated by the addition of one of the following agonists to platelet-rich plasma (PRP) or washed platelets: ADP, arachidonic acid (forming thromboxane A2) or U 46619, collagen, thrombin or TRAP, serotonin, epinephrine, PAF. The formation of platelet aggregates with stirring leads to changes in optical density that are monitored photometrically, usually for 4 min. The test has been developed originally by Born (1962a, and 1962b) and is used to evaluate quantitatively the effect of compounds on induced platelet aggregation *in vitro* or *ex vivo*. For *in vitro* studies, human PRP is preferred.

PROCEDURE

The test is carried out either *ex vivo* or *in vitro*. There are other commercial sources for the various agonists listed.

Table 2 Materials and solutions

Anticoagulating substances	
Hirudin (Sigma) or PPAK	200 µg/ml
Trisodium citrate	0.11 M
ACD solution	
Citric acid	38 mM
Sodium citrate	75 mM
Glucose	124 mM
Platelet-aggregating substances (final concentrations in the test)	
ADP: for reversible or biphasic aggregation	0.1–5 µM
ADP: for irreversible aggregation (Sigma)	3–10 µM
Sodium arachidonate (Biodata)	0.3–1 mM
Calcium ionophore A 23187 (Calbiochem)	10 µM
Collagen (Hormonchemie)	3 µg/ml
PAF-acether (C 16-PAF, Bachem)	0.1 µM
Thrombin (Sigma)	0.02–0.05 IU/ml
TRAP (SFLLRNP, Bachem)	1–10 µM
U 46619 (ICN)	1–10 µM
Ristocetin	0.1–1 mg/ml
GPRP (fibrin antipolymerant, Bachem)	0.5 mM
4-channel aggregometer (PAP 4, Bio Data)	

For *ex vivo* assays, mice, rats, or guinea pigs from either sex receive the test compound or the vehicle (for controls) by oral, intraperitoneal or intravenous administration. At the end of the absorption time, blood is collected by caval venipuncture under pentobarbital sodium anesthesia and xylazine (8 mg/kg i.m.) premedication.

From rabbits (Chinchilla strain, weighing 3 kg), blood is withdrawn by cardiopuncture under xylazine (20 mg/kg i.m.) sedation. The first blood sample (control) is collected before administration of the test compound, the second sample at the end of the absorption time of the test agent.

For *in vitro* assays, human blood is collected from the antecubital vein of adult volunteers who had not received any medication for the last two weeks.

PREPARATION OF PRP, PPP, AND WP

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Freshly collected venous blood is anticoagulated with hirudin (1 volume + 9 volumes of animal blood) or ACD solution (1 volume + 9 volumes of human blood) and centrifuged at 150 × *g* for 15 min to obtain platelet-rich plasma (PRP). The PRP-supernatant is carefully removed, and the rest is further centrifuged at 1500 × *g* for 10 min to obtain platelet-poor plasma (PPP). PRP is diluted with PPP to a platelet count of 3×10^8/ml before use in the aggregation assays. To obtain washed platelets (WP), 8.5 volumes of human blood are collected into 1.5 volumes of ACD and centrifuged as for PRP. PRP is acidified to a pH of 6.5 by addition of approximately 1 ml ACD to 10 ml PRP. Acidified PRP is centrifuged for 20 min at 430*g*. The pellet is resuspended in the original volume with Tyrode's solution (mM: NaCl 120, KCl 2.6, $NaHCO_3$ 12, NaH_2PO_4 0.39, HEPES 10, glucose 5.5; albumin 0.35%) and set to platelet count of 3×10^8/ml.

For *ex vivo* assays, duplicate samples of 320µl PRP from drug-treated and vehicle control subjects (for rabbits: control samples before drug administration) are inserted into the aggregometer at 37°C under continuous magnetic stirring at 1000 rpm. After the addition of 40 µl physiological saline and 40 µl aggregating agent, changes in optical density are monitored continuously at 697 nm.

For *in vitro* assays, 40 µl of the test solution are added to samples of 320 µl PRP or WP from untreated subjects. The samples are inserted into the aggregometer and incubated at 37°C for 2 min under continuous magnetic stirring at 1000 rpm. After the addition of 40 µl aggregating agent, changes in optical density are monitored continuously at 697 nm either for 4 min or until constant values for aggregation are achieved. In cases of thrombin activation of PRP, glycine-proline-aspartate-proline (GPRP) is added in order to avoid fibrin formation. In order to measure deaggregation, experimental compounds are added to stimulated PRP at 70 or 100% of control aggregation and monitoring is performed for further 10 min. Deaggregation is measured by the decrease of light transmission (see Haskel et al. 1989).

Studies should be completed within 3 hours after blood withdrawal.

EVALUATION

The transmission maximum serves as a scale for platelet aggregation (0% = transmission of PRP, 100% = transmission of PPP).

For *in vitro* assays:

1. Percent inhibition of platelet aggregation is determined in concentration groups relative to vehicle controls. Statistical significance is evaluated by means of the unpaired Student's t-test.
2. IC_{50} values are determined from the non-linear curve fitting of concentration-effect relationships.

IC_{50} is defined as the concentration of test drug for half maximal inhibition of aggregation.

3. Percent deaggregation is determined at 10 min after addition of compound; IC_{50} is calculated from the concentration-effect relationship.

For *ex vivo* assays:

1. Mean values for aggregation in dosage groups are compared to the vehicle control groups (for rabbits: control values before drug administration). Statistical significance is evaluated by means of the Student's *t*-test (paired for rabbits; unpaired for others).
2. ED_{50} values are determined from the dose-response curves. ED_{50} is defined as the dose of drug leading to 50% inhibition of aggregation in the animals.

CRITICAL ASSESSMENT OF THE METHOD

The assay, introduced by Born (1962a and 1962b), has become a standard method in clinical diagnosis of platelet function disorders and of aspirin intake. Furthermore, the method is used in the discovery of antiplatelet drugs with the advantage of rapid measurement of a functional parameter in intact human platelets. However, processing of platelets during the preparation of PRP, washed or filtered platelets from whole blood results in platelet activation and separation of large platelets. Additionally, there is no standardization among the different laboratories due to variation in use of different tubes, different final agonist concentrations, and other technical differences.

MODIFICATIONS OF THE METHOD

Several authors have described modifications of the assay procedure. Breddin et al. (1975) described spontaneous aggregation of platelets from vascular patients in a rotating cuvette. Klose et al. (1975) measured platelet aggregation under laminar flow conditions using a thermostated cone-plate streaming chamber in which shear rates are continuously augmented and platelet aggregation is measured from light transmission through a transilluminating system. Marguerie et al. (1979, 1980) developed a method of measuring two phases of platelet aggregation after gel filtration of a platelet suspension (see below). Lumley and Humphrey (1981) described a method to measure platelet aggregation in whole blood (see below). Fratantoni and Poindexter (1990) performed aggregation measurements using a microtiter plate reader with specific modification of the agitation of samples. Comparison of the 96-well microtiterplate method with conventional aggregometry showed similar dose-response curves for thrombin, ADP, and arachidonic acid.

Ammit and O'Neil (1991) used a quantitative bioassay of platelet aggregation for rapid and selective measurement of platelet-activating factor. Mousa et al. (1994, 1998) and others utilized this assay for *in vitro* screening and *ex vivo* and antiplatelet efficacy in various species and in humans for platelet GPIIb/IIIa antagonists.

Yamanaka et al. (2005) performed platelet aggregations assays using an eight channel aggregometer (NBS HEMA TRACER 801, Nikobioscience, Tokyo, Japan) to test structure-activity relationships of potent GPIb/IIIA antagonists.

Francischetti et al. (2000) used the Microplate Reader for studying a platelet aggregation inhibitor from the salivary gland of the blood-sucking bug, *Rhodnius prolixus*.

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B.2.5 Platelet Aggregation After Gel Filtration (Gel-Filtered Platelets, GFP)

PURPOSE AND RATIONALE

Triggering of platelet activation by low concentrations of ADP, epinephrine or serotonin—so-called weak platelet agonists—in plasma- and fibrinogen-free platelet suspensions does not result in platelet aggregation unless exogenous fibrinogen is added. As opposed to this, platelet aggregation induced by thrombin, collagen or prosta-glandin-endoperoxide—so-called strong agonists—is independent of exogenous fibrinogen because these substances lead to the secretion of intracellular platelet ADP and fibrinogen. Studies of platelet aggregation in gel-filtered platelets are performed in cases where the adhesive ligand fibrinogen or vWF is needed in a defined concentration or where plasma proteins could negatively interfere with the effect of compounds. The assay is mostly used to evaluate the influence of compounds on platelet GPIIb-IIIa or other integrins or on GPIb-IX-V. Mousa et al. (1994, 1998) and others have extensively utilized this assay to determine the impact of plasma protein binding on antiplatelet efficacy by comparing inhibition of platelet aggregation in GFP versus PRP.

PROCEDURE

Preparation of Gel-Filtered Platelets

The entire procedure is performed in plastic (polystyrene) tubes at room temperature according to Marguerie et al. (1979).

Blood is drawn from healthy adult volunteers who had no medication for the last two weeks. Venous blood (8.4 ml) is collected into 1.4 ml ACD-solution and centrifuged for 10 min at 120 *g*. The platelet-rich plasma (PRP) is carefully removed, the pH adjusted to 6.5 with ACD-solution, and centrifuged at 285 *g* for 20 min. The resulting pellet is resuspended in Tyrode's buffer (approx. 500 µl buffer/10 ml PRP). The platelet suspension is applied immediately to a Sepharose CL 2B column; equilibration and elution at 2 ml/min flow rate is done with Tyrode's buffer without hirudin and apyrase. Platelets are recovered in the void volume. Final platelet suspension is adjusted to 4×10^8/ml. Gel filtered platelets (GFP) are kept at room temperature for 1 h until the test is started.

Table 3 Materials and solutions

Acid-citrate-dextrose (ACD) solution	
Citric acid	0.8%
Sodium citrate	2.2%
Glucose	2.45%
Hirudin	0.6 U/ml
Tyrode's solution	
NaCl	137 mM
KCl	2.7 mM
$MgCl_2$	5.5 mM
NaH_2PO_4	3.0 mM
HEPES	3.5 mM
Glucose	5.5 mM
Albumin	0.2%
Hirudin	0.06 U/ml
Apyrase	40 µg/ml
pH	7.2
ADP	10 µM
Thrombin	0.02–0.05 U/ml
$CaCl_2$	0.5 mM
Fibrinogen (American Diagnostica)	1 mg/ml
von Willebrand factor	10 µg/ml
Sepharose CL 2B (Pharmacia)	
Acrylic glass column (Reichelt Chemietechnik, 3 cm inner diameter, 18 cm length)	
Aggregometer (PAP 4, Biodata)	

Experimental Course

For the aggregation studies, GFP in Tyrode's buffer is incubated with $CaCl_2$ (final concentration 0.5 mM) with or without fibrinogen (final conc. 1 mg/ml) in polystyrene tubes. After 1 min, 20 µl of the test compound or the vehicle (controls) are added, and the samples are incubated for another 2 min. After the addition of 20 µl platelet agonist, changes in light transmission are recorded. The whole procedure is done under continuous magnetic stirring at 37°C (1000 rpm) in the aggregometer. Samples with added $CaCl_2$ but without fibrinogen identify proper exclusion of plasma proteins if neither spontaneous aggregation occurs nor aggregation in the presence of weak agonists. Full aggregatory response of GFP to 10 µM ADP shows intact platelets (with only minor pre-activation with gel filtration).

EVALUATION

The transmission maximum serves as a scale for platelet aggregation. Each test compound is assayed with at least two different donor-GFPs; in the case of an anti-aggregating effect, the test is performed with 4–6 GFPs.

Mean values of the dosage groups are compared to the controls. Statistical significance is evaluated by means of the Student's *t*-test.

The percent inhibition of platelet aggregation in the dosage groups is calculated relative to the vehicle controls.

IC_{50} values (50% inhibition of aggregation) are determined from the concentration-effect curves.

For detailed methodology and evaluation of different agents see Marguerie et al. (1979 and 1980), Markell et al. (1993), and Mousa et al. (1994 and 1998).

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B.2.6 Platelet Aggregation in Whole Blood

PURPOSE AND RATIONALE

The method uses a whole blood platelet counter that counts single platelets and does not require their separation from other blood cell types. Platelet aggregation is induced in anti-coagulated human whole blood samples by the addition of the aggregating agents arachidonic acid or collagen. The number of platelets is determined in drug-treated and vehicle control samples; the percentage of inhibition of aggregation and IC_{50} values are calculated in dosage groups. The effect of compounds on other blood cells that secondarily can influence platelet aggregation is included in this test system. The method has been described by Lumley and Humphrey (1981) and by Cardinal and Flower (1980).

PROCEDURE

The entire procedure is performed in plastic (polystyrene) tubes and carried out at room temperature. Blood is drawn from healthy adult volunteers who had not received medication for the last 2 weeks; 9 ml venous blood is anti-coagulated with 1 ml of sodium citrate and kept in a closed tube at room temperature for 30–60 min until the start of the test.

Table 4 Materials and solutions

Anticoagulant: sodium citrate to induce platelet aggregation	3.8%
Sodium arachidonate (Biodata)	3.6×10^{-4} M
Collagen (Hormonchemie)	10 µg/ml
Serono Hematology System 9000 or Sysmex Micrcellcounter F 800	

For the aggregation studies, 10 µl test substance or vehicle (control) is added to 480 µl citrated blood. Samples in closed tubes are pre-incubated for 5 min in a 37°C water shaker bath at 75 strokes/min. Then, 10 µl aggregating agent are added, and samples are incubated for another 10 min. The number of platelets (platelet count) is determined in 10 µl samples immediately before and 10 min after the addition of the aggregating agent ('initial platelet count', '10-min-platelet count' after adding platelet agonist ± antagonist) in a hematology cell counter.

The following samples for the determination of the platelet count are prepared in duplicate:

- control aggregation = spontaneous aggregation (without aggregating agent): 480 µl blood + 20 µl vehicle. Blood samples with >20% spontaneous aggregation are not used to test for induced aggregation.
- maximal aggregation: 480 µl blood + 10 µl vehicle + 10 µl aggregating agent. Values represent the maximal induced aggregation rate of the blood sample.
- test substance aggregation: 480 µl blood + 10 µl test substance + 10 µl aggregating agent.

EVALUATION

From the samples for maximal aggregation (vehicle), the percentage of maximal aggregation is calculated according to the following formula:

$$\%\ \text{maximal aggregation} = 100 - \frac{\text{10-min-platelet count} \times 100}{\text{initial plateletcount}}$$

From the samples for test substance induced aggregation, the percentage of aggregation in dosage groups is

calculated according to the following formula:

$$\%\ \text{aggregation} = \frac{\text{10-min-platelet count} \times 100}{\text{initial platelet count}}$$

IC_{50} values (50% inhibition of aggregation) are determined from the dose-response curves (log concentration test substance versus % inhibition of aggregation).

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B.2.7 Platelet Micro- and Macro-Aggregation Using Laser Scattering

PURPOSE AND RATIONALE

- A new highly sensitive method to study platelet aggregation based on the measurement of mean radius or particle size makes it possible to record kinetics of formation of micro- and macro-aggregates in real time
- Sensitivity in measurements of spontaneous aggregation is higher than in routine light transmittance.

A new platelet aggregometer (AG-10; Kowa, Japan) that uses a laser-light-scattering beam has been introduced (Tohgi et al. 1996). Platelet aggregates, the size of which can be measured as total voltage of light-scatter intensity at 1.0-sec intervals for a 10-min period, can be divided into 3 ranges: small aggregates (diameter 9 to 25 µm), medium (26 to 50 µm), and large (>50 µm). Using laser scatter aggregation, it was found that young smokers had an increased number of small platelet aggregates, which cannot be detected with a conventional aggregometer based on the turbidometric method (Matsuo et al. 1997). This device detects platelet aggregation in the small-aggregate size range by the addition of unfractionated heparin (UFH), and the aggregates are disaggregable in incubation with protamine sulfate. When platelet aggregation induced by UFH at a final concentration of 0.5 U/mL was observed in 36 normal subjects with no history of heparin exposure, 13 had a positive response in excess of 0.5 V of light intensity in the small-aggregate size range. In chronic hemodialysis patients in whom heparin had been used regularly for many years, a positive response with heparin-induced aggregates was noted in 37 of 59 patients, which was increased compared with that of normal subjects. The light intensity in the small-aggregate size range was enhanced during heparinized dialysis. In patients with a positive heparin response, the intensity of aggregates after heparin was significantly increased compared with that in nonresponders to heparin. Also, we obtained the same results by this system: that enhanced platelet aggregation response to heparin was not inhibited by aspirin or argatroban but was inhibited by anti-glycoprotein IIb/IIIa antibodies or small molecule antagonists. The findings of enhanced platelet aggregation during heparin infusion could be directly obtained without the addition of ADP or TRAP using laser aggregometry (Xiao and Théroux 1998).

LIMITATIONS

This technique can not be applied in whole blood yet but can be used with PRP, washed platelet, or GFP

MODIFICATIONS OF THE METHOD

For functional characterization of an acid platelet aggregation inhibitor and hypotensive phopsholipase A_2 from *Bothrops jararacussu* snake venom, Andrião-Escarso et al. (2002) measured platelet aggregation in rabbit blood using a whole blood Lumi-Aggregometer.

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B.2.8 Fibrinogen Receptor Binding

PURPOSE AND RATIONALE

The assay is used to evaluate the binding characteristics of drugs at the fibrinogen receptor. A constant concentration of the radioligand ^{125}I-fibrinogen (30–50 nM) is incubated with increasing concentrations of a non-labeled test drug (0.1 nM–1 mM) in the presence of gel-filtered human platelets. If the test drug exhibits any affinity to fibrinogen receptors, it is able to compete with the radioligand for receptor binding sites. Thus, the lower the concentration range of the test drug, in which the competition reaction occurs, the more potent the test drug. Platelets are activated with 10 mmol/l ADP to stimulate the ^{125}I-fibrinogen binding at the GPIIb/IIIa receptor.

PROCEDURE

Preparation of Gel-Filtered Platelets

From a healthy volunteer, 200 ml blood is collected. Ana aliquot of 8.4 ml blood is mixed with 1.4 ml ACD-buffer in polystyrol tubes and centrifuged at $150 \times g$ for 15 min. The resulting platelet-rich plasma (PRP) is collected, and an aliquot is taken for platelet counting. Then, 10 ml PRP are mixed with 1 ml ACD-buffer (ACD-PRP, pH ~ 6.5); 5 ml portions of ACD-PRP are transferred to plastic tubes and centrifuged at $1500 \times g$ for 15 min. The resulting supernatant is decanted, and each pellet is resuspended in 500 µl Tyrode buffer C. An aliquot is taken for platelet counting to calculate the loss of platelets. The platelet suspension is then transferred to a Sepharose-packed column that has been eluted with approx. 100 ml degassed Tyrode buffer B (2 ml/min). The column is closed and eluted with degassed Tyrode buffer B (2 ml/min). The first platelets appear after 18–20 min and are then collected for 10 min in a closed plastic cup. Gel-filtered platelets (GFP) are set to 4×10^8 platelets/ml with Tyrode buffer B and kept at room temperature until the start of the test (Mousa et al. 1994 and 2001).

EXPERIMENTAL COURSE

For each concentration, samples are tested in triplicate (test tubes No. 72708, Sarstedt).The total volume of each incubation sample is 500 µl. The concentration of ^{125}I-fibrinogen is constant for all samples (10 µg/500 µl).

COMPETITION EXPERIMENTS

The competition reaction is characterized by one buffer value (bidistilled water) and various concentrations of non-labeled fibrinogen or test compound.

- 100 µl ^{125}I-fibrinogen
- 100 µl non-labeled fibrinogen or test drug (various concentrations, 10^{-10}–10^{-3} M)
- 5 µl ADP.

Non-specific-binding: The non-specific binding of ^{125}I-fibrinogen is defined as the radioligand binding in the presence of 10^{-5} M of non-labeled fibrinogen.

The binding reaction is started by adding 250 µl GFP (4×10^8 platelets/ml). The samples are incubated for 30 min at room temperature. Subsequently, a 100 µl aliquot of the incubation sample is transferred to a microtainer tube containing 400 µl glucose solution. The tubes are centrifuged at $1{,}500 \times g$ for 2 min to separate ^{125}I-fibrinogen bound at the platelet glycoprotein IIb–IIIa receptor from free radioligand. The supernatant is carefully decanted and is allowed to run off for approx. 30 min. Radioactivity of the platelet pellets is counted for 1 min in a gamma counter with an efficiency of 65.3%.

MATERIALS AND SOLUTIONS

See Table 5.

EVALUATION

The quantity of the specific ^{125}I-fibrinogen binding results from the difference between the total and the non-specific binding.

Platelet glycoprotein IIb-IIIa receptor binding is given as fmol ^{125}I-fibrinogen/10^8 platelets or ^{125}I-fibrinogen molecules bound per platelet.

The dissociation constant (K_i) of the test drug is determined from the competition experiment of ^{125}I-fibrinogen versus non-labeled drug by a computer-supported analysis of the binding data.

$$K = \frac{K_D^{125}\mathrm{I} \times IC_{50}}{K_D^{125}\mathrm{I} + [^{125}\mathrm{I}]}$$

IC_{50} = concentration of the test drug, which displaces 50% of the specifically glycoprotein IIb-IIIa receptor bound ^{125}I-fibrinogen in the competition experiment.

[^{125}I] = concentration of ^{125}I-fibrinogen in the competition experiment.

$K_D{}^{125}$I = dissociation constant of ^{125}I-fibrinogen, determined from the saturation experiment.

The K_i-value of the test drug is the concentration, at which 50% of the fibrinogen receptors are occupied by the test drug.

For detailed methodology and evaluations of various mechanisms and agents see the following selected

Table 5 Materials and solutions

Solutions for platelet preparation		
Stock solution I	Citrate	0.8%
	Sodium citrate	2.2%
Stock solution II	NaCl	120 mM
	KCl	2.8 mM
	NaH_2PO_4	10.0 mM
	HEPES	10.0 mM
ACD-buffer	Stock solution I	2.45%
	+ glucose	
	+ hirudin	0.06 U/ml
Tyrode buffer A	Stock solution II	
	+ $NaHCO_3$	12 mM
Tyrode buffer B	Stock solution II	
	+ $NaHCO_3$	12 mM
	+ glucose	5.5 mM
	+ bovine albumin	0.35%
Tyrode buffers A and B are degassed by aspiration for approx. 1 h after setting the pH to 7.2.		
Tyrode buffer C	Tyrode buffer B (degassed)	
	+ apyrase	40 µg/ml
	+ hirudin	0.06 U/ml
Chromatography column	Acryl glass column (200 × 170 mm, 30 mm diameter), closed with 3 perlon filters, pore sizes 63, 90 and 230 µm, and gauze 50 µm filled with degassed Sepharose CL2B-suspen-sion (Pharmacia LKB); equilibrated with 500 ml degassed Tyrode buffer A (2 ml/min)	
Incubation buffer		
Stock solution	NaCl	120 mM
	KCl	2.6 mM
	NaH_2PO_4	0.39 mM
	HEPES	10.0 mM
	$CaCl_2$	0.5 mM
Incubation buffer, pH 7.2	stock solution	
	+ $NaHCO_3$	12 mM
	+ glucose	5.5 mM
	+ human albumin	0.35%
Glucose solution (in incubation buffer)		
Radioligand	^{125}I-fibrinogen specific activity 3.7 Mbq/mg fibrinogen (100 µCi/mg fibrinogen) (Amersham), 1 mg radio-labeled fibrinogen is dissolved in 10 ml incubation buffer	
Non-labeled fibrinogen (mw 340000, grade L, Sigma; in bidistilled water)		10^{-3}–10^{-10} M
ADP (in incubation buffer)		10 µM
Gamma-counter (1282 Compugamma CS, LKB)		

references: Bennett, Vilaire (1979); Kornecki et al. (1981); Marguerie et al. (1979 and 1980); Mendelsohn et al. (1990), and Mousa et al. (1994).

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B.2.9 Euglobulin Clot Lysis Time

PURPOSE AND RATIONALE

The euglobulin lysis time is used as an indicator for the influence of compounds on the fibrinolytic activity in rat blood according to Gallimore et al. (1971). The euglobulin fraction of plasma is separated from inhibitors of fibrinolysis by acid precipitation and centrifugation. Euglobulin predominantly consists of plasmin, plasminogen, plasminogen activator and fibrinogen. By addition of thrombin to this fraction, fibrin clots are formed. The lysis time of these clots is determined as a measurement of the activity of activators of fibrinolysis (e. g. plasminogen activators). Thus, compounds that stimulate the release of tissue-type plasminogen activator from the vessel wall can be detected.

PROCEDURE

Rats are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium and placed on a heating pad (37°C). At the same time, the test solution or the vehicle (controls) is administered intravenously or intraperitoneally. Twenty-five minutes later, the animals receive another intraperitoneal injection of 12 mg/kg sodium pentobarbital to keep them in deep narcosis for 45 min.

Plasma Preparation

After the test compound is absorbed, blood is withdrawn from the inferior caval vein exposed by a midline excision. Blood (1.8 ml) is removed with a plastic syringe containing 0.2 ml 3.8% sodium citrate solution. The sample is thoroughly mixed, transferred to a plastic tube and immediately immersed in ice. Plasma is prepared by centrifugation at 2000 *g* for 10 min at 2°C.

Euglobulin Preparation

A 0.5 ml portion of plasma is added to 9.5 ml of ice-cold distilled water; the pH is brought to 5.3 by the addition of 0.13 ml of 1% acetic acid. The diluted plasma is kept on ice for 10 min, and the precipitated euglobulin fraction is collected by centrifugation at 2000 *g* for 10 min at 2°C. The supernatant is discharged, and the remaining fluid is removed by drying the tube on a filter paper for 1 min. The euglobulin precipitate is dissolved in 1 ml of 0.12 M sodium acetate solution.

Euglobulin Lysis Assay

Aliquots (0.45 ml) of the euglobulin solution are transferred to test tubes, and 0.05 ml thrombin (Test Thrombin, Behring Werke) (25 U/ml) are added. The tubes are transferred to a water bath at 37°C. The time interval between the addition of thrombin and the complete lysis of the clots is measured. For details see Gallimore et al. (1971) and Singh et al. (2005).

EVALUATION

The lysis time (min) is determined. Euglobulin lysis test (ELT) is shortened when activators of fibrinolysis are increased.

Percent lysis time is calculated in dosage treated groups as compared to controls.

Statistical evaluation is performed by means of the Student's *t*-test.

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B.2.10 Flow Behavior of Erythrocytes

PURPOSE AND RATIONALE

The deformation of erythrocytes is an important rheological phenomenon in blood circulation according to Teitel (1977) and Nash (1990). It allows the passage of normal red cells through capillaries with diameters

smaller than that of the discoid cells and reduces the bulk viscosity of blood flowing in large vessels. In the following test, the initial flow of filtration is taken as a criterion for erythrocyte deformability. A prolonged time of filtration can be due to 2 basic pathologic phenomena: an increased rigidity of the individual red cells or an increased tendency of the cells to aggregate. To simulate decreased red blood cell deformability, the erythrocytes are artificially modified by one (or by the combination) of the following stress factors:

- addition of calcium ions (increase in erythrocyte rigidity)
- addition of lactic acid (decrease in pH value)
- addition of 350–400 mmol NaCl (hyperosmolarity)
- storing the sample for at least 4 h (cellular ageing, depletion of ADP)

The following procedure can be used to evaluate the effect of test compounds on the flow behavior of erythrocytes.

PROCEDURE

Apparatus

Erythrocyte filtrometer MF 4 (Fa. Myrenne, 52159 Roetgen, Germany) Membrane filter (Nuclepore Corp.) pore diameter: 5–10 µm, pore density: 4×10^5 pores/cm^2.

Ex Vivo

Blood is collected from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.2–2.5 kg, or from Wistar rats weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, blood is incubated at 37°C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with K-EDTA (1 mg/ml blood) or heparin (5 IU/ml heparin sodium) and centrifuged at $250 \times g$ for 7 min. The supernatant (plasma) and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in autologous plasma containing 0.25% human albumin, and the haematocrit value is fixed at 10%. The red blood cells are altered by one or several of the stress factors mentioned above.

A sample of 2 ml of the stressed suspension is applied to the filtrometer and the initial flow rate is determined. The filtration curve is plotted automatically.

EVALUATION

The cumulative volume of the filtered suspension is recorded per time unit (10 min)

The slope of the curve is determined at different time intervals.

The initial flow rate (10% of the cell suspension having passed the filter) is recorded.

Statistics

Data of each set are first tested for normal distribution using the Kolmogoroff/Smirnow test. The normal distribution hypothesis is eliminated if the data having a significance level of 5% are not normally distributed. In case that both data sets to be compared are normally distributed, the F-test is applied. The hypothesis of homogeneity of variance of both test series is eliminated when the significance level for homogeneity of variance is 5%. The *t*-test for paired and non-paired data is performed when homogeneity of variance is present. In any case, a paired difference test (for paired data) or the U-test (for non-paired data) is likewise carried out (paired of difference test = Wilcoxon test; U-test = Wilcoxon-Mann-Whitney or Mann-Whitney test, respectively).

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B.2.11 Filterability of Erythrocytes

PURPOSE AND RATIONALE

The Single Erythrocyte Rigidometer (SER) allows the measurement of deformability of individual red blood cells by determining their passage time through a pore under constant shear stress. In this test, the passage times of single erythrocytes through one pore in a synthetic membrane are determined according to Kiesewetter et al. (1982), Roggenkamp et al. (1983), and Seiffge et al. (1986). The pore in the membrane practically represents a capillary with defined diameter and length. The driving pressure is produced by the constant shear stress. The passage of the red blood cells is measured with the help of an electrical device. A constant current of 50–200 nA is applied. When an erythrocyte passes through the pore, the current is interrupted. The test is used to detect compounds that improve filterability of erythrocytes. To simulate decreased red blood cell deformability, the erythrocytes

are artificially modified either by one or by a combination of the following stress factors:

- addition of calcium ions (increase in erythrocyte rigidity)
- addition of lactic acid (decrease in pH value)
- addition of 350–400 mmol NaCl (hyperosmolarity)
- storing the sample for at least 4 h (cellular ageing, depletion of ADP).

PROCEDURE

Apparatus

Single erythrocyte rigidometer (Myrenne, 52159 Roetgen, Germany)

Data: driving pressure: $dp = 70$ Pa (dog, rabbit, rat), $dp = 100$ Pa (man); wall shear stress: $\tau = 3$ Pa.

Single pore membrane: length: 30 µm; diameter: 3.5 µm (rat), 4.0 µm (rabbit, dog), 4.5 µm (man)

Ex Vivo

Blood is collected from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.2–2.5 kg, from Wistar rats weighing 150–300 g, or from humans. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, the blood samples are incubated at 37°C for 5 or 30 min.

The blood samples are mixed with K-EDTA (1 mg/ml blood) or heparin (5 IE/ml heparin sodium) to prevent clotting. The blood is centrifuged at $250 \times g$ for 7 min. The plasma and the buffy coat are removed and discarded. The packed erythrocytes are resuspended in filtrated HEPES-buffer containing 0.25% human albumin, and the haematocrit value is fixed to <1%. The red blood cells are altered by one or several stress factors mentioned above. A sample of 2 ml of the stressed suspension is applied to the measuring device, and the passage time of a population of 250 erythrocytes (tm) is determined. Cells remaining in the pore for more than 100 ms (tm >100 ms) lead to a rheological occlusion.

Untreated red blood cell suspensions serve as control.

EVALUATION

The mean passage time of 250 single erythrocytes and the number of rheological occlusions/250 erythrocytes is determined.

Statistics

The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes described above.

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B.2.12 Erythrocyte Aggregation

PURPOSE AND RATIONALE

The aggregation of red blood cells into rouleaux and from rouleaux into 3-dimensional cell networks is a rheological parameter that decisively influences the flow behavior of blood, especially in disturbed microcirculation. In the following procedure, an apparatus (erythrocyte aggregometer) is used to measure erythrocyte aggregation. The transparent measuring chamber (cone/plate configuration) is transilluminated by light of a defined wave length. The intensity of the transmitted light, which is modified by the aggregation process, is recorded. The method can be used to determine the effect of test compounds on erythrocyte aggregation according to Kiesewetter et al. (1982) and Schmid-Schoenbein et al. (1973).

PROCEDURE

Apparatus

Selective Erythrocyte Rigidometer (Fa. Myrenne, 52159 Roetgen, Germany)

Ex Vivo

Blood is collected from Beagle dogs weighing 12–20 kg, from rabbits weighing 1.2–2.5 kg, or from Wistar rats weighing 150–300 g. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, the blood sample is incubated at 37°C for 5 or 30 min.

Blood is obtained from the test subjects by venipuncture and mixed with K-EDTA (1 mg/ml) or heparin (5 IU/ml heparin sodium) to prevent clotting. Erythrocyte aggregation is determined in whole blood. A sample of 40 µl blood is transferred to the measuring device. The red cells are dispersed at a shear rate of 600/s. After 20 s, flow is switched to stasis, and the extent of erythrocyte aggregation is determined photometrically.

EVALUATION

Statistics

The statistical significance is evaluated according to the procedure described for flow behavior of erythrocytes (see above).

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B.2.13 Determination of Plasma Viscosity

PURPOSE AND RATIONALE

One of the principal methods for measuring viscosity is based on the rate of flow of a liquid through an orifice according to Harkness (1971). In this test, a defined volume of plasma is transferred into a capillary viscometer, and the efflux time required for the plasma to flow from the upper to the lower mark is measured. Using this procedure, the effect of test compounds on the viscosity of blood plasma can be determined. The test can be carried out either *ex vivo* or *in vitro.*

PROCEDURE

Ex Vivo

Beagle dogs weighing 12–20 kg, rabbits weighing 2.0–3.0 kg, or Wistar rats weighing 150–300 g of either sex are used as test animals. Likewise, the test procedure can be performed in humans. The test subjects receive the test compound by oral, subcutaneous or intravenous administration 15, 60, 90 or 180 min before the withdrawal of blood.

In Vitro

Following addition of the test compound, plasma (obtained as described below) is incubated at 37°C for 5 or 30 min.

Freshly collected venous blood is anticoagulated with 1 mg/ml blood K^+-EDTA or heparin sodium (5 IU/ml blood) and centrifuged at $250 \times g$ for 5 min. The supernatant (plasma) is removed, and a sample of 0.9 ml plasma is transferred into a capillary viscometer (Coulter Harkness, Coulter Electr., LTD, England) provided with a glass capillary of 0.5 mm inside diameter. The temperature during measurement is 37°C. The flow time, t, required for the plasma to flow through the capillary is measured. Untreated plasma serves as control.

EVALUATION

The viscosity of each sample can be determined using the following formula:

$$\eta = K \times t \times \rho$$

where η = viscosity of plasma, K = calibration constant of viscometer, t = flow time of 0.9 ml plasma, and ρ = density of plasma.

The change in viscosity relative to the control group is determined.

Statistical evaluation is carried out using the Student's t-test.

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B.3 In Vitro Models of Thrombosis

PURPOSE AND RATIONALE

There is abundant evidence suggesting that platelets play a pivotal role in the pathogenesis of arterial

thrombotic disorders, including unstable angina (UA), myocardial infarction (MI) and stroke. The underlying pathophysiological mechanism of these processes has been recognized as the disruption or erosion of a vulnerable atherosclerotic plaque leading to local platelet adhesion and subsequent formation of partially or completely occlusive platelet thrombi.

The specific platelet surface receptors that support these initial adhesive interactions are determined by the local fluid dynamic conditions of the vasculature and the extracellular matrix constituents exposed at the sites of vascular injury. Konstantopoulos et al. (1998) and Alveriadou et al. (1993) demonstrated that under high shear conditions, the adhesion of platelets to exposed subendothelial surfaces of atherosclerotic or injured vessels presenting collagen and von Willebrand factor (vWF) is primarily mediated by the platelet glycoprotein (GP)Ib/IX/V complex. This primary adhesion to the matrix activates platelets, leading ultimately to platelet aggregation mediated predominantly by the binding of adhesive proteins such as fibrinogen and vWF to GPIIb/IIIa. In addition, direct platelet aggregation in the bulk phase under conditions of abnormally elevated fluid shear stresses, analogous to those occurring in atherosclerotic or constricted arterial vessels, as shown by Turitto (1982), may be important. Shear-induced platelet aggregation is dependent upon the availability of vWF and the presence of both GPIb/IX and GPIIb/IIIa on the platelet membrane. It has been postulated that at high shear stress conditions, the interaction of vWF with the GPIb/IX complex is the initial event leading to platelet activation, which also triggers the binding of vWF to GPIIb/IIIa to induce platelet aggregate formation.

A variety of methods have been utilized to assess the *ex vivo* and/or *in vitro* efficacy of platelet antagonists, including photometric aggregometry, whole blood electrical aggregometry and particle counter methods, as described in the above segments. In photometric aggregometry, a sample is placed in a stirred cuvette in the optical light path between a light source and a light detector. Aggregate formation is monitored by a decrease in turbidity, and the extent of aggregation is measured as percent of maximal light transmission. The major disadvantage of this technique is that it cannot be applied in whole blood since the presence of erythrocytes interferes with the optical responses. Furthermore, it is insensitive to the formation of small aggregates. Particle counters are used to quantitate the size and the number of particles suspended in an electrolyte solution by monitoring the electrical current between two electrodes immersed in the solution. Aggregation in this system is quantitated by counting the platelets before and after stimulation, and it is usually expressed as a percentage of the initial count, as shown by Jen and McIntire (1984). However, the disadvantage of this technique is that it cannot distinguish platelets and platelet aggregates from other blood cells of the same size. Thus, one is limited to counting only a fraction of single platelets, as well as aggregates that are much larger than erythrocytes and leukocytes. The technique of electrical aggregometry allows the detection of platelet aggregates as they attach to electrodes immersed in a stirred cuvette of whole blood or platelet suspensions. Such an attachment results in a decrease in conductance between the two electrodes that can be quantitated in units of electrical resistance. However, a disadvantage of this method is that it is not sensitive in the detection of small aggregates, as demonstrated by Sweeney et al. (1989).

This segment discusses two complementary *in vitro* flow models of thrombosis that can be used to accurately quantify platelet aggregation in anticoagulated whole blood specimens and to evaluate the inhibitory efficacy of platelet antagonists: (1) a viscometric-flow cytometric assay to measure direct shear-induced platelet aggregation in the bulk phase, as demonstrated by Konstantopolous et al. (1995); and (2) a parallel-plate perfusion chamber coupled with a computerized videomicroscopy system to quantify the adhesion and subsequent aggregation of human platelets in anticoagulated whole blood flowing over an immobilized substrate (e. g. collagen I), as shown by Konstantopolous et al. (1995), and Mousa et al. (2002). Furthermore, Mousa et al. (2002) demonstrated a third *in vitro* flow assay in which surface-anchored platelets are pre-incubated with a GPIIb/IIIa antagonist, and unbound drug is washed away prior to the perfusion of THP-1 monocytic cells, thereby enabling one to distinguish agents with markedly distinct affinities and receptor-bound lifetimes.

PROCEDURE

Isolation of Human Platelets

The steps described in subsequent subheadings outline the procedure for isolation and purification of platelets from whole blood obtained by venipuncture from human volunteers. Obtain blood sample by venipuncture from an antecubital vein into polypropylene syringes containing either sodium citrate (0.38% final concentration) or heparin (10 U/ml final concentration). Centrifuge anticoagulated whole blood at $160 \times g$ for 15 min to prepare platelet-rich plasma (PRP).

Isolation of Washed Platelets

PRP specimens are subjected to a further centrifugation (1,100 *g* for 15 min) in the presence of 2 μM PGE_1 (Evangelista et al. 1996).

The platelet pellet is resuspended in HEPES-Tyrode buffer containing 5 mM EGTA and 2 μM PGE_1.

Platelets are then washed via centrifugation (1,100 *g* for 10 min), resuspended at 2×10^8/mL in HEPES-Tyrode buffer, and kept at room temperature for no longer than 4 hours before use in aggregation/adhesion assays.

MATERIALS

- Anticoagulant solution (sodium citrate, porcine heparin, PPACK etc.)
- Fluorescently labeled platelet-specific antibody
- Dulbecco's phosphate-buffered saline (D-PBS) (with and without Ca^{2+}/Mg^{2+}).
- Formaldehyde
- Type I collagen, from bovine Achille's tendon
- 0.5 mol/L glacial acetic acid in water
- Glass coverslips (24 × 50 mm; Corning; Corning, NY)
- Silicone sheeting (gasket) (0.005-in or 0.010-in thickness; Specialty Manufacturing Inc; Saginaw, MI)
- Quinacrine dihydrocholride
- Prostaglandin E_1 (PGE_1) and EGTA
- Thrombin
- Bovine serum albumin
- HEPES-Tyrode buffer (129 mM NaCl, 9.9 mM $NaHCO_3$, 2.8 mM KCl, 0.8 mM K_2PO_4, 0.8 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$, 10 mM HEPES, 5.6 mM dextrose)
- 3-aminopropyltriethoxysilane (APES)
- Acetone
- 70% nitric acid in water
- THP-1 monocytic cells
- Platelet antagonists such as abciximab

EVALUATIONS

The methods described below (Sections 2.1–2.3) outline different dynamic adhesion/aggregation assays used to assess the *in vitro* and/or *ex vivo* efficacy of platelet antagonists: (1) a viscometric-flow cytometric assay to measure shear-induced platelet-platelet aggregation in the bulk phase, (2) a perfusion chamber coupled with a computerized videomicroscopy system to visualize in real time and quantify (a) the adhesion and subsequent aggregation of platelets flowing over an immobilized substrate (e. g. extracellular matrix protein) and (b) free-flowing monocytic cell adhesion to immobilized platelets.

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B.3.1 Cone-and-Plate Viscometry Under Shear-Flow Cytometry

PURPOSE AND RATIONALE

The cone-and-plate viscometer is an *in vitro* flow model used to investigate the effects of bulk fluid shear stress on suspended cells. Anticoagulated whole blood specimens (or isolated cell suspensions) are placed between the two platens (both of stainless steel) of the viscometer. Rotation of the upper conical platen causes a well-defined and uniform shearing stress to be applied to the entire fluid medium, as described by Konstantopolous et al. (1998). The shear rate (γ) in this system can be readily calculated from the cone angle and the speed of the cone using the formula:

$$\gamma = \left(\frac{2\pi\omega}{60\theta_{cp}} \right)$$

where γ is the shear rate in sec^{-1}, ω is the cone rotational rate in revolutions per minute (rev/min) and θ_{cp} is the cone angle in radians. The latter is typically in the range of 0.3 to 1.0°. The shear stress, τ, is proportional to shear rate, γ, as shown by: $\tau = \mu \cdot \gamma$, where μ is the viscosity of the cell suspension (the viscosity of anticoagulated whole blood is ~0.04 cp at 37°C). This type of rotational viscometer is capable of generating shear stresses from ~2 dyn/cm^2 (venous level) to greater than 200 dyn/cm^2 (stenotic arteries).

PROCEDURE

Single platelets and platelet aggregates generated upon shear exposure of blood specimens are differentiated from other blood cells on the basis of their characteristic forward-scatter and fluorescence (by the use of fluorophore-conjugated platelet specific antibodies) profiles by flow cytometry, as described by Konstantopolous et al. (1995). This technique requires no washing or centrifugation steps that may induce artifactual platelet activation, and it allows the study of platelet function in the presence of other blood elements. The procedure used to quantify platelet aggregation induced by shear stress is as follows:

- Incubate anticoagulated whole blood with platelet antagonist or vehicle (control) at 37°C for 10 min.
- Place a blood specimen (typically ~500 µl) on the stationary platen of a cone-and-plate viscometer maintained at 37°C.
- Take a small aliquot (~3 µl) from the pre-sheared blood sample, fix it with 1% formaldehyde in D-PBS (~30 µl).
- Expose the blood specimen, in the presence or absence of a platelet antagonist, to well-defined shear levels (typically 4000 sec^{-1} to induce significant platelet aggregation in the absence of a platelet antagonist) for prescribed periods of time (typically 30 to 60 sec).
- Take a small aliquot (~3 µl) from the sheared blood specimen, and immediately fix it with 1% formaldehyde in D-PBS (~30 µl).
- Incubate the fixed blood samples with a saturating concentration of a fluorescently labeled platelet-specific antibody, such as anti-GPIb(6D1)-FITC, for 30 min in the dark.
- Dilute specimens with 2 ml of 1% formaldehyde, and analyze them by flow cytometry.

Flow cytometric analysis is used to distinguish platelets from other blood cells on the basis of their characteristic forward scatter and fluorescence profiles, as shown in Fig. 1. Data acquisition is then carried out on each sample for a set period (usually 100 sec), thereby allowing equal volumes for both the pre-sheared and sheared specimens to be achieved. As a result, the percent platelet aggregation can be determined by the disappearance of single platelets into the platelet aggregate region using the formula: $\%\ Platelet\ Aggregation = (1 - Ns/Nc \times 100)$, where *Ns* represents the single platelet population of the sheared specimen and *Nc* represents the single platelet population of the pre-sheared specimen. By comparing the extents of platelet aggregation in the presence and absence of a platelet antagonist, its antiplatelet efficacy can be readily determined.

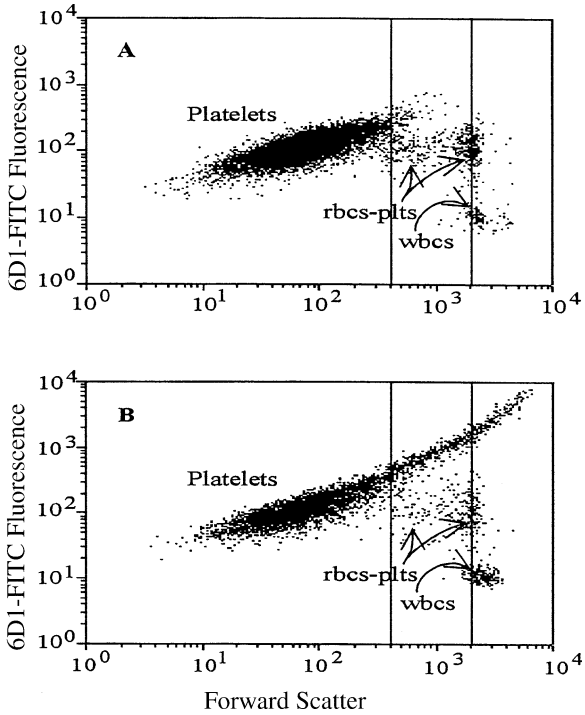

Figure 1 Quantification of shear-induced platelet aggregation by flow cytometry. Panel A corresponds to an unsheared blood specimen. Panel B corresponds to a blood specimen that has been subjected to a pathologically high level of shear stress for 30 sec. As can be seen in the figure, there are three distinct cell populations. The upper population consists of platelets and platelet aggregates. The "rbcs-plts" population corresponds to platelets associated with erythrocytes and leukocytes. The "wbcs" population consists of some leukocytes that have elevated levels of FITC autofluorescence. The left vertical line separates single platelets (≤ 4.5 µm in diameter) from platelet aggregates, whereas the right vertical line separates "small" from "large" platelet aggregates. The latter were defined to be larger than 10 µm in equivalent sphere diameter

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B.3.2 Platelet Adhesion and Aggregation Under Dynamic Shear

PURPOSE AND RATIONALE

The steps described and outlined an *in vitro* flow model of platelet thrombus formation, which can be used to evaluate the *ex vivo* and/or *in vitro* efficacy of platelet antagonists. Thrombus formation may be initiated by platelet adhesion from rapidly flowing blood onto exposed subendothelial surfaces of injured vessels containing collagen and von Willebrand factor (vWF), with subsequent platelet activation and aggregation. Konstantopolous et al. (1995) described the use of a parallel-plate flow chamber that provides a controlled and well-defined flow environment based on the chamber geometry and the flow rate through the chamber. The wall shear stress, τ_w, assuming a Newtonian and incompressible fluid, can be calculated using the formula:

$$\tau_w = \frac{6\mu Q}{wh^2}$$

where Q is the volumetric flow rate, μ is the viscosity of the flowing fluid, h is the channel height, and b is the channel width. A flow chamber typically consists of a transparent polycarbonate block, a gasket whose thickness determines the channel depth, and a glass coverslip coated with an extracellular matrix protein such as type I fibrillar collagen. The apparatus is held together by vacuum. Shear stress is generated by flowing fluid (e. g. anticoagulated whole blood or isolated cell suspensions) through the chamber over the immobilized substrate under controlled kinematic conditions using a syringe pump. Mousa et al. (2002) combined the parallel-plate flow chamber with a computerized epi-fluorescence videomicroscopy system that enables one to visualize in real time and separately quantify the adhesion and subsequent aggregation of human platelets in anticoagulated whole blood (or isolated platelet suspensions) flowing over an immobilized substrate.

PROCEDURE

Preparation of Collagen-Coated Surfaces

- Dissolve 500 mg collagen type I from bovine Achille's tendon into 200 ml of 0.5 mol/L acetic acid in water, pH 2.8.
- Homogenize for 3 hours.
- Centrifuge the homogenate at 200*g* for 10 min, collect supernatant, and measure collagen concentration by a modified Lowry analysis.
- Coat glass coverslips with 200 µl of fibrillar collagen I suspension on all but first 10 mm of the slide length (coated area = 12.7 × 23), and place in a humid environment at 37°C for 45 min.
- Rinse excess collagen with 10 ml of D-PBS maintained at 37°C before assembly into the flow chamber (Folie et al. 1988).

Platelet Perfusion Studies

- Add the fluorescent dye quinacrine dihydrochloride to anticoagulated whole blood samples at a final concentration of 10 µM immediately after blood collection.
- Prior to the perfusion experiment, incubate blood with either a platelet antagonist or vehicle (control) at 37°C for 10 min.
- Perfuse anticoagulated whole blood through the flow chamber for 1 min at wall shear rates ranging from 100 sec^{-1} (typical of venous circulation) to 1500 sec^{-1} (mimicking partially constricted arteries) for prescribed periods of time (e. g. 1 min). Platelet-substrate interactions are monitored in real time using an inverted microscope equipped with an epifluorescent illumination attachment and silicon-intensified target video camera, and recorded on videotape. The microscope stage and flow chamber are maintained at 37°C by an incubator heating module and incubator enclosure during the experiment.

EVALUATION

Videotaped images are digitized and computer analyzed at 5, 15 and 60 sec for each perfusion experiment. The number of adherent individual platelets in the microscopic field of view during the initial 15 sec of flow is determined by image processing and used as the measurement of platelet adhesion that initiates platelet thrombus formation. The number of platelets in each individual thrombus is calculated as the total thrombus intensity (area × fluorescence intensity) divided by the average intensity of single platelets determined in the 5-sec images. By comparing the extents of platelet aggregation in the presence and absence of a platelet antagonist, its antiplatelet efficacy can be de-

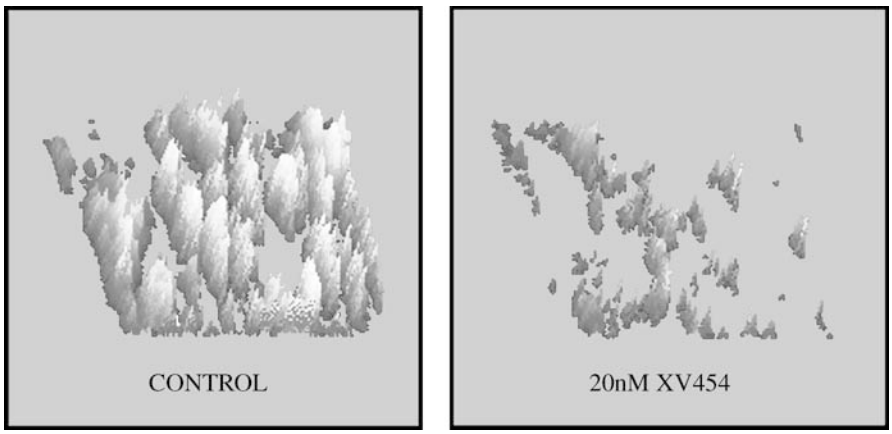

Figure 2 Three-dimensional computer-generated representation of platelet adhesion and subsequent aggregation on collagen I/von Willebrand factor from normal heparinized blood perfused in the absence (control) or presence of a GPIIb/IIIa antagonist (XV454) at 37°C for 1 min at 1,500 sec^{-1}

termined (Fig. 2). Along these lines, any potential inhibitory effects of a platelet antagonist on platelet adhesion can be readily assessed.

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Mousa SA, Abulencia JP, McCarty OJ et al (2002) Comparative efficacy between glycoprotein IIb/IIIa antagonists roxifiban and orbofiban in inhibiting platelet responses in flow models of thrombosis. J Cardiovasc Pharmacol 39:552–560

B.3.3 Cell Adhesion to Immobilized Platelets: Parallel-Plate Flow Chamber

PURPOSE AND RATIONALE

In this assay, immobilized platelets are pretreated with a GPIIb/IIIa antagonist, and any unbound drug is washed away before the perfusion of monocytic THP-1 cells. Mousa et al. (1998) demonstrated that agents with slow platelet off-rates such as XV454 ($t_{1/2}$ of dissociation = 110 min; K_d = 1 nM) and abciximab ($t_{1/2}$ of dissociation = 40 min; K_d = 9.0 nM) that are distributed predominantly as receptor-bound entities with little unbound in the plasma, can effectively block these heterotypic interactions as shown by Abulencia et al. (2001) and by Mousa et al. (2002). In contrast, agents with relatively fast platelet dissociation rates such as orbofiban ($t_{1/2}$ of dissociation = 0.2 min; K_d >110 nM), whose antiplatelet efficacy depends on the plasma concentration of the active drug, do not exhibit any inhibitory effects, as described by Mousa et al. (2002).

PROCEDURE

Preparation of 3-Aminopropyltriethoxysilane (APES)-Treated Glass Slides

- Soak glass coverslips overnight in 70% nitric acid.
- Wash coverslips with tap water for 4 hours.
- Dry coverslips by washing once with acetone, followed by immersion in a 4% solution of APES in acetone for 2 min.
- Repeat the step above, followed by a final rinse of the glass coverslips with acetone.
- Wash coverslips three times with water, and allow them to dry overnight.

Immobilization of Platelets on 3-APES-Treated Glass Slides

- Layer washed platelets or platelet-rich plasma (2×10^8 cells/ml) on the surface of a coverslip at ~30 µl/cm^2.
- Allow platelets to bind to APES-treated coverslip in a humid environment at 37°C for 30 min.

Monocytic THP-1 Cell-Platelet Adhesion Assay

- Assemble the platelet-coated coverslip on a parallel-plate flow chamber, which is then mounted on the stage of an inverted microscope equipped with a CCD camera connected to a VCR and TV monitor.
- Perfuse the antiplatelet antagonist at the desirable concentration or vehicle (control) over surface-bound platelets, and incubate for 10 min. The extent of platelet activation can be further modulated by the presence of chemical agonists such as thrombin (0.02–2 U/ml) during the 10 min incubation. The microscope stage and flow chamber are maintained

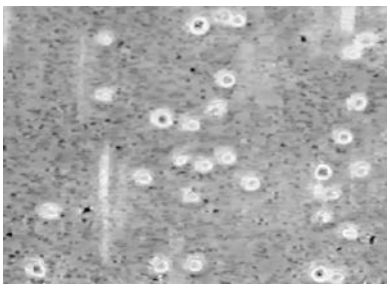

Figure 3 Phase-contrast photomicrograph of THP-1 cells (phase bright objects) attached to a layer of thrombin-treated platelets (phase dark objects) after THP-1 cell perfusion for 3 min at a shear stress level of 1.5 dyn/cm^2

at 37°C by an incubator heating module and incubator enclosure during the experiment.

- In some experiments, unbound platelet antagonist is removed by a brief washing step (4 min) prior to the perfusion of the cells of interest over the platelet layer. In others, the desirable concentration of the platelet antagonist is continuously maintained in the perfusion buffer during the entire course of the experiment.
- Perfuse cells (e. g. THP-1 monocytic cells, leukocytes, tumor cells, protein-coated beads, etc.) over surface-bound platelets, either in the presence or absence of a platelet antagonist (see above), at the desirable flow rate for prescribed periods of time. THP-1 cell binding to immobilized platelets is monitored in real time and recorded on videotape.
- Determine the extent of THP-1 cell tethering, rolling and stationary adhesion to immobilized platelets, as well as the average velocity of rolling THP-1 cells. By comparing the corresponding extents of THP-1 cell tethering, rolling and stationary adhesion to immobilized platelets in the presence and absence of a platelet antagonist (Fig. 3), its antiplatelet efficacy can be determined as shown by Mousa et al. (1995).

EVALUATION

Low-speed centrifugation results in the separation of platelets (top layer) from larger and more dense cells such as leukocytes and erythrocytes (bottom layer). To minimize leukocyte contamination in PRP specimens, slowly aspirate the uppermost two thirds of the platelet layer. Furthermore, certain rare platelet disorders, such as Bernard-Soulier Syndrome (BSS), are characterized by larger than normal platelets which must therefore be isolated by allowing whole blood to gravity separate for 2 hours post-venipuncture.

The mechanical force most relevant to platelet-mediated thrombosis is shear stress. The normal time-averaged levels of venous and arterial shear stresses range between 1–5 dyn/cm^2 and 6–40 dyn/cm^2, respectively. However, fluid shear stress may reach levels well over 200 dyn/cm^2 in small arteries and arterioles partially obstructed by atherosclerosis or vascular spasm. The cone-and-plate viscometer and parallel-plate flow chamber are two of the most common devices used to simulate fluid mechanical shearing stress conditions in blood vessels.

Due to the large concentration of platelets and erythrocytes in whole blood, small aliquots (~3µl) of pre-sheared and post-sheared specimens must be obtained and processed prior to the flow cytometric analysis. This will minimize an artifact produced as a platelet and an erythrocyte pass through the light beam of a flow cytometer at the same time.

The "rbcs-plts" population represents 3–5% of the displayed cells. A small fraction (~5%) of this population seems to be leukocyte-platelet aggregates, as evidenced by the use of an anti-CD45 monoclonal antibody. The remaining events correspond to erythrocytes associated with platelets. However, it appears that the majority of the latter population is an artifact generated by the simultaneous passage of a platelet and an erythrocyte through the beam of a flow cytometer. This concept is corroborated by the fact that further dilution of pre-sheared and sheared blood specimens and/or reduction of the sample flow rate during the flow cytometric analysis results in a dramatic relative decrease of the "rbcs-plts" population.

The collagen density remaining on glass coverslips after D-PBS rinsing can be measured by the difference in weight of 20 clean uncoated slides versus 20 collagen-treated slides.

Experiments are optimally monitored ~100–200 µm downstream from the collagen/glass interface using a 60 × FLUOR objective and 1 × projection lens, which gives a 3.2×10^4 µm^2 field of view. A field of view closer to the interface may lead to non-reproducible results due to variations in the collagen layering in that region. In contrast, positions farther downstream are avoided in order to minimize the effects of upstream platelet adhesion and subsequent aggregation on both the fluid dynamic environment as well as bulk platelet concentration.

The digitization of a background image (at the onset of perfusion prior to platelet adhesion to the collagen I surface) and its subtraction from a subsequent image acquired 5 sec after an initial platelet adhesion event allows the determination of the fluorescence in-

tensity emitted by a single platelet. The intensity level of each single platelet is measured as a mean gray level between 0 (black) and 255 (white) through the use of an image processing software (e. g. OPTIMAS; Agris-Schoen Vision Systems, Alexandria, VA), and is multiplied by its corresponding area (total number of pixels covered by each single platelet). The aforementioned products are then averaged for all single platelet events detected at the 5-sec time point, thus enabling us to calculate the average intensity of single platelets.

A single field of view ($10 \times 0.55\,mm^2$) is monitored during the 3 min period of the experiment; at the end, five additional fields of view ($0.55\,mm^2$) are monitored for 15 sec each. The following parameters can be quantified: (a) the number of total interacting cells per mm^2 during the entire 3-min perfusion experiment; (b) the number of stationary interacting cells per mm^2 after 3 min of shear flow; (c) the percentage of total interacting cells that are stationary after 3 min of shear flow; and (d) the average rolling velocity (μm/sec) of interacting cells. The number of interacting cells per mm^2 is determined manually by reviewing the videotapes. Stationary interacting cells per mm^2 are considered as those that move <1-cell radius within 10 sec at the end of the 3-min attachment assay. To quantify their number, images can be digitized from a videotape recorder using an imaging software package (e. g. OPTIMAS). Rolling velocities can be computed as the distance traveled by the centroid of the rolling THP-1 cell divided by the time interval using image processing.

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B.4 In Vivo or Ex Vivo Models

PURPOSE AND RATIONALE

The general understanding of the pathophysiology of thrombosis is based on the observations of Virchow in 1856. He proposed three factors responsible for thrombogenesis: obstruction of blood flow, changes in the properties of blood constituents (hypercoagulability), and vessel wall injury. Experimental models of thrombosis focus on one, two or all three factors of Virchow's triad. Therefore, they differ with respect to the prothrombotic challenge—either stenosis, stasis, vessel wall injury (mechanical, electrical, chemical, photochemical, laser light), insertion of foreign surface, or injection of a prothrombotic factor, and they differ with respect to the vessel type and with respect to the animal species.

Roughly, two types of models can be differentiated (Didisheim 1972; Kaiser et al. 1999; Mousa et al. 1998; Perzborn et al. 2005): (1) models in which thrombi are produced in veins by stasis and/or injection of a procoagulant factor, resulting in fibrin-rich "red" venous type thrombi; or (2) models in which thrombi are produced in arteries by vessel wall injury and/or stenosis, resulting in platelet-rich "white" mural thrombi. But the differentiation is not strict because platelets and the coagulation system influence each other. Drugs preventing fibrin formation may well act in arterial models and vice versa. Thrombosis models are usually performed in healthy animals. The underlying chronic diseases in humans, namely atherosclerosis or thrombophilias, are not included in the models. Thus, any model is limited regarding its clinical relevance. The pharmacological effectiveness of a new antithrombotic drug should be studied in more than one animal model. In spite of these limitations, animal models predict clinical effectiveness of drugs for the treatment and prevention of thrombotic diseases fairly well. A list of such drugs is presented in a recent review by Leadley et al. (2000). Furthermore, the clinical usefulness of an antithrombotic drug is determined by its safety/efficacy ratio regarding the bleeding risk. Assessment of a parameter of the hemostatic system should therefore be included in the models if possible.

The development of antithrombotic agents requires pre-clinical assessment of the biochemical and pharmacologic effects of these drugs. It is important to note that the second- and third-generation antithrombotic drugs are devoid of *in vitro* anticoagulant effects, yet *in vivo*, by virtue of endogenous interactions, these drugs produce potent antithrombotic actions. The initial belief that an antithrombotic drug must exhibit *in vitro* anticoagulant activity is no longer valid. This important scientific observation has been possible only because of the availability of animal models.

Several animal models utilizing species such as rats, rabbits, dogs, pigs and monkeys have been made available for routine use. Other animal species such as the hamster, mouse, cat and guinea pig have also been uti-

lized. Species variations are an important consideration in selecting a model and interpreting the results as these variations can result in different antithrombotic effects. Rats and rabbits are the most commonly used species in which both arterial and venous thrombosis has been investigated. Both pharmacologic and mechanical means have been used to produce a thrombogenic effect in these models. Both rat and rabbit models for studying bleeding effects of drugs have also been developed. The rabbit ear blood loss model is most commonly used to test the hemorrhagic effect of drugs. The rat tail bleeding models have also been utilized for the study of several antithrombotic drugs.

These animal models have been well established and can be used for the development of antithrombotic drugs. It is also possible to use the standardized bleeding and thrombosis models to predict the safety and efficacy of drugs. Thus, in addition to the evaluation of *in vitro* potency, the endogenous effect of antithrombotic drugs can also be investigated. Such standardized methods can be recommended for inclusion in pharmacopoeial screening procedures. Numerous models have now been developed to mimic a variety of clinical conditions where antiplatelet and antithrombotic drugs are used, including myocardial infarction, stroke, cardiopulmonary bypass, trauma, peripheral vascular diseases and restenosis. While dog and primate models are relatively expensive, they have also provided useful information on the pharmacokinetics and pharmacodynamics of antithrombotic drugs. The primate models, in particular, have been extremely useful, as the hemostatic pathways in these species are comparable to those in humans. The development of such agents as the specific glycoprotein IIb/IIIa inhibitor antibodies relies largely on these models. These models are, however, of pivotal value in the development of antithrombotic drugs and provide extremely useful data on the safety and efficacy of new drugs developed for human usage.

PROCEDURE

Animal Models of Thrombosis

In most animal models of thrombosis, healthy animals are challenged with thrombogenic (pathophysiologic) stimuli and/or physical stimuli to produce thrombotic or occlusive conditions. These models are useful for the screening of antithrombotic drugs.

I. Stasis-Thrombosis Model: Since its introduction by Wessler et al. (1959), the rabbit model of jugular stasis thrombosis has been extensively used for the pharmacologic screening of antithrombotic agents. This model has also been adapted for use in rats (Meuleman et al. 1991). In the stasis thrombosis model, a hypercoagulable state is mimicked by administration of one of a number of thrombogenic challenges, including human serum (Carrie et al. 1994), thromboplastin (Walenga et al. 1986), activated prothrombin complex concentrates (Vlasuk et al. 1991), factor Xa (Millet et al. 1994) and recombinant relipidated tissue factor (Callas et al. 1995). This administration serves to produce a hypercoagulable state. Diminution of blood flow achieved by ligating the ends of the vessel segments serves to augment the prothrombotic environment. The thrombogenic environment produced in this model simulates venous thrombosis where both blood flow and the activation of coagulation play a role in the development of a thrombus.

II. Models Based on Vessel Wall Damage: The formation of a thrombus is not solely induced by a plasmatic hypercoagulable state. In the normal vasculature, the intact endothelium provides a nonthrombogenic surface over which the blood flows. Disruption of the endothelium not only limits the beneficial effects enumerated above, but also exposes subendothelial tissue factor and collagen that serve to activate the coagulation and platelet aggregation processes, respectively. Endothelial damage can be induced experimentally by physical means (clamping, catheter), chemical means (FITC, Rose Bengal, ferrous chloride), thermal injury or electrolytic injury.

EVALUATION

Each setting in the design of an animal model can answer specific question in relation to certain thrombotic disorders in human. However, the ultimate model of human thrombosis is in human.

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B.4.1 Stenosis- and Mechanical Injury-Induced Coronary Thrombosis: Folts Model

PURPOSE AND RATIONALE

Thrombosis in stenosed human coronary arteries is one of the most common thrombotic diseases leading to unstable angina, acute myocardial infarction or sudden death. Treatment with angioplasty, thrombolysis, or by-pass grafts can expose new thrombogenic surfaces and re-thrombosis may occur. The mechanisms responsible for this process include interactions of platelets with the damaged arterial wall and platelet aggregation.

In order to study new drugs for their antithrombotic potential in coronary arteries, Folts and Rowe (1974) developed the model of periodic acute platelet thrombosis and cyclic flow reductions (CFRs) in stenosed canine coronary arteries. Uchida et al. described a similar model in 1975. The model includes various aspects of unstable angina pectoris (i. e. critical stenosis, vascular damage, downstream vasospasm induced by vasoconstrictors released or generated by platelets). The cyclic variations in coronary blood flow are a result of acute platelet thrombi that may occlude the vessel but that either embolize spontaneously or can easily be embolized by shaking the constricting plastic cylinder. They are not a result of vasospasm (Folts et al. 1982). Clinically, aspirin can reduce the morbidity and mortality of coronary thrombotic diseases but its effect is limited. Similarly, CFRs in the Folts model are abolished by aspirin but the effect can be reversed by increases in catecholamines and shear forces (Folts and Rowe 1988). As part of an expert meeting on animal models of thrombosis, a review of the Folts model has been published (Folts 1991).

Five different protocols are described in the following section for the induction of coronary thrombosis.

Coronary Thrombosis Induced by Stenosis

The described preparations are characterized by episodic, spontaneous decreases in coronary blood flow interrupted by restorations of blood flow. CFRs, which are alterations in coronary blood flow, are associated with transient platelet aggregation at the site of the coronary constriction and abrupt increase in blood flow after embolization of platelet-rich thrombi.

Damage of the vessel wall is produced by placing a hemostatic clamp on the coronary artery; a fixed amount of stenosis is produced by an externally applied obstructive plastic cylinder upon the damaged part of the vessel. In dogs, the stenosis is critical, i. e. the reactive hyperemic response to a 10-sec occlusion is abolished (protocol 1); in pigs, the stenosis is subcritical, i. e. there is a partial reactive hyperemia left (Just and Schönafinger 1991; protocol 2).

For some animals, especially for young dogs, damage of the vessel wall and stenosis is not sufficient to induce thrombotic cyclic flow variations. In these cases, an additional activation of platelets by infusion of epinephrine (protocol 3) is required, leading to the formation of measurable thrombi. In another preparation (protocol 4), thrombus formation is induced by subcritical stenosis without prior clamping of the artery and infusion of platelet activating factor (PAF), according to the model described by Apprill et al. (1985). In addition to these protocols, coronary spasms induced by released platelet components can influence coronary blood flow. Therefore, this model includes the main pathological factors of unstable angina pectoris.

Coronary Thrombosis Induced by Electrical Stimulation

In this preparation, coronary thrombosis is induced by delivery of low-amperage electrical current to the inti-

mal surface of the artery, according to the method described by Romson et al. (1980a). In contrast to the stenosis protocols, an occluding thrombosis is formed gradually without embolism after some hours (protocol 5). As a consequence of this time course, the thrombi formed are of the mixed type and contain more fibrin than the platelet thrombi with critical stenosis.

PROCEDURE

Coronary Thrombosis Induced by Stenosis

Protocol 1: Critical Stenosis Dogs of either sex weighing 15–40 kg, at least 8 months of age, are anesthetized with pentobarbital sodium (bolus of 30–40 mg/kg and continuous infusion of approx. 0.1 mg/kg/min); respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth or fifth intercostal space, the pericardium is opened and the left circumflex coronary artery (LCX) is exposed. An electromagnetic or Doppler flowprobe is placed on the proximal part of the LCX to measure coronary blood flow. Distal to the flowprobe, the vessel is squeezed with a 2-mm hemostatic clamp for 5 sec. A small cylindrical plastic constrictor, 2–4 mm in length and with an internal diameter of 1.2–1.8 mm (depending on the size of the LCX) is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times (2–5 times) until the appropriate narrowing of the vessel is achieved, and cyclic flow variations are observed. In case of an occlusion of the artery without spontaneous embolization of the formed thrombus, reflow is induced by shortly lifting the vessel with a thread placed beneath the stenotic site.

Only dogs with regularly repeated CFRs of similar intensity within a pre-treatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application. CFRs are registered for 2–4×60 min and compared to pre-treatment values.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

Protocol 2: Subcritical Stenosis Male castrated pigs (German landrace, weighing 20–40 kg) are anesthetized with ketamine (2 mg/kg i.m.), metomidate (10 mg/kg i.p.) and xylazine (1–2 mg/kg i.m.). In order to maintain the stage of surgical anesthesia, animals receive a continuous i.v. infusion of 0.1–0.2 mg/kg/min pentobarbital sodium. Respiration is maintained through a tracheal tube using a positive pressure respirator. The heart is exposed through a left thoracotomy at the fourth and fifth intercostal space, the pericardium is opened and the left descending coronary artery (LAD) is exposed. An electromagnetic or Doppler flowprobe is placed on the proximal part of the LAD to measure coronary blood flow. Distal to the flowprobe, the vessel is squeezed with a 1-mm hemostatic clamp for 5 sec. A small cylindrical plastic constrictor, 2 mm in length, is then placed around the artery at the site of the damage. Usually, the constrictor has to be changed several times until the appropriate narrowing of the vessel is achieved, which produces cyclic flow reductions. CFRs are similar to those in dogs; pigs, however, show a reactive hyperemic response. If embolization does not occur spontaneously, the formed thrombus is released at reduction of blood flow by shortly lifting the vessel with forceps.

Only pigs with regularly repeated CFRs of similar intensity within a pre-treatment phase of 60 min are used in these experiments.

The test substance is then administered by intravenous bolus injection or continuous infusion, or by intraduodenal application. CFRs are registered for 2×60 min and compared to pre-treatment values.

Protocol 3: Stenosis+Epinephrine Infusion If protocol 1 does not lead to CFRs, additionally epinephrine (0.2 µg/kg/min) is infused into a peripheral vein for twice over 60 minutes (60 min before and 60 min following drug administration). CFRs are registered and compared in the 60 min post-drug phase to the 60 min pre-drug phase.

Protocol 4: Stenosis+PAF Infusion The LCX is stenosed without prior mechanical wall injury. This preparation does not lead to thrombus formation (subcritical stenosis). For the induction of CFRs, PAF (C 16-PAF, Bachem) (0.2 nmol/kg/min) is infused into a cannulated lateral branch of the coronary artery.

After 30 min, PAF infusion is terminated and blood flow returns to its normal, continuous course. Thirty minutes later, the test substance is concomitantly administered and a second PAF infusion is started for 30 min.

CFRs are registered and compared in the drug-treated, second PAF phase to the pre-drug, first PAF phase.

Coronary Thrombosis Induced by Electrical Stimulation

Protocol 5 The LCX is punctuated distal to the flow probe with a chrome-vanadium-steel electrode (3-

mm length, 1-mm diameter). The electrode (anode) is placed in the vessel in contact with the intimal lining and connected over a Teflon coated wire to a 9 V battery, a potentiometer and an amperemeter. A disc electrode (cathode) is secured to a subcutaneous thoracal muscle layer to complete the electrical circuit. The intima is stimulated with 150 µA for 6 hours. During this time, an occluding thrombosis is gradually formed.

The test substance or the vehicle (control) is administered either at the start of the electrical stimulation or 30 min following the start.

The time interval until the thrombotic occlusion of the vessel occurs and the thrombus size (wet weight measured immediately after removal at the end of the experiment) are determined.

Prior to testing, preparations for additional hemodynamic measurements are performed (see below).

For all protocols, the following preparations and measurements are performed:

- To measure peripheral arterial blood pressure (BP [mm Hg]), the right femoral artery is cannulated and connected to a Statham pressure transducer.
- Left ventricular pressure (LVP [mm Hg]) is determined by inserting a microtip-catheter via the carotid artery retrogradely.
- Left ventricular end-diastolic pressure (LVEDP [mm Hg]) is evaluated through sensitive amplification of the LVP.
- Contractility (LV dp/dt max [mm Hg/s]) is determined from the initial slope of the LVP curve.
- Heart rate (min^{-1}) is determined from the pulsatile blood pressure curve.
- The ECG is recorded in lead II.
- Arterial pH and concentrations of blood gases are kept at physiological levels by adjusting respiration and infusion of sodium bicarbonate.
- Blood hematocrit values (37%–40%) and number of erythrocytes are kept constant by infusion of oxypolygelatine in dogs and electrolyte solution in pigs.
- Body temperature is monitored with a rectal thermistor probe and kept constant by placing the animals on a heated metal pad with automatic regulation of temperature.
- Template buccal mucosal bleeding time is carried out using the Simplate$^{\hat{a}}$ device.

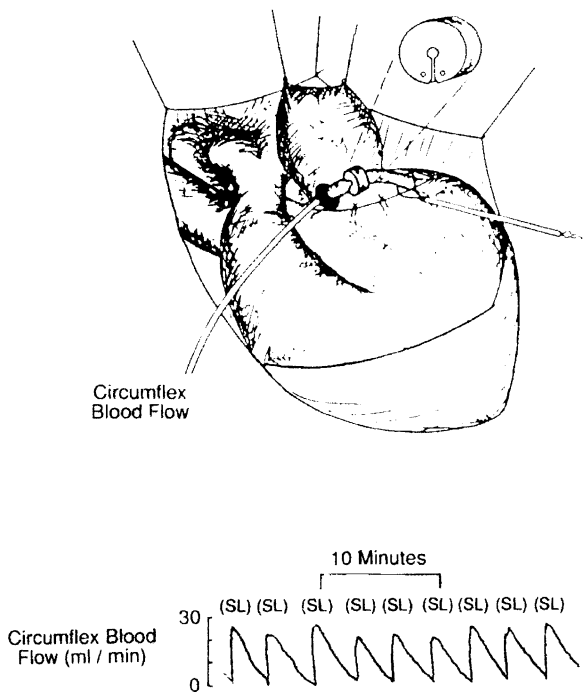

Figure 4 Technique for monitoring platelet aggregation in the partially obstructed left circumflex coronary artery of the dog. Electromagnetic flow probes measure blood flow. Partial obstruction of the coronary artery with a plastic Lexan cylinder results in episodic cyclical reductions in coronary blood flow that are due to platelet-dependent thrombus formation. Every 2–3 mm the thrombus must be mechanically shaken loose to restore blood. For detailed application of the Folts model, see Folts, Rowe (1974, 1988); and Folts et al. (1976, 1982)

EVALUATION

For all protocols, the mean maximal reduction of blood pressure (systolic/diastolic [mm Hg]) is determined.

Protocols 1–4 The following parameters are determined to quantify stenosis-induced coronary thrombosis:

- Frequency of CFRs = cycle number per time
- Magnitude of CFRs = cycle area (mm^2)

Percent change in cycle number and cycle area after drug treatment is calculated compared to pre-treatment controls.

Statistical significance is assessed by the paired Student's t-test.

Protocol 5 The following parameters are determined to quantify electrically-induced coronary thrombosis:

- Occlusion time (min) = time to zero blood flow
- Thrombus size (mg) = wet weight of the thrombus immediately after removal.

Percent change in mean values for occlusion time and thrombus size in drug-treated groups is compared to the control group.

Statistical significance is assessed by the non-paired Student's *t*-test.

CRITICAL ASSESSMENT

Both, the stenosis (Folts) and the electrical (Romson/Lucchesi) model of coronary thrombosis are widely used to study the role of mediators in the thrombotic process and the effect of new antithrombotic drugs. Bush and Patrick (1986) reviewed the role of the endothelium in arterial thrombosis and the effect of some inhibitors and mediators in the Folts model, e. g. thromboxane, prostacyclin, cyclooxygenase, serotonin, NO-donors and other vasodilators. The effect of an NO-donor could be reversed by the NO-scavenger oxyhemoglobin indication that indeed NO was responsible for the anti-thrombotic action (Just and Schönafinger 1991). Recent mechanisms of antithrombotic drug action that have been studied in either of the two coronary thrombosis models are the oral GP IIb/IIIa antagonist DMP 728 (Mousa et al. 1996); the LMWH enoxaparin (Leadley et al. 1998), which inhibited CFRs in contrast to unfractionated heparin; the thrombin inhibitors PEG-hirudin (Ruebsamen and Kirchengast 1998) and melagatran (Mehta et al. 1998); an anti-P-selectin antibody (Ikeda et al. 1999); and activated protein C (Jackson et al. 2000).

The clinical relevance of studies in the Folts model has been questioned because the model is very sensitive to antithrombotic compounds. However, the lack of a reversal of the effect by epinephrine or increase in degree of stenosis differentiates any new drug from aspirin. Electrical coronary thrombosis is less sensitive: e. g. aspirin has no effect, and with some drugs higher dose levels are required; however, in principle, most drug mechanisms act in both models if at all.

MODIFICATIONS OF THE METHOD

Romson et al. (1980b) described a simple technique for the induction of coronary artery thrombosis in the conscious dog by delivery of low-amperage electric current to the intimal surface of the artery.

Benedict et al. (1986) modified the electrical induction of thrombosis by use of two Doppler flow probes proximal and distal to the needle electrode in order to measure changes in blood flow velocity. The electrical current was stopped at 50% increase in flow velocity, and thrombosis then occurred spontaneously. The important role of serotonin was demonstrated by increases in coronary sinus serotonin levels just prior to occlusion.

Warltier et al. (1987) described a canine model of thrombin-induced coronary artery thrombosis, as well as the effects of intracoronary streptokinase on regional myocardial blood flow, contractile function, and infarct size.

Al-Wathiqui et al. (1988) described the induction of cyclic flow reduction in the coronary, carotid, and femoral arteries of conscious chronically instrumented dogs.

The method of Folts thrombosis has also been applied to carotid arteries in monkeys. Coller et al. (1989) induced CFRs in carotid arteries of anesthetized cynomologus monkeys and showed abolition by the GP IIb/IIIa antibody abciximab.

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B.4.2 Stenosis- and Mechanical Injury-Induced Arterial and Venous Thrombosis: Harbauer-Model

PURPOSE AND RATIONALE

Harbauer et al. (1984) and Harbauer and Allendorf (1988) first described a venous model of thrombosis induced by mechanical injury and stenosis of the jugular vein. In a modification, both arterial and venous thrombosis is produced in rabbits by stenosis of the carotid artery and the jugular vein with simultaneous mechanical damage of the endothelium. This activates platelets and the coagulation system and leads to changes in the bloodstream pattern. As a consequence, occluding thrombi are formed as detected by blood flow measurement. The dominant role of platelets in this model is shown by the inhibitory effect of an antiplatelet serum in both types of vessels (Just 1986). The test is used to evaluate the antithrombotic capacity of compounds in an *in vivo* model of arterial and venous thrombosis where thrombus formation is highly dependent on platelet activation.

PROCEDURE

Male Chinchilla rabbits weighing 3–4 kg receive the test compound or the vehicle (controls) by oral, intravenous or intraperitoneal administration. The first ligature (vein, preparation see below) is performed at the end of absorption (i.p. approx. 30 min, p.o. approx. 60 min, i.v. variable).

Sixty-five minutes before stenosis, the animals are sedated by intramuscular injection of 8 mg/kg xylazine (Rompun) and anesthetized by intravenous injection of 30–40 mg/kg pentobarbital sodium 5 min later. During the course of the test, anesthesia is maintained by continuous infusion of pentobarbital sodium (30–40 mg/kg/h) into one femoral vein. A Statham pressure transducer is placed into the right femoral artery for continuous measurement of blood pressure. Spontaneous respiration is maintained through a tracheal tube. One jugular vein and one carotid artery are exposed on opposite sides. Small branches of the vein are clamped to avoid blood flow in spite of vessel occlusion. Electromagnetic or Doppler flow probes are placed on the vein (directly central to the vein branching) and on the artery (as far central as possible). Blood flow (ml/min) is measured continuously. After reaching steady state (approx. 15–30 min), a metal rod with a diameter of 1.3 mm is placed on the jugular vein (2 cm central to the vein branching) and a ligature is tightened. After 1 min, the rod is removed from the ligature. Immediately thereafter (approx. 1.5 min), the carotid artery is damaged by briefly squeezing it with forceps. Then a small plastic constricting cylinder 1.2 mm wide and 2 mm long is placed around the site of the endothelial damage.

In addition, the template bleeding time is measured at various time interval before and after drug treatment (depending on the route of administration) in the shaved inner ear using the Simplate device. Care is taken to select parts of the skin without larger vessels.

EVALUATION

Percent thrombus formation (= thrombosis incidence) is judged by determination of the number of occluded vessels (blood flow = 0).

Percent inhibition of thrombosis incidence is calculated in dosed groups as compared to vehicle controls. Thrombosis incidence is always 100% in vehicle controls.

Statistical significance is assessed by means of the Fisher exact test.

If initial values for blood flow do not significantly differ in dosage and control groups, the area below the blood flow curves is measured by planimetry in addition, and mean values in dosed groups are compared to controls by means of the unpaired Student's *t*-test. Mean values of occlusion times [min] in dosage and

control groups are calculated and compared by means of the t-test.

The maximal change in systolic and diastolic blood pressure during the period of stenosis as compared to the initial values before drug administration is determined. There is no standardized assessment score. As an example, a reduction of systolic blood pressure by 30 mm Hg and of diastolic blood pressure by 20 mm Hg is quoted as a strong reduction in blood pressure.

CRITICAL ASSESSMENT OF THE METHOD

Two main factors of arterial thrombosis are essential in this model: high-grade stenosis and vessel wall damage. In the absence of either, no thrombus is found. The occlusive thrombus is formed fast and in a highly reproducible manner. In both vessels thrombus formation is equally dependent on platelet function, as shown by antiplatelet serum. Therefore, the jugular vein thrombosis in this model differs from stasis-induced deep vein thrombosis with predominant fibrin formation. On the other hand, these occlusive thrombi are more stable than the pure platelet thrombi in the Folts model since carotid blood flow cannot be restored by shaking the constrictor. The following antithrombotic drugs are effective: (i) antiplatelet drugs like ticlopidine, prostacyclin/iloprost, NO-donors (SNP, molsidomine) but not aspirin, thromboxane-synthase-inhibitors; (ii) anticoagulants like hirudin, high-dose heparin, warfarin; and (iii) streptokinase/t-PA (Bevilacqua et al. 1991, Just 1986). In contrast, drugs that only lower blood pressure—such as hydralazine, clonidine, and prazosin—have no effect on thrombus formation in this model.

MODIFICATIONS OF THE METHOD

Bevilacqua et al. (1991) performed the same model in rabbit carotid arteries but compared the procedure in one artery before drug treatment with the contralateral artery after drug treatment. Heparin, the synthetic thrombin inhibitor FPRCH2Cl, iloprost and t-PA inhibited carotid occlusion in this model but not aspirin.

Spokas and Wun (1992) produced venous thrombosis in the vena cava of rabbits by vascular damage and stasis. The vascular wall was damaged by crushing with hemostat clamps. A segment of the vena cava was looped with two ligatures, 2.5 cm apart. At 2 h after ligation, the isolated venous sac was dissected and the clot removed for determination of dry weight.

Lyle et al. (1995) searched for an animal model mimicking the thrombotic reocclusion and restenosis occurring in several cases after successful coronary angioplasty in man. The authors developed a model of angioplasty-induced injury in atherosclerotic rabbit femoral arteries. Acute ^{111}indium-labelled platelet deposition and thrombosis were assessed 4 hours after balloon injury in arteries subjected to prior endothelial damage (air desiccation) and cholesterol supplementation (1 month). The effects of inhibitors of factor X_a or platelet adhesion, heparin, and aspirin on platelet deposition were studied.

Thrombosis Induced by Cooling

Lindenblatt et al. (2005), Meng (1975), Meng and Seuter (1977) and Seuter et al. (1979) described a method to induce arterial thrombosis in rats by chilling of the carotid artery. Rats were anesthetized; the left carotid artery was exposed and occluded proximal by means of a small clamp. The artery was placed for 2 min into a metal groove which was cooled to –15°C. The vessel was compressed by a weight of 200 g. In addition, a silver clip was fixed to the vessel distally from the injured area to produce a disturbed and slow blood flow. After 4 min, the proximal clamp was removed and the blood flow reestablished in the injured artery. In the rabbit, slightly different conditions were used: the chilling temperature was –12°C for a period of 5 min, and the compressing weight was 500 g. The wound was closed, and the animal was allowed to recover from anesthesia. Antithrombotic compounds were administered in various doses at different time intervals before surgery. After 4 hours, the animals received heparin and were reanesthetized. The lesioned carotid artery was removed and thrombus wet-weight was immediately measured.

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B.4.3 Electrical-Induced Thrombosis

PURPOSE AND RATIONALE

The use of electrical current to induce thrombosis in hamster and dog has been described in the early 1950s by Lutz et al. (1951) and Sawyer et al. (1953a, b). In general, two different approaches exist. One method produces electrical damage by means of two externally applied hook-like electrodes (Hladovec 1973, Philp et al. 1978). The other method uses a needle electrode that is advanced through the walls of the blood vessels and positioned in their lumen; the second electrode is placed into a subcutaneous site completing the circuit (Salazar 1961, Romson et al. 1980, Benedict et al. 1986).

PROCEDURE

Anaesthetized rats weighing 200–300 g are intubated, and a femoral artery is cannulated for administration of drugs. One carotid artery is isolated from surrounding tissues over a distance of 10–15 mm.

A pair of rigid stainless-steel wire hook-like electrodes with a distance of 4 mm are adjusted to the artery by means of a rack and pinion gear manipulator. The artery is raised slightly away from the surrounding tissue. Isolation of the electrodes is achieved by the insertion of a small piece of parafilm under the artery. Blood flow is measured with an ultrasonic Doppler flowmeter (Transonic, Ithaca NY, USA); the flow probe (1RB) is placed proximal to the damaged area.

Thrombus formation is induced in the carotid arteries by the application of an electrical current (350 V, DC, 2 mA) delivered by an electrical stimulator (Stoelting Co, Chicago, Cat. No 58040) for 5 min to the exterior surface of the artery.

EVALUATION

- Blood flow before and after induction of thrombus for 60 min
- Time to occlusion (min): the time between onset of the electrical current and the time at which blood flow decreases under 0.3 ml/min
- Patency of the blood vessel over 30 min.

CRITICAL ASSESSMENT OF THE METHOD

The electrical-induced thrombus is composed of densely packed platelets with some red cells. Moreover, the electrical injury causes extensive damage to intimal and subintimal layers. The endothelium is completely destroyed, and this damage extends to subendothelial structures including smooth muscle cells. The deep damage could reduce the possibility of discrimination between drugs on the basis of their antithrombotic activity. However, Philp et al. (1978) could show that unfractionated heparin completely blocked thrombus formation, whereas other antiplatelet agents displayed differentiated antithrombotic action. He concluded that this relatively simple model of arterial thrombosis might prove a useful screening test for drugs with antithrombotic potential.

MODIFICATIONS OF THE METHOD

The technique described by Salazar et al. (1961) uses a stainless steel electrode that is inserted into a coronary artery in the dog and that delivers anodal current to the intravascular lumen. The electrode is positioned under fluoroscopic control, which complicates the method. The technique was modified by Romson et al. (1980). They placed the electrode directly into the coronary artery of open-chest anaesthetized dogs.

Rote et al. (1993, 1994) used a carotid thrombosis model in dogs. A calibrated electromagnetic flow meter was placed on each common carotid artery proximal to both the point of insertion of an intravascular electrode and a mechanical constrictor. The external constrictor was adjusted with a screw until the pulsatile flow pattern decreased by 25% without altering the mean blood flow. Electrolytic injury to the intimal surface was accomplished with the use of an intravascular electrode composed of a Teflon-insulated silver-coated copper wire connected to the positive pole of a 9-V nickel-cadmium battery. The cathode was connected to a subcutaneous site. Injury was initiated in the right carotid artery by application of a 150 µA continuous pulse anodal direct current to the intimal sur-

face of the vessel for a maximum duration of 3 hours or for 30 min beyond the time of complete vessel occlusion, as determined by the blood flow recording. Upon completion of the study on the right carotid, the procedure for induction of vessel wall injury was repeated on the left carotid artery after administration of the test drug.

Benedict et al. (1986) introduced a procedure in which anodal current is discontinued when mean distal coronary flow velocity increased by approximately 50%, reflecting disruption of normal flow by the growing thrombus. Occlusive thrombosis occurred within 1 hour after stopping the electrical current. It was observed that the final phase of thrombosis occurred independently of electrical injury.

A ferret model of acute arterial thrombosis was developed by Schumacher et al. (1996). A 10-min anodal electrical stimulation of 1 mA was delivered to the external surface of the carotid artery while measuring carotid blood flow. This produced an occlusive thrombus in all vehicle-treated ferrets within 41 ± 3 min, with an average weight of 8 ± 1 mg. Thrombus weight was reduced by aspirin or a thromboxane receptor antagonist.

Guarini (1996) produced a completely occlusive thrombus in the common carotid artery of rats by applying an electrical current to the arterial wall (2 mA for 5 min) while simultaneously constricting the artery with a hemostatic clamp placed immediately downstream from the electrodes.

Sturgeon et al. (2006) adapted the Folts and the electric methods of arterial thrombosis in small animals. Mousa et al. (1999) used the same animal pre- and post-treatment by using left and right arterial sides.

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B.4.4 $FeCl_3$-Induced Thrombosis

PURPOSE AND RATIONALE

A variety of chemical agents has been used to induce thrombosis in animals. Topical $FeCl_3$ was described by Reimann-Hunziger (1944) and recently by Wang et al. (2006) as thrombogenic stimulus in veins. Kurz et al. (1990) showed that the thrombus produced with this method in the carotid arteries of rats is composed of platelets and red blood cells enmeshed in a fibrin network. This model is used as a simple and reproducible test for evaluation of antithrombotic (Broersma et al. 1991) and profibrinolytic test compounds (van Giezen et al. 1997).

PROCEDURE

Rats weighing between 250 and 300 g are anaesthetized with Inactin (100 mg/kg), and a polyethylene catheter (PE-205) is inserted into the trachea via a tracheotomy to facilitate breathing. Catheters are also placed in the femoral artery for blood samples and measurement of arterial blood pressure and in the jugular vein for administration of test agents. The right carotid artery is isolated and an ultrasonic Doppler flowprobe (probe 1RB, Transonic, Ithaca NY, USA) is placed on the vessel to measure blood flow. A small piece of Parafilm "M" (American Can Co, Greenwich,

CT) is placed under the vessel to isolate it from surrounding tissues throughout the experiment.

The test agent is administered by gavage or as an intravenous injection at a defined time prior to initiation of thrombus formation. Thrombus formation is induced by the application of filter paper (2 × 5 mm), saturated with 25% $FeCl_3$ solution, to the carotid artery. The paper is allowed to remain on the vessel 10 min before removal. The experiment is continued for 60 min after the induction of thrombosis. At that time, the thrombus is removed and weighed.

EVALUATION

- Blood flow before and after induction of thrombus for 60 min
- Time to occlusion (min): the time between $FeCl_3$ application and the time at which blood flow decreases under 0.3 ml/min
- Thrombus weight after blotting the thrombus on filter paper

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B.4.5 Thrombin-Induced Clot Formation in Canine Coronary Artery

PURPOSE AND RATIONALE

A canine model of thrombin-induced clot formation was developed by Gold et al. (1984) in which localized coronary thrombosis was produced in the LAD. This is a variation of the technique described by Collen et al. (1983) who used radioactively labeled fibrinogen to monitor the occurrence and extent of thrombolysis of rabbit jugular veins clots. The vessel was intentionally de-endothelialized by external compression with blunt forceps. Snare occluders were then placed proximal and distal to the damaged site, and thrombin (10 U) was injected into the isolated LAD segment in a small volume via a previously isolated side branch. Autologous blood (0.3–0.4 ml) mixed with calcium chloride (0.05 M) also was injected into the isolated LAD segment, producing a stasis-type red clot superimposed on an injured blood vessel. The snares were released 2–5 min later, and total occlusion was confirmed by selective coronary angiography. This model of coronary artery thrombosis relies on the conversion of fibrinogen to fibrin by thrombin. The fibrin-rich thrombus contains platelets, but at no greater concentration than in a similar volume of whole blood. Once the thrombus is formed, it is allowed to age for 1–2 hours, after which a thrombolytic agent can be administered to lyse the thrombus and restore blood flow.

PROCEDURE

In the initial study described by Gold et al. (1984), recombinant t-PA was characterized for its ability to lyse 2-hour-old thrombi. Tissue plasminogen activator was infused at doses of 4.3, 10, and 25 µg/kg/min, i.v, and resulted in reperfusion times of 40, 31, and 13 min, respectively. Thus, in this model of canine coronary thrombosis, t-PA exhibited dose-dependent coronary thrombolysis. Furthermore, it is possible to study the effect of different doses of t-PA on parameters of systemic fibrinolytic activation, such as fibrinogen, plasminogen, and a_2-antiplasmin, as well as to assess myocardial infarct size. For example, Kopia et al. (1988) demonstrated that streptokinase (SK) elicited dose-dependent thrombolysis in this model.

Subsequently, Gold et al. (1986 and 1988) modified the model to study not only reperfusion but also acute reocclusion. Clinically, reocclusion is a persistent problem after effective coronary thrombolysis, which is reported to occur in 15–45% of patients (Goldberg et al. 1985). Thus, an animal model of coronary reperfusion and reocclusion would be important from the standpoint of evaluating adjunctive therapies to t-PA to hasten and/or increase the response rate to thrombolysis as well as prevent acute reocclusion.

Thrombin-Induced Rabbit Femoral Artery Thrombosis: Localized thrombosis can also be produced in rabbit peripheral blood vessels such as the femoral artery by injection of thrombin, calcium chloride, and fresh blood via a side branch (Shebuski et al. 1988).

Either femoral artery is isolated distal to the inguinal ligament and traumatized distally from the lateral circumflex artery by rubbing the artery with the

jaws of forceps. An electromagnetic flow probe is placed distal to the lateral circumflex artery to monitor femoral artery blood flow (FABF). The superficial epigastric artery is cannulated for induction of the thrombus and subsequent infusion of thrombolytic agents. Localized thrombi distal to the lateral circumflex artery with snares approximately 1 cm apart are induced by the sequential injection of thrombin, $CaCl_2$ (1.25 mmol), and a volume of blood sufficient to distend the artery. After 30 min, the snares are released and FABF is monitored for 30 mm to confirm total obstruction of flow by the thrombus.

EVALUATION

The model of thrombin-induced clot formation in the canine coronary artery was modified such that a controlled high-grade stenosis was produced with an external constrictor. Blood flow was monitored with an electromagnetic flow probe. In this model of clot formation with superimposed stenosis, reperfusion in response to t-PA occurs with subsequent reocclusion. The monoclonal antibody against the human GPIIb/IIIa receptor developed by Coller et al. (1983) and tested in combination with t-PA in the canine thrombosis model hastened t-PA-induced thrombolysis and prevented acute reocclusion (Yasuda et al., 1988). These actions *in vivo* were accompanied by abolition of ADP-induced platelet aggregation and markedly prolonged bleeding time.

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B.4.6 Laser-Induced Thrombosis

PURPOSE AND RATIONALE

Thrombus formation in rat or rabbit mesenteric arterioles or venules is induced by laser beams. The test can be performed in normal or pretreated (induction of arteriosclerosis or adjuvant arthritis) animals. The mediators for thrombus formation in this method are platelet adhesion to the injured endothelial vessel wall on one hand and ADP-induced platelet aggregation on the other. Most probably, ADP is primarily released by laser beam-lysed erythrocytes, due to the fact that erythrocyte hemoglobin exerts strong adsorbability to frequencies emitted by laser beams. There is a further, secondary, aggregation stimulus following the release reaction induced by the platelets themselves.

PROCEDURE

Apparatus

- 4 W argon laser (Spectra Physics, Darmstadt, FRG); wave length: 514.5 nm; energy below the objective: 15 mW; duration of exposure: 1/30 or 1/15 sec
- Microscope ICM 405, LD-Epipland 40/0.60 (Zeiss, Oberkochen, FRG)
- Video camera (Sony, Trinicon tube)
- Recorder (Sony, U-matic 3/4")
- Videoanalyzer and correlator to determine blood flow velocity

In Vivo Experiment

Male Sprague Dawley or spontaneously hypertensive stroke-prone Wistar or Lewis rats with adjuvant induced arthritis weighing 150–300 g or New Zealand rabbits with arteriosclerosis induced by cholesterol feeding for 3 months are used. The animals receive the test compound by oral, intravenous, intraperitoneal, or subcutaneous administration. Control animals are treated with vehicle alone. Prior to thrombus induction, the animals are pretreated by s.c. injection of 0.1 mg/kg atropine sulfate solution and anaesthetized

by intraperitoneal administration of 100 mg/kg ketamine hydrochloride and 4 mg/kg xylazine.

Thrombus formation is induced 15, 30, 60 or 90 min post dosing. Investigations are performed in arterioles or venules of 13±1 µm in diameter of the fat-free ileocaecal portion of the mesentery. During the test procedure, the mesenterium is superfused with physiological saline solution or degassed paraffin liquid (37°C). The ray of the argon laser is led into the inverted ray path of the microscope by means of a ray adaptation and adjusting device. The frequency of injuries is 1 per 2 min. The exposure time for a single laser shot is 1/30 or 1/15 sec. The number of injuries necessary to induce a defined thrombus is determined. All thrombi formed during the observation period with a minimum length of 13 µm or an area of at least 25 μm^2 are evaluated. All measuring procedures are photographed by a video system.

Standard compounds:

- acetylsalicylic acid (10 mg/kg, per os)
- pentoxifylline (10 mg/kg, per os)

For detailed description and evaluation of various agents and mechanisms see the following references: Arfors et al. (1968), Herrmann (1983), Seiffge and Kremer (1984 and 1986), Seiffge and Weithmann (1987), and Weichert et al. (1983).

EVALUATION

The number of laser shots required to produce a defined thrombus is determined. Mean values and SEM are calculated.

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B.4.7 Photochemical-Induced Thrombosis

PURPOSE AND RATIONALE

In 1977, Rosenblum and El-Sabban reported that ultraviolet light can produce platelet aggregation in cerebral microvessels of the mouse after intravascular administration of sodium fluorescein. They found that in contrast to heparin, both aspirin and indomethacin prolonged the time to first platelet aggregate. Herrmann (1983) provided a detailed study in which he showed that scavengers of singlet oxygen, not of hydroxyl radicals, inhibited platelet aggregation induced by the photochemical reaction. He postulated that by exciting the intravascularly administrated fluorescein, singlet oxygen damages endothelial cells, which subsequently leads to platelet adhesion and aggregation.

PROCEDURE

Studies are performed in mesenteric arteries of 15–30 µm diameter in anesthetized rats. After intravenous injection of fluorescein isothiocyanate-dextran 70 (FITC-dextran, Sigma, 10%, 0.3 ml), the FITC-dextran in arterioles is exposed to ultraviolet light (wavelength of excitation 490 nm, wavelength of emission 510 nm).

EVALUATION

Thrombus formation is quantitated by determining the time between onset of excitation and appearance of the first platelet aggregate adhering to the vessel wall.

CRITICAL ASSESSMENT OF THE METHOD

In contrast to other thrombosis induction methods, photochemically induced thrombosis can be easily used in smaller animals. Thrombi are composed primarily of platelets; however, the primary target of the photochemical insult is the endothelial cells by means of oxygen radical damage.

MODIFICATIONS OF THE METHOD

Matsuno et al. (1991) report a method to induce thrombosis in the rat femoral artery by means of a photochemical reaction after injection of a fluorescent dye (rose Bengal, 10 mg/kg i.v.) and transillumination with a filtered xenon lamp (wave length: 540 nm). Blood flow is monitored by a pulsed Doppler flow meter. Occlusion is achieved after approximately 5–6 min. Pretreatment with heparin dose-dependently prolongs the time required to interrupt the blood flow. The model also enables one to study thrombolytic mechanisms, which had been evaluated with t-PA. A comparative

data for hirudin in various models was carried out by Just et al. (1991).

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B.4.8 Foreign-Surface-Induced Thrombosis

The presence of foreign materials in the circulation produces activation of the coagulation and the platelet system. Various prothrombotic surfaces have been used to develop experimental animal models. In contrast to many other thrombosis models, the thrombosis induced by foreign surfaces does not presuppose endothelial damage.

B.4.8.1 Wire Coil-Induced Thrombosis

PURPOSE AND RATIONALE

A classical method to produce thrombosis is based on the insertion of wire coils into the lumen of blood vessels. The model was first described by Stone and Lord (1951) in aorta of dogs and was further modified to be used in arterial coronary vessels of opened-chest dogs. The use in venous vessels was described by Kumada et al. (1980).

The formation of thrombotic material around the coil is reproducible and can be easily standardized to study pharmacological agents (Just and Schönafinger 1991, Mellott et al. 1993, Rübsamen and Hornberger 1996).

Venous thrombosis is produced in rats by insertion of a stainless steel wire coil into the inferior caval vein. Platelets as well as plasmatic coagulation are activated on the wire coil. Thrombus formation onto the wire is quantitated by measuring the protein content of the thrombotic material isolated. The kinetics of thrombus formation show an increase in weight and protein content within the first 30 min, followed by a steady state between thrombus formation and endogenous thrombolysis leading to a constant protein content of thrombi between 1 and up to 48 hours following implantation of the wire coil. Thrombosis incidence in untreated control animals in this model is 100%. The test is used to evaluate antithrombotic and thrombolytic properties of compounds in an *in vivo* model of venous thrombosis in rats.

PROCEDURE

Male Sprague-Dawley rats weighing 260–300 g receive the test compound or the vehicle (controls) by oral, intravenous or intraperitoneal administration. At the end of absorption (i.v. 1 min, i.p. 30 min, p.o. 60 min), the animals are anesthetized by intraperitoneal injection of 1.3 g/kg urethane. Through a midline incision the caudal caval vein is exposed and a stainless steel wire coil (a dental pate carrier, Zipperer size 40(st), Zdarsky Erler KG, München) is inserted into the lumen of the vein just below the left renal vein branching by gently twisting of the wire toward the iliac vein. The handle of the carrier is cut off so as to hold the back end of the wire at the vein wall. The incision is sutured and the animal is placed on its back on a heating pad (37°C). The wound is reopened after 2 hours; the wire coil is carefully removed together with the thrombus on it and rinsed with 0.9% saline. The thrombotic material is dissolved in 2 ml alkaline sodium carbonate solution (2% Na_2CO_3 in 0.1 N NaOH) in a boiling water bath for 3 min. The protein content is determined in 100 µl aliquots by the colorimetric method of Lowry. See figure below (Fig. 5).

Thrombolysis

In addition to the described preparation, for continuous infusion of a thrombolytic test solution a polyethylene catheter is inserted in the jugular vein. One and a half hours after implantation of the wire coil, the test compound or the vehicle (controls) is infused for up to 2.5 hours. The wire coil is then removed and the protein content of thrombi is determined (see above). Bernat et al. (1986) demonstrated the fibrinolytic activity of urokinase and streptokinase-human plasminogen complex in this model.

EVALUATION

Thrombosis incidence (= number of animals with thrombi in dosage groups as compared to vehicle controls) is assessed.

The mean protein content (mg) of the thrombotic material in dosage groups and vehicle controls is deter-

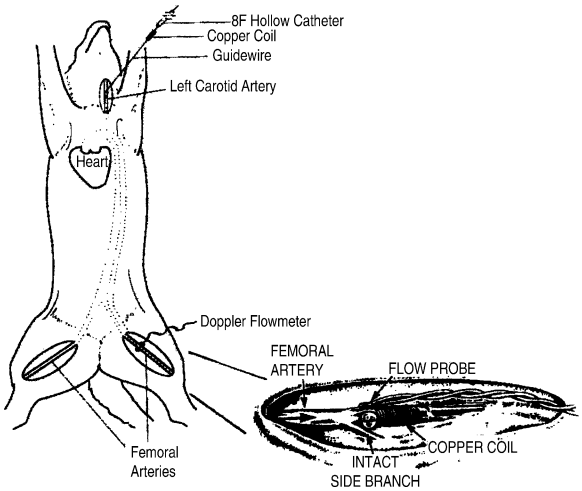

Figure 5 Schematic diagram of the canine femoral artery copper coil model of thrombolysis. A thrombogenic copper coil is advanced to either femoral artery via the left carotid artery. By virtue of the favorable anatomical angles of attachment, a hollow polyurethane catheter advanced down the left carotid artery nearly always enters the descending aorta, and with further advancement, into either femoral artery without fluoroscopic guidance. A flexible, Teflon-coated guidewire is then inserted through the hollow catheter and the latter is removed. A copper coil is then slipped over the guidewire and advanced to the femoral artery (see inset). Femoral artery flow velocity is measured directly and continuously with a Doppler flow probe placed just proximal to the thrombogenic coil and distal to a prominent sidebranch, which is left patent to dissipate any dead space between the coil and the next proximal sidebranch. Femoral artery blood flow declines progressively to total occlusion over the next 10–12 mm after coil insertion

mined. Percent change in protein content is calculated in dosage groups as compared to controls.

Statistical significance is assessed by means of the unpaired Student's *t*-test.

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B.4.8.2 Eversion Graft-Induced Thrombosis

PURPOSE AND RATIONALE

The eversion graft model for producing thrombosis in the rabbit artery was first described by Hergrueter et al. (1988) and later modified by Jang et al. (1989, 1990) and Gold et al. (1991). A 4- to 6-mm segment of the rabbit femoral or the dog left circumflex artery is excised, everted and then reimplanted into the vessel by end-to-end anastomoses. After restoration of the blood flow, a platelet-rich occlusive thrombus forms rapidly, leading to complete occlusion of the vessel. This model mimics a deep arterial injury since the adventitial surface is a non-endothelial tissue containing tissue factor and collagen. The rabbit model described here uses a carotid graft inserted into the femoral graft to avoid vasoconstriction often occurring in the inverted femoral segments.

PROCEDURE

In anaesthetized New Zealand White rabbits, the right carotid artery is exposed. After double ligation, a 3-mm segment of the artery is excised, everted and immersed in prewarmed (37°C) isotonic saline. Thereafter, the right femoral artery is exposed and occluded by means of a double-occluder (2-cm distance). The femoral artery is transected and the everted graft from the carotid artery is inserted by end-to-end anastomosis using 12 sutures with 9–0 nylon (Prolene, Ethicon, Norderstedt, Germany) under a surgical microscope (Wild M650, Leitz, Heerbrugg, Switzerland). Perfusion of the graft is measured by means of an ultrasonic flowmeter (Model T106, Transonic, Ithaca, NY, USA). The flow probe is positioned 2 cm distal from the graft. After a stabilization period of 15 min, the test substance is given intravenously through the catheterized right jugular vein. Ten minutes after substance administration, the vessel clamps are released and the blood flow is monitored by the flowmeter for 120 min.

Arterial blood is collected from the left carotid artery at baseline (immediately before substance administration), 10 min, 60 min and 120 min after substance administration.

EVALUATION

- Time until occlusion (time after restoring of vessel

blood flow until occlusion of the vessel, indicated by a flow less than 3.0 ml/min)
- Patency (time during which perfusion of graft is measured related to an observation period of 120 min after administration of test compounds).

Statistical Analysis

Time until occlusion and patency are expressed as median and the interquartile range/2 (IQR/2). Significant differences ($p < 0.05$) are calculated by the non-parametric Kruskal-Wallis test.

CRITICAL ASSESSMENT OF THE METHOD

The eversion graft is very thrombogenic, although technically difficult and time consuming. The deep occlusive thrombi can prevented only by intraarterially administered thrombolytics or aggressive antithrombotic treatments such as recombinant hirudin at high dosages, or PEG-hirudin. The adventitial surface is a non-endothelial tissue containing tissue factor and collagen. Thus, both the coagulation system and blood platelets are activated.

MODIFICATIONS OF THE METHOD

Gold et al. (1991) modified the model to be used in thoracotomized dogs in partial obstructed left circumflex coronary arteries. The combination of reduced blood flow due to the constrictor, along with an abnormal non-endothelial surface, produces total thrombotic occlusion within 5 min.

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B.4.8.3 Arteriovenous Shunt Thrombosis

PURPOSE AND RATIONALE

A method for the direct observation of extracorporal thrombus formation was introduced by Rowntree and Shionoya (1927) and extensively utilized by others (Rushkin et al. 2003; Tang et al. 2003). These first studies could provide evidence that anticoagulants like heparin and hirudin do inhibit thrombus development in arteriovenous shunts. Since today, the A-V-shunt thrombosis models have been often used to evaluate the antithrombotic potential of new compounds in different species including rabbits (Knabb et al. 1992), rats (Hara et al. 1995), pigs (Scott et al. 1994), dogs and cats (Best et al. 1938), and non-human primates (Yokoyama et al. 1995).

PROCEDURE

Rats are anaesthetized and fixed in supine position on a temperature-controlled heating plate to maintain body temperature. The left carotid artery and the right jugular vein are catheterized with short polyethylene catheters. The catheters are filled with isotonic saline solution and clamped. The two ends of the catheters are connected with a 2-cm glass capillary with an internal diameter of 1 mm. This glass capillary provides the thrombogenic surface. At a defined time after administration of the test compound, the clamps that are occluding the A-V-shunt are opened.

The measurement of the patency of the shunt is performed indirectly with a NiCrNi-thermocouple, which is fixed distal to the glass capillary. If blood is flowing, the temperature rises from room temperature to body temperature. In contrast, decreases of temperature indicate the formation of an occluding thrombus. The temperature is measured continuously over 30 min after opening of the shunt.

CRITICAL ASSESSMENT OF THE METHOD

It has been shown by Best et al. (1938) that the thrombi formed in the AV-shunt are to a greater part white arterial thrombi. This might be due to the high pressure and shear rate inside the shunts; in those cases, the thrombi tend to be more arterial in character (Chi et al. 1999).

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B.4.8.4 Thread-Induced Venous Thrombosis

PURPOSE AND RATIONALE

Compared to the arterial system, it seems to be more difficult to develop a thrombosis model in venous blood vessels with respect to reproducibility and variability (Chi et al. 1999). Complete stasis together with a thrombogenic stimulus (Wessler-type) is used by numerous investigators to evaluate the effect of compounds on venous thrombosis. Hollenbach et al. (1994) developed a rabbit model of venous thrombosis by inducing cotton threads into the abdominal vena cava of rabbits. The cotton threads serve as a thrombogenic surface, and a thrombus forms around it, growing to a maximum mass after 2–3 hours. The prolonged non-occlusive character of thrombogenesis in this model focuses on progression of thrombus formation rather than initiation. Therefore, the conditions more closely resemble pathophysiology in humans because blood continues to flow throughout the experiment (Chi et al. 1999).

PROCEDURE

Rabbits weighing between 2.5 and 3.5 kg are anaesthetized with isofluorane inhalation anesthesia, and a polyethylene catheter is inserted into the left carotid artery. A polyethylene tube (PE 240, inner diameter 1.67 mm) of 14 cm length is filled with isotonic saline, and a copper wire with 5 fixed cotton threads (length 6 cm) is inserted into the tube (after determination of the net weight of the cotton threads). A laparotomy is performed and the vena cava and iliac vein are dissected free from surrounded tissue. The test agent is administered by a rabbit intragastric tube 60 min (depending on the *ex vivo* study) prior to initiation of thrombus formation. Blood samples will be measured at 60, 90, 120, 150 and 210 min after oral administration of the test compound.

Thrombus formation is induced by the inserting the thrombosis catheter into the caval vein via the iliac vein (7 cm). Then the copper wire is pushed forward 3 cm to liberate the cotton threads into the vessel lumen. One hundred fifty minutes after thrombus initiation, the caval segment containing the cotton threads and the developed thrombus will be removed, longitudinally opened and the content blotted on filter paper. After weighing the cotton thread with the thrombus, the net thread weight will be subtracted to determine the corrected thrombus weight.

EVALUATION

- Corrected thrombus weight after blotting the thrombus on filter paper and subtraction of the net weight of the cotton thread
- Mean arterial blood pressure
- APTT, HepTest, antiFIIa and antiFXa activity.

CRITICAL ASSESSMENT OF THE METHOD

The composition of the cotton-threaded thrombus shows a composition of fibrin together with tightly aggregated and distorted erythrocytes, thus being in accordance with human deep-vein thrombosis structure. Non-occlusive thrombus formation has been successfully inhibited by heparins, prothrombinase complex inhibitors and thrombin inhibitors (Hollenbach et al. 1994, 1995).

MODIFICATIONS OF THE METHOD

In addition to the originally described method, it is possible to measure blood flow by means of an ultrasonic flow probe, attached distally to the position of the cotton threads on the vein.

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B.4.8.5 Thrombus Formation on Superfused Tendon

PURPOSE AND RATIONALE

In all models that include vessel wall damage, blood gets in contact with adhesive proteins of the subendothelial matrix, i. e. von Willebrand factor, collagens, fibronectin, laminin and others. Gryglewski et al. (1978) described an *in vivo* method where blood of an unanesthetized animal is in contact *ex vivo* with a foreign surface consisting mainly of collagen. The foreign surface is produced out of the tendon of another animal species. After superfusion of the tendon, blood is recirculated to the unanesthetized animal. The method aims at the quantitation of the antiplatelet potency of drugs based on the formation of platelet thrombi onto the surface of the tendons or of aortic strips from atherosclerotic rabbits.

PROCEDURE

Blood was withdrawn from the carotid artery of anesthetized and heparinized cats by a roller pump at a speed of 6 ml/min. After a passage through a warmed jacket (37°C), blood was separated into 2 streams, each flowing at a speed of 3 ml/min superfusing in parallel 2 twin strips of the central part of longitudinally cut rabbit Achilles tendon (30 × 3 mm). The blood superfusing the strips dripped into collectors and by its gravity was returned to the venous system of the animals through the left jugular vein. The tissue strips were freely suspended in air and the upper end was tied to an auxotonic lever of a smooth muscle/heart Harvard transducer, while the lower end was loaded with a weight (1–2 g) to keep the lever with its counterweight in a neutral position. When superfused with blood, the strips were successively covered with clots changing the weight of the strips. The weight changes were continuously recorded. After a control period of 30 min, the formed thrombi were gently removed and fixed in formalin for histological examination. Then, the strips were superfused with Tyrode solution and the animals injected with the antithrombotic drug. After 10 min, blood superfusion was renewed for another 30 min.

EVALUATION

The ratio of an increase in weight of the strips after the drug treatment to the increase in weight before drug treatment was considered as an index of antiaggregatory activity.

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B.4.9 Stasis-Induced Thrombosis (Wessler Model)

PURPOSE AND RATIONALE

The "Wessler model" is a classical method of inducing venous thrombosis in animals. Wessler (1952, 1953, 1955a & b, 1957) and Wessler et al. (1959) combined local venous stasis with hypercoagulability produced by injection of human or dog serum into the systemic circulation of dogs or rabbits. The jugular vein of these animals is occluded by clamps 1 min after the injection of the procoagulatory stimulus into the circulation. Within a few minutes after clamping, a red clot is formed in the isolated venous segment. Fareed et al. (1985) summarized a variety of substances that can be used as pro-coagulatory stimuli. Aronson and Thomas (1985) found an inverse correlation between the duration of stasis and the amount of the hypercoagulating agents to produce the clot.

PROCEDURE

Anaesthetized rabbits are fixed in supine position on a temperature-controlled (37°C) heating table. Following cannulation of both carotid arteries (the left in cranial direction) and the right femoral vein, segments of 2 cm length of the two external jugular veins are exposed and isolated between two loose sutures. Then, 0.3 ml/kg calcium thromboplastin (SIGMA, Deisenhofen, Germany, FRG) is administered via the left carotid artery. Meticulous care is taken to maintain a standard injection time of 30 sec followed by injection of 0.5 ml physiological saline within 15 sec; 45 sec later, both jugular vein segments are occluded by distal and proximal sutures. Stasis is maintained for 30 min. Blood samples are taken immediately before occlusion and 30 sec before end of stasis. After excision, the occluded vessel segments are placed on a soaked sponge and opened by a longitudinal incision.

EVALUATION

The size of the clots is assessed using a score system: (0: blood only; 1: very small clot piece[s], filling out at most 1/4 of the vessel; 2: larger clot piece[s], filling out at most 1/2 of the vessel; 3: very large clot[s], filling out at most 3/4 of the vessel; 4: one large clot, filling out the whole vessel). The scores of the left and the right jugular vein are added, forming the thrombus size value of one animal. Additionally, the thrombus weight is measured after blotting the thrombus on filter paper.

Thrombus score is expressed as median (minimum–maximum). Thrombus weight is given as mean ± SEM. For the statistical evaluation of the antithrombotic effect, the nonparametric U-Test of Mann and Whitney (thrombus score) or Student's *t*-test for unpaired samples (thrombus weight) is used. Significance is expressed as $p < 0.05$.

CRITICAL ASSESSMENT OF THE METHOD

Breddin (1989) described the Wessler model because of its static character as the retransformation of an *in vitro* experiment into a very artificial test situation. One of the major drawbacks is the relative independence of platelet function and hemodynamic changes that largely influence thrombus formation *in vivo*. However, the model has been shown to be very useful for evaluation of the antithrombotic effect of compounds like heparin and hirudin.

MODIFICATIONS OF THE METHOD

There are a number of different procoagulant agents that had been used to induce thrombosis in this model, such as human serum, Russel viper venom, thromboplastin, thrombin, activated prothrombin complex concentrates and factor X_a (Aronson and Thomas 1985, Fareed et al. 1985). The sensitivity and accuracy of the model can be improved by injecting iodinated fibrinogen into the animals before injecting the thrombogenic agent and then measuring the specific radioactivity in the clot.

The general drawback of the Wessler model is the static nature of the venous thrombus development. To overcome this problem some investigators have developed more dynamic models with reperfusion of the occluded vessel segments after clot development. Depending on the time of test compound administration (pre- or post-thrombus initiation), the effect on thrombus growth and fibrinolysis can be evaluated. Levi et al. (1992) have used this model to assess the effects of a murine monoclonal anti-human PAI-1 antibody, and Biemond et al. (1996) compared the effect of thrombin-and factor X_a-inhibitors with a low molecular weight heparin.

Venous reperfusion model: New Zealand white rabbits weighing 2.5 kg are anesthetized with 0.1 ml atropine, 1.0 mg/kg diazepam, and 0.3 ml Hypnorm (Duphar, 10 mg/ml fluanisone and 0.2 ml fentanyl). Further anesthesia is maintained with 4 mg/kg i.v. thiopental. The carotid artery is cannulated after exposition through an incision in the neck. The jugular vein is dissected free from tissue, and small side branches are ligated over a distance of 2 cm. The vein is clamped proximally and distally to isolate the vein segment. Citrated rabbit blood (from another rabbit) is mixed with ^{131}I-radiolabeled fibrinogen (final radioactivity, approximately 25 mCi/ml). Then, 150 µl of this blood is aspirated in a 1-ml syringe containing 25 µl thrombin (3.75 IU) and 45 µl 0.25 mol $CaCl_2$, and 200 µl of the clotting blood is immediately injected into the isolated segment. The vessel clamps are removed 30 min after clot injection, and blood flow is restored. ^{125}I-radio-labeled fibrinogen (approximately 5 µCi) is injected through the cannula in the carotid artery, (in case of the fibrinolysis studies immediately followed by 0.5 mg/kg recombinant tissue-type plasminogen activator). For each dosage group, four thrombi are analyzed. The extent of thrombolysis is assessed by measurement of the remaining ^{131}I-fibrinogen in the clot and compared with the initial clot radioactivity. The comparison between blood and thrombus ^{125}I-radioactivity reveals the extent of thrombus growth (blood volume accreted to the blood). The thrombus lysis and extension are monitored 60 or 120 min after thrombus formation and are expressed as percentage of the initial thrombus volume. Statistics is performed as variance analysis and the Newman-Keuls test. Statistical significance is expressed at the level of $p < 0.05$.

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B.4.10 Disseminated Intravascular Coagulation (DIC) Model

PURPOSE AND RATIONALE

DIC is another model that is also used widely in rats and mice. It is a model of systemic thrombosis or disseminated intravascular coagulation (DIC), which is induced by tissue factor, endotoxin (lipopolysaccharide), or FXa (Herbert et al. 1996, Yamazaki et al. 1994, Sato et al. 1998). After systemic administration of the thrombogenic stimulus, this model can be performed with or without mechanical vena caval stasis. When stasis is used, the major parameter is the thrombus mass, but when stasis is not used, the readouts are fibrin degradation products, fibrinogen, platelet count, PT, and APTT, among others. As shown by the many and varied parameters, when used without stenosis, the post-experimental analysis can be time-consuming and technically demanding. Although rodents are useful as a primary efficacy model, limitations such as the ability to withdraw multiple blood samples over the course of the experiment and the difference in activity of at least some FXa inhibitors in human compared to rat plasma *in vitro* require that compounds be characterized further in more advanced *in vivo* models of thrombosis.

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B.4.11 Microvascular Thrombosis in Trauma Models

PURPOSE AND RATIONALE

Successful replantation of amputated extremities is dependent in large degree on maintaining the microcirculation. A number of models have been developed in which blood vessels are subjected to crush injury with or without vascular avulsion and subsequent anastomosis (Fu et al. 1997, Korompilias et al. 1997, Stockmans et al. 1997). In the model of Stockmans (1997), both femoral veins are dissected from the surrounding tissue. A trauma clamp, which has been adjusted to produce a pressure of 1,500 g/mm^2, is positioned parallel to the long axis of the vein. The anterior wall of the vessel is grasped between the walls of the trauma clamp and the two endothelial surfaces are rubbed together for a period of 30 sec as the clamp is rotated. Formation and dissolution of platelet-rich mural thrombi are monitored over a period of 35 min by transillumination of the vessel. By using both femoral veins, the effect of drug therapy can be compared to control in the same animal, minimizing intra-animal variations.

The models of Korompilias et al. (1997) and Fu et al. (1997) examine the formation of arterial thrombosis in rats and rabbits, respectively. In these models, either the rat femoral artery or the rabbit central ear artery is subjected to a standardized crush injury. The vessels are subsequently divided at the midpoint of the crushed area and then anastomosed. Vessel patency is evaluated by milking the vessel at various time points post-anastomosis. These models have been used to demonstrate the effectiveness of topical administration of LMWH in preventing thrombotic occlusion of the vessels. Such models, while effectively mimicking the clinical situation, are limited by the necessity of a high degree of surgical skill to effectively anastomose the crushed arteries.

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B.4.12 Cardiopulmonary Bypass Models

PURPOSE AND RATIONALE

Cardiopulmonary bypass (CPB) models have been described in baboons (Van Wyk et al. 1998), swine (De-

wanjee et al. 1996) and dogs (Henny et al. 1985). In each model, the variables that can affect the hemostatic system—such as anesthesia, shear stresses caused by the CPB pumps and the exposure of plasma components and blood cells to foreign surfaces (catheters, oxygenators, etc)—are comparable to that observed with human patients. With these models, it is possible to examine the potential usefulness of novel anticoagulants in preventing thrombosis under relatively harsh conditions where both coagulation and platelet function are altered. The effectiveness of direct thrombin inhibitors (Van Wyk et al. 1998), LMWHs (Murray 1985) and heparinoids (Henny et al. 1985) has been compared to standard heparin. Endpoints have included the measurement of plasmatic anticoagulant levels, the histological determination of microthrombi deposition in various organs, the formation of blood clots in the components of the extracorporeal circuit and the deposition of radiolabeled platelets in various organs and on the components of the extracorporeal circuit. These models, therefore, can be used to assess the antithrombotic potential of new agents for use in CPB surgery and also to assess the biocompatability of components used to maintain extracorporeal circulation. For detailed protocols and evaluations see Callas et al. (1995), Carrie et al. (1994), Fu et al. (1997), Korompilias et al. (1997), Meuleman et al. (1991), Millet et al. (1994), Stockmans et al. (1997), Vlasuk et al. (1991), Walenga et al. (1987), and Wessler et al. (1959).

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B.4.13 Extracorporeal Thrombosis Models

PURPOSE AND RATIONALE

These models employ passing blood over a section of damaged vessel (or other selected substrates) and recording the thrombus accumulation on the damaged vessel histologically or by scintigraphic detection of radiolabeled platelets or fibrin (Badimon and Badimon 1989). This model is interesting because the results can be directly compared to the *in vivo* deep arterial injury model (Wysokinski et al. 1996) results and to results from a similar extracorporeal model used in humans (Dangas et al. 1998; Ørvim et al. 1995). Dangas et al. (1998) used this model to characterize the antithrombotic efficacy of abciximab, a monoclonal antibody-based platelet glycoprotein IIb/IIIa inhibitor, after administration to patients undergoing percutaneous coronary intervention. They demonstrated that abciximab reduces both the platelet and fibrin components of the thrombus, thereby providing further insight into the unique long-term effectiveness of short-term administration of this drug. Ørvim et al. (1995) also used this model in humans to evaluate the antithrombotic efficacy of rTAP, but instead of evaluating the compound after administration of rTAP to the

patient, the drug was mixed with the blood immediately as it flowed into the extracorporeal circuit prior to flowing over the thrombogenic surface. By changing the thrombogenic surface, they were able to determine that rTAP was more effective at inhibiting thrombus formation on a tissue-factor coated surface compared to a collagen-coated surface. These results suggest that optimal antithrombotic efficacy requires an antiplatelet approach along with an anticoagulant. Although this model does not completely represent pathological intravascular thrombus formation, the use of this "human model" of thrombosis may be very useful in developing new drugs because it directly evaluates the *ex vivo* antithrombotic effect of a drug in flowing human blood.

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B.4.14 Experimental Thrombocytopenia or Leucocytopenia

PURPOSE AND RATIONALE

Intravenous administration of collagen, arachidonic acid, ADP, platelet activating factor (PAF) or thrombin activates thrombocytes leading to a maximal thrombocytopenia within a few minutes. The effect is reinforced by additional injections of epinephrine. Activation of platelets leads to intravasal aggregation and temporary sequestration of aggregates in the lungs and other organs. Depending on the dose of agonist, this experimentally induced reduction of the number of circulating platelets is reversible within 60 min after induction. Following administration of PAF, a leucocytopenia is induced in addition. The assay is used to test the inhibitory capacity of drugs against thrombocytopenia or leucocytopenia as a consequence of *in vivo* platelet or leukocyte stimulation.

PROCEDURE

Male guinea pigs (Pirbright White) weighing 300–600 g, or male NMRI mice (25–36 g), or Chinchilla rabbits of either sex weighing 2–3 kg are used. Animals receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (p.o. 60 min, i.p. 30 min, i.v. variable), the marginal vein of the ear of rabbits is cannulated and the thrombocytopenia-inducing substances collagen or arachidonic acid are injected slowly. Blood is collected from the ear artery.

Guinea pigs, hamsters, or mice are anesthetized with pentobarbital sodium (i.p.) and Rompun (i.m.) and placed on an electrically warmed table at 37°C. The carotid artery is cannulated for blood withdrawal and the jugular vein is cannulated to administer the thrombocytopenia-inducing substances collagen+adrenaline (injection of the mixture of both within 10 sec) or PAF or thrombin. In mice, collagen + adrenaline are injected into a tail vein.

Approximately 50–100 µl blood is collected into potassium-EDTA-coated tubes at times –1, 1 and 2 min (guinea pigs and mice) or 5, 10 and 15 min (rabbits) following the injection of the inducer. The number of platelets and leukocytes is determined within 1 hour after withdrawal in 10 µl samples of whole blood using a microcellcounter suitable for blood of various animal species.

EVALUATION

The percentage of thrombocytes (or leukocytes) is determined in vehicle control and dosage groups at the different times following injection of the inducer relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are taken as 100%.

Percent inhibition of thrombocytopenia (or leucocytopenia) is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

CRITICAL ASSESSMENT OF THE METHOD

The method of collagen+epinephrine-induced thrombocytopenia is presently widely used to study the phenotype of mice knocked out for a specific gene with suspected role in hemostasis/thrombosis. A recent example is the *Gas* 6 –/– mouse (Angelillo-Scherrer et al. 2001) and mice lacking the gene for the G protein G(z) (Yang et al. 2000). The advantage of the method for this purpose is the simple experimental procedure

and the small volume of blood necessary. In general, application of the method in small animals (mice, hamsters) needs only small amounts of drug substance. The model is a useful first step of *in vivo* antithrombotic efficacy of antiplatelet drugs.

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B.4.15 Collagenase-Induced Thrombocytopenia

PURPOSE AND RATIONALE

Intravenous administration of the proteolytic enzyme collagenase leads to formation of endothelial gaps and to exposure of deeper layers of the vessel wall. This vascular endothelial injury is mainly involved in triggering thrombus formation by activation of platelets through contact with the basal lamina. As a consequence, thrombocytopenia is induced, which is maximal within 5–10 min following collagenase injection and reversible within 30 min after induction. The model is used to test the inhibitory capacity of compounds against thrombocytopenia in a model of collagenase-induced thrombocytopenia in rats as an alternative to the model described before.

PROCEDURE

Male Sprague-Dawley rats weighing 260–300 g are used. The animals receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), rats are anesthetized with pentobarbital sodium (i.p.). One carotid artery is cannulated for blood withdrawal and one jugular vein is cannulated for inducer injection. The animals receive an intravenous injection of heparin and 20 min later, approximately 100 µl blood is collected (initial value). Ten minutes later, the thrombocytopenia-inducing substance collagenase is administered intravenously.

At times 5, 10, 20 and 30 min following the injection of collagenase, samples of approximately 100 µl blood are collected into potassium-EDTA-coated tubes. The number of platelets is determined in 10 µl samples of whole blood within 1 hour after blood withdrawal, using a microcellcounter. See Völkl and Dierichs (1986) for details.

EVALUATION

The percentage of platelets is determined in vehicle control and dosage groups at the different times following injection of collagenase relative to the initial value of control or dosage group, respectively. Calculated percent values of controls are set at 100%.

Percent inhibition of thrombocytopenia is calculated in dosage groups relative to controls.

Statistical significance is evaluated by means of the unpaired Student's *t*-test.

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B.4.16 Reversible Intravital Aggregation of Platelets

PURPOSE AND RATIONALE

Isotopic labeling of platelets can be employed to monitor platelet aggregation and desegregation *in vivo*. Adenosine diphosphate (ADP), platelet activating factor (PAF), arachidonic acid, thrombin and collagen are known to induce platelet aggregation. In the following procedure, labelled platelets are continuously monitored in the thoracic (A) and abdominal (B) region of test animals. Administration of aggregation promoting agents produces an increase in counts in A and a fall in counts in B. This observation implies that platelets are being aggregated within the vascular system and accumulate in the pulmonary microvasculature. The *in vivo* method can be used to evaluate platelet antiaggregatory properties of test compounds.

PROCEDURE

Preparation of Labeled Platelets

Blood is obtained from rats by cardiopuncture. After centrifugation at 240 *g* for 10 min, the platelet-rich plasma (PRP) is transferred into a tube and suspended in calcium-free Tyrode solution containing 250 ng/ml PGE_1. The suspension is centrifuged at 640 *g* for 10 min. The supernatant is discarded and the sediment is suspended by gentle shaking with calcium-free Tyrode solution containing 250 ng/ml PGE_1. ^{51}Cr is added to 1 ml of the platelet suspension. Following a 20-min incubation period at 37°C, the suspension is again centrifuged at 640 *g* for 10 min. The supernatant

is removed, and the labeled platelets are finally resuspended in 1 ml calcium-free Tyrode solution containing 250 ng/ml PGE_1.

In Vivo Experiment

Male Sprague-Dawley or stroke-prone spontaneously hypertensive rats weighing 150–300 g are used. The animals are anaesthetized with pentobarbital sodium (30 mg/kg, i.p.). Following tracheotomy, the vena femoralis is exposed and cannulated. The labeled platelets are administered via the cannula. The circulating platelets are monitored continuously in the thoracic (A) and abdominal (B) region. The counts are collected using a dual-channel gamma spectrometer (Nuclear Enterprise 4681) incorporating a microcomputer (AM 9080A). One hour after administration of labeled platelets (when counts in A and B have stabilized), the aggregation-promoting agent (ADP, PAF, arachidonic acid, thrombin or collagen) is administered twice by intravenous injection. One hour is allowed to elapse between each i.v. injection.

The test compound is administered 2 hours after platelet injection concurrently with the fourth administration of the aggregating agent. Thirty minutes (ADP, PAF, arachidonic acid, thrombin), or 1 hour (collagen) after compound administration another control injection of the aggregating agent is given. This injection is either used as an additional control or it may reveal long-term efficacy of a test compound.

EVALUATION

The microcomputer continuously reveals information about aggregation and desegregation of labeled platelets.

The following parameters are recorded:
A = counts over thorax
B = counts over abdomen
Difference: *A*–*B*
ratio: *A/B*.

The time course of response is shown in a curve. The area under the curve is calculated by a computer program.

Statistical significance is calculated using the Student's *t*-test.

MODIFICATION OF THE METHOD

Oyekan and Botting (1986) described a method for monitoring platelet aggregation *in vivo* in rats, using platelets labeled with indium^{3+} oxine and recording the increase in radioactivity count in the lung after injection of adenosine diphosphate or collagen.

Smith et al. (1989) monitored continuously the intrathoracic content of intravenously injected ^{111}indium-labeled platelets in anesthetized guinea pigs using a microcomputer-based system.

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B.5 Bleeding Models

B.5.1 Subaqueous Tail Bleeding Time in Rodents

PURPOSE AND RATIONALE

The damage of a blood vessel results in the formation of a hemostatic plug, which is achieved by several different mechanisms including vascular spasm, formation of a platelet plug, blood coagulation, and growth of fibrous tissue into the blood clot.

A diagnostic parameter for specific defects of the hemostatic system and for the influence of drugs affecting hemostasis is the length of time that it takes for bleeding to stop from a standard incision, the so-called bleeding time.

Bleeding-time measurements in animals are used to evaluate the hemorrhagic properties of antithrombotic drugs. The transection of the tail of a rodent was first established by Döttl and Ripke (1936) and is commonly used in experimental pharmacology.

PROCEDURE

Anaesthetized rats are fixed in supine position on a temperature-controlled (37°C) heating table. Following catheterization of a carotid artery (for measurement of blood pressure) and a jugular vein, the test compound is administered. After a defined latency period, the tail of the rat is transected with a razor-blade mounted on a self-constructed device at a distance of 4 mm from the tip of the tail. Immediately after transection, the tail is immersed into a bath filled with isotonic saline solution (37°C).

EVALUATION

The time until bleeding stops is determined within a maximum observation time of 600 sec.

CRITICAL ASSESSMENT OF THE METHOD

There are numerous variables that can influence rodent's bleeding time measurements, as discussed by Dejana et al. (1979): position of the tail (horizontal or vertical), the environment (air or saline), temperature, anesthesia, or procedure of injury (Simplate method, transsection). All these variables are responsible for the different results reported in literature on compounds like aspirin and heparin under different assay condition (Stella et al. 1975; Minsker and Kling 1977).

Furthermore, it is impossible to transect exactly one blood vessel, because the transected tail region consists of a few major arteries and veins with mutual interaction between one another.

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B.5.2 Arterial Bleeding Time in Mesentery

PURPOSE AND RATIONALE

Arterial bleeding is induced by micropuncture of small arteries in the area supplied by the mesenteric artery. Bleeding is arrested in living blood vessels by the formation of a hemostatic plug due to the aggregation of platelets and to fibrin formation. In this test, compounds are evaluated that inhibit thrombus formation, thus prolonging arterial bleeding time. The test is used to detect agents which interfere with primary hemostasis in small arteries.

PROCEDURE

Male Sprague-Dawley rats weighing 180–240 g receive the test compound or the vehicle (controls) by oral, intraperitoneal or intravenous administration. After the end of the absorption time (i.p. 30 min, p.o. 60 min, i.v. variable), the animals are anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. Rats are placed on an electrically warmed table at 37°C.

The abdomen is opened by a mid-line incision and the mesentery is lifted to display the mesenteric arteries. The mesentery is draped over a plastic plate and superfused continuously with Tyrode's solution maintained at 37°C. Bleeding times are determined with small mesenteric arteries (125–250 µm external diameter) at the junction of mesentery with intestines. Adipose tissue surrounding the vessels is carefully cut with a surgical blade.

Arteries are punctured with a hypodermic needle (25 gauge: 16 × 5/10 mm). The bleeding time of the mesenteric blood vessels is observed through a microscope at a magnification of 40×. The time in seconds is determined from the puncturing until the bleeding is arrested by a hemostatic plug.

EVALUATION

1. Mean values of bleeding times are determined for each dosage group (4–6 animals, 4–6 punctures each) and compared to the controls.
2. The significance of the results is assessed with the unpaired Student's *t*-test.
3. The percent prolongation of bleeding time in dosage groups relative to the vehicle controls is calculated.

For further details on methods and evaluations of various mechanisms or agents see the following: Butler et al. (1982), Dejana et al. (1979), and Zawilska et al. (1982).

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B.5.3 Template Bleeding Time Method

PURPOSE AND RATIONALE

The template bleeding time method is used to produce a standardized linear incision into the skin of humans to detect abnormalities of primary hemostasis due to deficiencies in the platelet or coagulation system. The method has been modified with the development of a spring-loaded cassette with two disposable blades (Simplate II, Organon Teknika, Durham, NC). These template devices ensure reproducibility of length and depth of dermal incisions. Forsythe and Willis (1989) described a method that enables the Simplate tech-

nique to be used as a method to analyze the bleeding time in the oral mucosa of dogs.

PROCEDURE

The dog is positioned in sternal or lateral recumbency. A strip of gauze is tied around both the mandible and maxilla as a muzzle. The template device is placed evenly against the buccal mucosa, parallel to the lip margin, and triggered. Simultaneously, a stopwatch is started. Blood flow from the incision is blotted using circular filter paper (Whatman No. 1, Fisher Scientific Co, Clifton, NJ) held directly below, but not touching the wounds. The position of the filter paper is changed every 15 sec. The end point for each bleeding is determined when the filter paper no longer develops a red crescent.

EVALUATION

The time from triggering the device until blood no longer appears on the paper is recorded as the bleeding time. The normal range lies between 2 to 4 min.

CRITICAL ASSESSMENT OF THE METHOD

The template bleeding time varies considerably between laboratories as well as between species and strains. Therefore, it is important to perform the incisions and the blotting in an identical fashion. Prolonged bleeding times in dogs have been recognized with thrombocytopenia, von Willebrand's disease, uremia, treatment with aspirin, anticoagulants, and dextran (Forsythe and Willis 1989, Klement et al. 1998). Brassard and Meyers (1991) describe the buccal mucosa bleeding time as a test that is sensitive to platelet adhesion and aggregation deficits. Generally, results of antithrombotic drugs in bleeding time models in animals do not exactly predict bleeding risks in clinical situations. But the models allow comparison between drugs with different actions (Dejana et al. 1979, Lind 1991).

MODIFICATIONS OF THE METHOD

The Simplate device can also be used to perform incisions at the shaved inner ear of rabbits, taking care to avoid major vessels. The normal range of bleeding time in anaesthetized rabbits is approximately 100 sec.

Klement et al. (1998) described another ear bleeding model in anaesthetized rabbits. The shaved ear was immersed in a beaker containing saline at 37°C. Five full-thickness cuts were made with a no. 11 Bard-Parker scalpel blade, avoiding major vessels, and the ear was immediately re-immersed in saline. At different times thereafter (5 to 30 min) aliquots of the saline solution were removed, red cells were sedimented and lysed, and cyanohemoglobin was determined as a measure of blood loss. In this study, hirudin produced more bleeding than standard heparin.

A cuticle bleeding time (toenail bleeding time) measurement in dogs has been described by Giles et al. (1982). A guillotine-type toenail clipper is used to sever the apex of the nail cuticle. A clean transection of the nail is made just into the quick, to produce a free flow of blood. The nail is left to bleed freely. The time until bleeding stops is recorded as the bleeding time. Several nails can be cut at one time to ensure appropriate technique. The normal range lies between 2 to 8 min.

Kubitza et al. (2005) and others extended the use of the model in humans.

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B.6 Genetic Models of Hemostasis and Thrombosis

PURPOSE AND RATIONALE

Recent advances in genetic molecular biology have provided tools allowing scientists to design genetically altered animals that are deficient in certain proteins involved in thrombosis and hemostasis (so-called "knock-outs", or "nulls") (Carmeliet and Collen 1999, Pearson and Ginsburg 1999). These animals have been extremely useful for identifying and validating novel targets for therapeutic intervention. That is, by examin-

ing the phenotype (e.g, spontaneous bleeding, platelet defect, prolonged bleeding after surgical incision, etc.) of a specific knock-out strain, scientists can identify the role of the knocked-out protein. Then if the phenotype is favorable (e.g, not lethal), pharmacological agents can be designed to mimic the knock-out. More recently, novel gene medicine approaches have also benefited greatly from the availability of these models, as discussed below. The following section briefly summarizes some of the major findings in thrombosis and hemostasis using genetically altered mice and concludes with an example of how these models have been used in the drug discovery process.

The majority of these gene knock-outs result in mice that develop normally, are born in the expected Mendelian ratios, and are viable (as defined by the ability to survive to adulthood). Although seemingly normal, these knock-out mice display alterations in hemostatic regulation, especially when challenged. Deletion of FVIII, FIX, vWF, and the β_3-integrin (Bi et al. 1996, Denis et al. 1998, Hodivala-Dilke et al. 1999, Wang et al. 1997) all result in mice that bleed upon surgical challenge, and despite some minor differences in bleeding susceptibility, these mouse knock-out models mirror the human disease states quite well (hemophilia A, hemophilia B, von Willebrand disease, and Glanzmann's thrombasthenia, respectively). In addition, deletion of some hemostatic factors results in fragile mice with severe deficiencies in their ability to regulate blood loss. Prenatally, these mice appear to develop normally, but they are unable to survive the perinatal period due to severe hemorrhage, in most cases due to the trauma of birth.

Genetic knock-outs have also been useful in dissecting the role of individual signaling proteins in platelet activation. Deletion of the β_3-integrin (Hodivala-Dilke et al. 1999) or of $G_{\alpha q}$ (Offermanns et al. 1997) results in dramatic impairment of agonist-induced platelet aggregation. Alteration of the protein-coding region in the β_3-integrin carboxy-tail, β_3-DiY, at sites that are thought to be phosphorylated upon platelet activation, also results in unstable platelet aggregation (Law et al. 1999). Deletion of various receptors such as thromboxane A_2, P-selectin, P2Y1, and PAR-3 demonstrate diminished responses to some agonists while other platelet responses are intact (Thomas et al. 1998, Subramaniam et al. 1996, Leon et al. 1999, Kahn et al. 1998). Deletion of PAR-3, another thrombin receptor in mice, has little effect on hemostasis. This indicated the presence of yet another thrombin receptor in platelets and led to the identification of PAR-4 (Kahn et al. 1998).

Given that knock-outs of prothrombotic factors yield mice with bleeding tendencies, it follows that deletion of factors in the fibrinolytic pathway results in increased thrombotic susceptibility in mice. Plasminogen (Bugge et al. 1995; Ploplis et al. 1995), tissue plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), and the combined t-PA/u-PA knock-out (Carmeliet et al. 1994) result in mice that demonstrate impaired fibrinolysis, susceptibility for thrombosis, vascular occlusion, and tissue damage due to fibrin deposition. Interestingly, due to fibrin formation in the heart, these mice may provide a good model of myocardial infarction and heart failure caused by thrombosis (Christie et al. 1999). Intriguingly, mice deficient in PAI-1, the primary inhibitor of plasminogen activator, demonstrate no spontaneous bleeding and a greater resistance to venous thrombosis due to a mild fibrinolytic state (Carmeliet et al. 1993), suggesting that inhibition of PAI-1 might be a promising approach for novel antithrombotic agents.

In addition to their role in the regulation of hemostasis, several of these genes are important in embryonic development. For example, deletion of tissue factor (Bugge et al. 1996; Toomey et al. 1996; Carmeliet et al. 1996), tissue factor pathway inhibitor (Huang et al. 1997), or thrombomodulin (Healy et al. 1995) results in an embryonic lethal phenotype. These and other (Connolly et al. 1996, Cui et al. 1996) hemostatic factors also appear to contribute to vascular integrity in the developing embryo. These data suggest that initiation of coagulation and generation of thrombin is important at a critical stage of embryonic development, yet other factors must contribute since some of these embryos are able to progress and survive to birth.

Clearly, genetically altered mice have provided valuable insight into the roles of specific hemostatic factors in physiology and pathophysiology. Results of these studies have provided rationale and impetus for attacking certain targets pharmacologically. These types of models have also provided excellent model systems for studying novel treatments for human diseases. For example, these models provided exceptional systems for studying gene therapy for hemophilia. Specifically, deletion of FIX, generated by specific deletions in the *FIX* gene and its promoter, results in mice that mimic the human phenotype of hemophilia B (Lin et al. 1997). When these mice are treated with adenoviral mediated transfer of human FIX, the bleeding diathesis is fully corrected (Kung et al. 1998). Similarly, selectively bred dogs that have a characteristic point mutation in the sequence encoding the catalytic domain of FIX also have a severe hemophilia B that is

phenotypically similar to the human disease (Evans et al. 1989). When adeno-associated virus-mediated canine FIX gene was administered to these dogs intramuscularly, therapeutic levels of FIX were measured for up to 17 months (Herzog et al. 1999). Clinically relevant partial recovery of whole blood clotting time and APTT was also observed over this prolonged period. These data provided support for initiating the first study of adeno-associated virus-mediated FIX gene transfer in humans (Kay et al. 2000). Preliminary results from this clinical study provided evidence for expression of FIX in the three hemophilia patients studied and also provided favorable safety data to substantiate studying this therapy at higher doses. Although it is likely that there are differences between the human disease and animal models of hemophilia (or other diseases), it is clear that these experiments have provided pharmacological, pharmacokinetic, and safety data that were extremely useful in developing this approach and designing safe clinical trials.

Gene therapy approaches to rescuing patients with bleeding diatheses are further advanced than gene therapy for thrombotic indications. However, promising preclinical data indicates that local overexpression of thrombomodulin (Waugh et al. 1999a) or tissue plasminogen activator (Waugh et al. 1999b) inhibits thrombus formation in a rabbit model of arterial thrombosis. Similarly, local gene transfer of tissue factor pathway inhibitor prevented thrombus formation in balloon-injured porcine carotid arteries (Zoldhelyi et al. 2000). These and other studies (Vassalli et al. 1997) suggest that novel gene therapy approaches will also be effective for thrombotic indications, but these treatments will need to be carefully optimized for pharmacokinetics, safety and, efficacy in laboratory animal studies prior to administration to humans.

Genetically Modified Animals

Development and application of animal models of thrombosis has played a crucial role in discovering and validating novel drug targets, selecting new agents for clinical evaluation, and providing dosing and safety information for clinical trials. In addition, these models have provided valuable information regarding the mechanisms of these new agents and the interactions between antithrombotic agents that work by different mechanisms. The development and application of small and large animal models of thrombosis to the discovery and development of novel antithrombotic agents is described in this review. The methods and major issues regarding the use of animal models of thrombosis, such as positive controls, appropriate pharmacodynamic markers of activity, safety evaluation, species-specificity, and pharmacokinetics, are highlighted. Finally, the use of genetic models of thrombosis/hemostasis is presented using gene-therapy for hemophilia as an example of how animal models have aided in the development of therapies that are presently being evaluated clinically.

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Healy AM, Rayburn HB, Rosenberg RD, Weiler H (1995) Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci USA 92:850–854

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Hodivala-Dilke KM, McHugh KP, Tsakiris DA et al (1999) β_3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 103:229–238

Huang ZF, Higuchi D, Lasky N, Broze GJJ (1997) Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 90:944–951

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Kung J, Hagstrom J, Cass D et al (1998) Human FIX corrects bleeding diathesis of mice with hemophilia B. Blood 91:784–790
Law DA, DeGuzman FR, Heiser P et al (1999) Integrin cytoplasmic tyrosine motif is required for outside-in αIIbβ3 signalling and platelet function. Nature 401:808–811
Leon C, Hechler B, Freund M et al (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor-null mice. J Clin Invest 104:1731–1737
Lin HF, Maeda N, Sithies O et al (1997) A coagulation factor IX-deficient mouse model for human hemophilia B. Blood 90:3962–3966
Offermanns S, Toombs CF, Hu YH, Simon MI (1997) Defective platelet activation in Gα_q-deficient mice. Nature 389:183–186
Pearson JM, Ginsburg D (1999) Use of transgenic mice in the study of thrombosis and hemostasis. In: Handbook of Experimental Pharmacology. Vol. 132, Antithrombotics. Berlin:Springer-Verlag, pp 157–174
Ploplis VA, Carmeliet P, Vazirzadeh S et al (1995) Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. Circulation 92:2585–2593
Subramaniam M, Frenette PS, Saffaripour S et al (1996) Defects in hemostasis in P-selectin-deficient mice. Blood 87:1238–1242
Thomas DW, Mannon RB, Mannon PJ et al (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A_2. J Clin Invest 102:1994–2001
Toomey JR, Kratzer KE, Lasky NM et al (1996) Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood 88:1583–1587
Vassalli G, Dichek DA (1997) Gene therapy for arterial thrombosis. Cardiovasc Res 35:459–469
Wang L, Zoppè M, Hackeng TM et al (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA 94:11563–11566
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Waugh JM, Kattash M, Li J et al (1999b) Gene therapy to promote thromboresistance: local overexpression of tissue plasminogen activator to prevent arterial thrombosis in an in vivo rabbit model. Proc Natl Acad Sci USA 96:1065–1070
Zoldhelyi P, McNatt J, Shelat HS et al (2000) Thromboresistance of balloon-injured porcine carotid arteries after local gene transfer of human tissue factor pathway inhibitor. Circulation 101:289–295

B.6.1 Knock-Out Mice

PURPOSE AND RATIONALE

Genetically modified animals, in particular knock-out mice, help in the understanding of the role of various factors in blood clotting, thrombolysis, and platelet function. They are useful to verify the mode of action of new drugs.

Factor I (Fibrinogen)

Phenotype

Born in normal appearance, ~10% die shortly after birth and another 40% around 1–2 months after birth due to bleeding, failure of pregnancy, blood samples failing to clot or support platelet aggregation *in vitro* (Suh et al. 1995).

REFERENCES AND FURTHER READING

Suh TT, Holmback K, Jensen NJ et al (1995) Resolution of spontaneous bleeding events but failure of pregnancy in fibrinogen-deficient mice. Genes Dev 9:2020–2033

Factor II (Prothrombin)

Phenotype

Partial embryonic lethality: 50% between embryonic day (E) 9.5–11.5; at least 1/4 survive to term, but fatal hemorrhage few days after birth; factor II important in maintaining vascular integrity during development as well as postnatal life (Sun et al. 1998, Xu et al. 1998).

REFERENCES AND FURTHER READING

Sun WY, Witte DP, Degen JL et al (1998) Prothrombin deficiency results in embryonic and neonatal lethality in mice. Proc Natl Acad Sci USA 95:7597–7602
Xu J, Wu Q, Westfield L et al (1998) Incomplete embryonic lethality and fatal neonatal hemorrhage caused by prothrombin deficiency in mice. Proc Natl Acad Sci USA 95:7603–7607

Factor V

Phenotype

Half of the embryos die at E9–10, possibly as a result of abnormal yolk sac vasculature, remaining 50% progress normally to term, but die from massive hemorrhage within 2 hours of birth, more severe in mouse than in human (Cui et al. 1996; Yang et al. 2000).

REFERENCES AND FURTHER READING

Cui J, O'Shea KS, Purkayastha A et al (1996) Fatal hemorrhage and incomplete block to embryogenesis in mice lacking coagulation factor V. Nature 384:66–68
Yang TL, Cui J, Taylor JM et al (2000) Rescue of fatal neonatal hemorrhage in factor V deficient mice by low level transgene expression. Thromb Haemost 83:70–77

Factor VII

Phenotype

Develop normally but suffer fatal perinatal bleeding (Rosen et al. 1997).

REFERENCES AND FURTHER READING

Rosen ED, Chan JCY, Idusogie E et al (1997) Mice lacking factor VII develop normally but suffer fatal perinatal bleeding. Nature 390:290–294

Factor VIII

Phenotype

Mild phenotype compared with severe hemophilia A in humans; no spontaneous bleeding, illness or reduced activity during the first year of life; have residual clotting activity (APTT), as shown by Bi et al. (1995).

REFERENCES AND FURTHER READING

Bi L, Lawler AM, Antonarakis SE et al (1995) Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. Nat Genet 10:119–121

Factor IX

Phenotype

Factor IX coagulant activities (APTT): +/+ 92%, +/– 53%, –/– <5%; bleeding disorder (extensive bleeding after clipping a portion of the tail, bleeding to death if not cauterized (Kundu et al. 1998, Wang et al. 1997).

REFERENCES AND FURTHER READING

Kundu RK, Sangiorgi F, Wu LY et al (1998) Targeted inactivation of the coagulation factor IX gene causes hemophilia B in mice. Blood 92:168–174

Wang L, Zoppe M, Hackeng TM et al (1997) A factor IX-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA 94:11563–11566

Factor X

Phenotype

Partial embryonic lethality (1/3 died on E11.5–12.5); fatal neonatal bleeding between postnatal day (P) 5–20, as shown by Dewerchin et al. (2000).

REFERENCES AND FURTHER READING

Dewerchin M, Liang Z, Moons L et al (2000) Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice. Thromb Haemost 83:185–190

Factor XI

Phenotype

APTT prolonged in –/– (158–200 sec) compared with +/+ (25–34 sec) and +/– (40–61 sec); no factor XI activity and antigen, did not result in intrauterine death, –/– similar bleeding as +/+ with a tendency to prolongation (Gailani et al. 1997).

REFERENCES AND FURTHER READING

Gailani D, Lasky NM, Broze GJ (1997) A murine model of factor XI deficiency. Blood Coagul Fibrinolysis 8:134–144

TF (Tissue Factor)

Phenotype

Abnormal circulation from yolk sac to embryo ~E8.5 leading to embryo wasting and death; TF has a role in blood vessel development (Bugge et al. 1996, Carmeliet et al. 1996, Toomey et al. 1996, 1997).

REFERENCES AND FURTHER READING

Bugge TH, Xiao Q, Kombrinck KW et al (1996) Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. Proc Natl Acad Sci U S A 93:6258–6263

Carmeliet P, Mackman N, Moons L et al (1996) Role of tissue factor in embryonic blood vessel development. Nature 383:73–75

Toomey JR, Kratzer KE, Lasky NM et al (1996) Targeted disruption of the murine tissue factor gene results in embryonic lethality. Blood 88:1583–1587

Toomey JR, Kratzer KE, Lasky NM, Broze GJ (1997) Effect of tissue factor deficiency on mouse and tumor development. Proc Natl Acad Sci USA 94:6922–6926

TFPI (Tissue Factor Pathway Inhibitor)

Phenotype

None survive the neonatal period; 60% die between E9.5–11.5 with signs of yolk sac hemorrhage (Huang et al. 1997).

REFERENCES AND FURTHER READING

Huang ZF, Higuchi D, Lasky N, Broze GJ (1997) Tissue factor pathway inhibitor gene disruption produces intrauterine lethality in mice. Blood 90:944–951

Thrombin Receptor

Phenotype

50% die at E9–10; 50% survive and become grossly normal adult mice with no bleeding diathesis; –/– platelets strongly respond to thrombin; –/– fibroblast loose their ability to respond to thrombin⟶ second TR must exist, as shown by Connolly et al. (1996) and by Darrow et al. (1996).

REFERENCES AND FURTHER READING

Connolly AJ, Ishihara H, Kahn ML et al (1996) Role of the thrombin receptor in development and evidence for a second receptor. Nature 381:516–519

Darrow AL, Fung-Leung WP, Ye RD et al (1996) Biological consequences of thrombin receptor deficiency in mice. Thromb Haemost 76:860–866

Thrombomodulin

Phenotype

Embryonic lethality before development of a functional cardiovascular system; die before E9.5 due to retardation of growth; TM+/– mice develop normal without thrombotic complications (Christie et al. 1999, Healy et al. 1995, 1998, Weiler-Guettler et al. 1998).

REFERENCES AND FURTHER READING

Christie PD, Edelberg JM, Picard MH et al (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104:533–539

Healy AM, Rayburn HB, Rosenberg RD, Weiler H (1995) Absence of the blood-clotting regulator thrombomodulin causes embryonic lethality in mice before development of a functional cardiovascular system. Proc Natl Acad Sci USA 92:850–854

Healy AM, Hancock WW, Christie PD et al (1998) Intravascular coagulation activation in a murine model of thrombomodulin deficiency: effects of lesion size, age, and hypoxia on fibrin deposition. Blood 92:4188–4197

Weiler-Guettler H, Christie PD, Beeler DL et al (1998) A targeted point mutation in thrombomodulin generates viable mice with a prethrombotic state. J Clin Invest 101:1983–1991

Protein C

Phenotype

KO mice appeared to develop normally macroscopically, but possessed obvious signs of bleeding and thrombosis; did not survive beyond 24 hours after delivery; microvascular thrombosis in the brain and necrosis in the liver; plasma clottable fibrinogen was not detectable, suggesting fibrinogen depletion and secondary consumptive coagulopathy (Jalbert et al. 1998).

REFERENCES AND FURTHER READING

Jalbert LR, Rosen ED, Moons L et al (1998) Inactivation of the gene for anticoagulant protein C causes lethal perinatal consumptive coagulopathy in mice. J Clin Invest 102:1481–1488

Plasminogen

Phenotype

Severe spontaneous thrombosis; reduced ovulation and fertility; cachexia and shorter survival; severe glomerulonephritis; impaired skin healing; reduced macrophage and keratinocyte migration (Bugge et al. 1995, Ploplis et al. 1995).

REFERENCES AND FURTHER READING

Bugge TH, Flick MJ, Daugherty CC, Degen JL (1995) Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. Genes Dev 9:794–807

Ploplis VA, Carmeliet P, Vazirzadeh S et al (1995) Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. Circulation 92:2585–2593

Alpha$_2$-Antiplasmin

Phenotype

Normal fertility, viability and development; no bleeding disorder; spontaneous lysis of injected clots; $\longrightarrow$ enhanced fibrinolytic potential; significant reduction of renal fibrin deposition after LPS (Lijnen et al. 1999).

REFERENCES AND FURTHER READING

Lijnen HR, Okada K, Matsuo O et al (1999) Alpha$_2$-antiplasmin gene deficiency in mice is associated with enhanced fibrinolytic potential without overt bleeding. Blood 93:2274–2281

T-PA (Tissue-Type Plasminogen Activator)

Phenotype

Extensive spontaneous fibrin deposition; severe spontaneous thrombosis; impaired neointima formation; reduced ovulation and fertility; cachexia and shorter survival; severe glomerulonephritis; abnormal tissue remodeling (Carmeliet et al. 1998, Christie et al. 1999).

REFERENCES AND FURTHER READING

Carmeliet P, Schoonjans L, Kieckens L et al (1998) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Christie PD, Edelberg JM, Picard MH et al (1999) A murine model of myocardial microvascular thrombosis. J Clin Invest 104:533–539

PAI-1 (Plasminogen Activator Inhibitor-1)

Phenotype

Reduced thrombotic incidence; no bleeding; accelerated neo-intima formation; reduced lung inflammation; reduced atherosclerosis. Detailed studies on PAI-1 are reported by Carmeliet et al. (1993), Eitzman et al. (1996), Erickson et al. (1990), Kawasaki et al. (2000), and Pinsky et al. (1998).

REFERENCES AND FURTHER READING

Carmeliet P, Stassen JM, Schoonjans L et al (1993) Plasminogen activator inhibitor-1 gene-deficient mice. II. Effects on hemostasis, thrombosis, and thrombolysis. J Clin Invest 92:2756–2760

Eitzman DT, McCoy RD, Zheng X et al (1996) Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. J Clin Invest 97:232–237

Erickson LA, Fici GJ, Lund JE et al (1990) Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. Nature 346:74–76

Kawasaki T, Dewerchin M, Lijnen HR et al (2000) Vascular release of plasminogen activator inhibitor-1 impairs fibrinolysis during acute arterial thrombosis in mice. Blood 96:153–60

Pinsky DJ, Liao H, Lawson CA et al (1998) Coordinated induction of plasminogen activator inhibitor-1 (PAI-1) and inhibition of plasminogen activator gene expression by hypoxia promotes pulmonary vascular fibrin deposition. J Clin Invest 102:919–928

TAFI (Thrombin Activatable Fibrinolysis Inhibitor)

Not described.

Vitronectin

Phenotype

Normal development, fertility, and survival; serum is completely deficient in "serum spreading factor" and PAI-1 binding activities; delayed arterial and venous thrombus formation (Eitzman et al. 2000, Zheng et al. 1995).

REFERENCES AND FURTHER READING

Eitzman DT, Westrick RJ, Nabel EG, Ginsburg D (2000) Plasminogenactivator inhibitor-1 and vitronectin promote vascular thrombosis in mice. Blood 95:577–580

Zheng X, Saunders TL, Camper SA et al (1995) Vitronectin is not essential for normal mammalian development and fertility. Proc Natl Acad Sci USA 92:12426–12430

Urokinase, U-PA (Urinary-Type Plasminogen Activator)

Phenotype

Single u-PA deficiency: viable, fertile, normal life span; occasionally spontaneous fibrin deposits in normal and inflamed tissue; higher incidence of endotoxin-induced thrombosis. Combined t-PA and u-PA deficiency: mice survive embryonic development; retarded growth, reduced fertility, shortened life span; spontaneous fibrin deposits more extensively and in more organs (Carmeliet et al. 1998, Heckel et al. 1990).

Transgenic mice carrying the u-PA gene linked to the albumin enhancer/promoter exhibit spontaneous intestinal and intraabdominal bleeding directly related to transgene expression in the liver and elevated plasma u-PA level; 50% die between 3 and 84 hours after birth; severe hypofibrinogenemia, loss of clotting function.

REFERENCES AND FURTHER READING

Carmeliet P, Schoonjans L, Kieckens L et al (1998) Physiological consequences of loss of plasminogen activator gene function in mice. Nature 368:419–424

Heckel JL, Sandgren EP, Degen JL et al (1990) Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. Cell 62:447–456

UPAR (Urinary-Type Plasminogen Activator Receptor)

Phenotype

Phenotype normal; attenuated thrombocytopenia and mortality associated with severe malaria (Bugge et al. 1995, 1996, Dewerchin et al. 1996, Piguet et al. 2000).

REFERENCES AND FURTHER READING

Bugge TH, Suh TT, Flick MJ et al (1995) The receptor for urokinase-type plasminogen activator is not essential for mouse development or fertility. J Biol Chem 270:16886–16894

Bugge TH, Flick MJ, Danton MJ et al (1996) Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc Natl Acad Sci USA 93:5899–5904

Dewerchin M, van Nuffelen A, Wallays G et al (1996) Generation and characterization of urokinase receptor deficient mice. J Clin Invest 97:870–878

Piguet PF, Da-Laperrousaz C, Vesin C et al (2000) Delayed mortality and attenuated thrombocytopenia associated with severe malaria in urokinase- and urokinase receptor-deficient mice. Infect Immun 68:3822–3829

Gas 6 (Growth Arrest-Specific Gene 6 Product)

Phenotype

Mice are viable, fertile, appear normal; do not suffer spontaneous bleeding or thrombosis; have normal tail bleeding time. Platelets fail to aggregate irreversibly to ADP, collagen, or U 46619. Arterial and venous thrombosis is inhibited and mice are protected from fatal thromboembolism after injection of collagen plus epinephrine (Angelillo-Scherrer et al. 2001).

REFERENCES AND FURTHER READING

Angelillo-Scherrer A, DeFrutos PG, Aparicio C et al (2001) Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. Nat Med 7:215–221

GPIbalpha (Glycoprotein Ib Alpha, Part of the GP Ib-V–IX Complex)

Phenotype

Bleeding, thrombocytopenia and giant platelets (similar to human Bernard Soulier syndrome). See Ware et al. (2000) for details.

REFERENCES AND FURTHER READING

Ware J, Russell S, Ruggeri ZM (2000) Generation and rescue of a murine model of platelet dysfunction: the Bernard-Soulier syndrome. Proc Natl Acad Sci USA 97:2803–2808

GPV (Glycoprotein V, Part of the GP Ib-V–IX Complex)

Phenotype

Increased thrombin responsiveness, GpV–/– platelets are normal in size, normal amounts in GpIb-IX, functional in vWF-binding; platelets are hyperresponsive to thrombin$\longrightarrow$ increased aggregation response; shorter bleeding time;$\longrightarrow$GpV = negative modulator of platelet function (Ramakrishnan et al. 1999).

REFERENCES AND FURTHER READING

Ramakrishnan V, Reeves PS, DeGuzman F et al (1999) Increased thrombin responsiveness in platelets from mice

lacking glycoprotein V. Proc Natl Acad Sci USA 96:13336–13341

GPIIb (Integrin Alpha IIb, Glycoprotein IIb, Part of the GP IIb–IIIa Complex)

Phenotype

Bleeding disorder similar to Glanzmann thrombasthenia in man; platelets failed to bind fibrinogen, to aggregate and to retract a fibrinogen clot; α-granules do not contain fibrinogen (Tronik-Le Roux et al. 2000).

REFERENCES AND FURTHER READING

Tronik-Le Roux D, Roullot V, Poujol C et al (2000) Thrombasthenic mice generated by replacement of the integrin alpha$_{IIb}$ gene: demonstration that transcriptional activation of this megakaryocytic locus precedes lineage commitment. Blood 96:1399–1408

GP IIIa (Integrin Beta3, Glycoprotein IIIa, Part of the GP IIb–IIIa Complex)

Phenotype

Viable, fertile, increased fetal mortality; features of Glanzmann thrombasthenia in man, e. g. defective platelet aggregation, clot retraction; spontaneous bleeding, prolonged bleeding times; dysfunctional osteoclasts, development of osteosclerosis with age (Hodivala-Dilke et al. 1999, McHugh et al. 2000).

REFERENCES AND FURTHER READING

Hodivala-Dilke KM, McHugh KP, Tsakiris DA et al (1999) Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 103:229–238

McHugh KP, Hodivala-Dilke K, Zheng MH et al (2000) Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. J Clin Invest 105:433–440

GP IIa (Glycoprotein IIa, Integrin Beta 1, Part of the GP Ia–IIa Complex)

Phenotype

Integrin beta1 null platelets from conditional knockout mice develop normally, platelet count is normal. Collagen induced platelet aggregation is delayed but otherwise normal; tyrosine phosphorylation pattern is normal but phosphorylation is delayed. Bleeding time in bone marrow chimeric mice is normal; no major *in vivo* defects (Nieswandt et al. 2001).

REFERENCES AND FURTHER READING

Nieswandt B, Brakebusch C, Bergmeier W et al (2001) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. EMBO J 20:2120–2130

VWF (von Willebrand Factor)

Phenotype

Factor VIII levels strongly reduced due to defective protection by vWF; highly prolonged bleeding time, hemorrhage, spontaneous bleeding; mice useful for investigating the role of vWF; delayed platelet adhesion in ferric-chloride-induced arteriolar injury (Denis et al. 1998, Ni et al. 2000).

REFERENCES AND FURTHER READING

Denis C, Methia N, Frenette PS et al (1998) A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. Proc Natl Acad Sci USA 95:952–959

Ni H, Denis CV, Subbarao S et al (2000) Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. J Clin Invest 106:385–392

Thromboxane A2 Receptor (TXA2r)

Phenotype

Mild bleeding disorder and altered vascular responses to TXA2 and arachidonic acid (Thomas et al. 1998).

REFERENCES AND FURTHER READING

Thomas DW, Mannon RB, Mannon PJ et al (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. J Clin Invest 102:1994–2001

Prostacyclin Receptor (PGI2r)

Phenotype

Viable, fertile, normotensive; increased susceptibility to thrombosis; reduced inflammatory and pain responses (Murata et al. 1997).

REFERENCES AND FURTHER READING

Murata T, Ushikubi F, Matsuoka T et al (1997) Altered pain reception and inflammatory response in mice lacking prostacyclin receptor. Nature 388:678–682

PECAM (Platelet: Endothelial Cell Adhesion Molecule)

Phenotype

Normal platelet aggregation; prolonged bleeding time as described by Duncan et al. (1999) and by Mahooti et al. (2000).

REFERENCES AND FURTHER READING

Duncan GS, Andrew DP, Takimoto H et al (1999) Genetic evidence for functional redundancy of platelet/endothelial cell adhesion molecule-1 (PECAM-1) CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. J Immunol 162:3022–3030

Mahooti S, Graesser D, Patil S et al (2000) PECAM-1 (CD 31) expression modulates bleeding tome *in vivo*. Am J Pathol 157:75–81

Pallid (Pa)

Phenotype

Among 13 hypopigment mouse mutants with storage pool deficiency, the pallid mouse is a model of the human Hermansky Pudlak syndrome (the beige mouse is a model of the Chediak Higashi syndrome). Pallid mice exhibit prolonged bleeding time, pigment dilution, kidney lysosomal enzyme elevation serum alpha 1 antitrypsin deficiency and abnormal otolith formation. The gene defective in pallid mice encodes the highly charged 172-amino acid protein pallidin, which interacts with syntaxin 13, a protein mediating vesicle docking and fusion (Huang et al. 1999).

REFERENCES AND FURTHER READING

Huang L, Kuo YM, Gitschier J (1999) The pallid gene encodes a novel, syntaxin 13-interacting protein involved in platelet storage pool deficiency. Nat Genet 23:329–332

G Alpha(q) (Guanyl Nucleotide Binding Protein G Alpha q)

Phenotype

Defective aggregation in response ADP, TXA2, thrombin, collagen; shape change normal (Offermans et al. 1997, Ohlmann et al. 2000).

REFERENCES AND FURTHER READING

Offermans S, Toombs CF, Hu YH, Simon MI (1997) Defective platelet activation in G alpha(q)-deficient mice. Nature 389:183–186

Ohlmann P, Eckly A, Freund M et al (2000) ADP induces partial platelet aggregation without shape change and potentiates collagen-induced aggregation in the absence of Galphaq. Blood 96:2134–2139

G z (Member of the Gi Family of G Proteins)

Phenotype

Impaired platelet aggregation to epinephrine; resistance to fatal thromboembolism; exaggerated response to cocaine, reduced effect of morphine and antidepressant drugs (Yang et al. 2000).

REFERENCES AND FURTHER READING

Yang J, Wu J, Kowalska MA et al (2000) Loss of signaling through G protein, Gz, results in abnormal platelet activation and altered responses to psychoactive drugs. Proc Natl Acad Sci USA 97:9984–9989

Phospholipase C Gamma

Phenotype

Viable, fertile, decreased mature B cells; defective B cell and mast cell function; defective Fc_{gamma} receptor signaling, therefore, loss of collagen-induced platelet aggregation (Wang et al. 2000).

REFERENCES AND FURTHER READING

Wang D, Feng J, Wen R et al (2000) Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity 13:25–35

CD39 (Vascular Adenosine Triphosphate Diphosphohydrolase)

Phenotype

Viable, fertile; prolonged bleeding times but minimally perturbed coagulation parameters; reduced platelet interaction with injured mesenteric vasculature *in vivo*. Platelets fail to aggregate to standard agonists *in vitro* associated with purinergic P2Y1 receptor desensitization; fibrin deposition at multiple organ sites (Enjyoji et al. 1999).

REFERENCES AND FURTHER READING

Enjyoji K, Sevigny J, Lin Y et al (1999) Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nat Med 5:1010–1017

Protein Kinase, cGMP-Dependent

Phenotype

Viable, fertile; unresponsive to cGMP and NO; defective VASP-phosphorylation; increased adhesion and aggregation of platelets *in vivo* in ischemic/reperfused mesenteric microcirculation; no compensation by cAMP kinase system (Massberg et al. 1999).

REFERENCES AND FURTHER READING

Massberg S, Sausbier M, Klatt P et al (1999) Increased adhesion and aggregation of platelets lacking cyclic guanosine 3′,5′-monophosphate kinase I. J Exp Med 189:1255–1264

Vasodilator-Stimulated Phosphoprotein (VASP)

Phenotype

Viable, fertile; mild platelet dysfunction with megakaryocyte hyperplasia, increased collagen/thrombin activation, impaired cyclic nucleotide mediated inhibition of platelet activation (Aszodi et al. 1999, Hauser et al. 1999).

REFERENCES AND FURTHER READING

Aszodi A, Pfeifer A, Ahmad M et al (1999) The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. EMBO J 18:37–48

Hauser W, Knobeloch KP, Eigenthaler M et al (1999) Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. Proc Natl Acad Sci USA 96:8120–8125

Arachidonate 12-Lipoxygenase (P-12LO)

Phenotype

Platelets exhibit a selective hypersensitivity to ADP, manifested as a marked increase in slope and percent aggregation in *ex vivo* assays and increased mortality in an ADP-induced mouse model of thromboembolism (Chen et al. 1994, Johnson et al. 1998).

REFERENCES AND FURTHER READING

Chen XS, Sheller JR, Johnson EN, Funk CD (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372:179–182

Johnson EN, Brass LF, Funk CD (1998) Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. Proc Natl Acad Sci USA 95:3100–3105

Arachidonate 5-Lipoxygenase (P-5LO)

Phenotype

Develop normally and are healthy. No difference in their reaction to endotoxin shock; however, they resist the lethal effects of shock induced by platelet-activating factor. Inflammation induced by arachidonic acid is markedly reduced (Chen et al. 1994, Johnson et al. 1998).

REFERENCES AND FURTHER READING

Chen XS, Sheller JR, Johnson EN, Funk CD (1994) Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 372:179–182

Johnson EN, Brass LF, Funk CD (1998) Increased platelet sensitivity to ADP in mice lacking platelet-type 12-lipoxygenase. Proc Natl Acad Sci USA 95:3100–3105

Thrombopoietin

Phenotype

TPO –/– and c-mpl –/–: both exhibit a 90% reduction in megakaryocyte and platelet levels, but even with these small platelet levels the mice do not have excessive bleeding; all platelets that are present are morphologically normal+functionally; *in vivo* TPO is required for control of megakaryocyte and platelet number but not for their maturation (Lawler et al. 1998).

REFERENCES AND FURTHER READING

Lawler J, Sunday M, Thibert V et al (1998) Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. J Clin Invest 101:982–992

Thrombospondin-1

Phenotype

Normal thrombin-induced platelet aggregation; increase in circulating number of white blood cells; TSP-1 is involved in normal lung homeostasis (Lawler et al. 1998).

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Table 6 Genetic models of thrombosis and hemostasis

Knock-out	Viable	Embryonic Development / Survival	References
Coagulation			
Protein C	No	Normal Perinatal death	Jalbert et al. 1998
Fibrinogen	Yes	Normal Perinatal death	Suh et al. 1995
Fibrinogen-QAGVD	Yes	Normal	Suh et al. 1995
fV	No	Partial embryonic loss Perinatal death	Cui et al. 1996
FVII	Yes	Normal Perinatal death	Rosen et al. 1997
fVIII	Yes	Normal	Bi et al. 1996
fIX	Yes	Normal	Wang et al. 1997
fXI	Yes	Normal	Gailani et al. 1997
Tissue factor	No	Lethal	Toomey et al. 1996 Bugge et al. 1996
TFPI	No	Lethal	Huang et al. 1997
vWF	Yes	Normal	Denis et al. 1998
Prothrombin	No	Partial embryonic lossr perinatal death	Xue et al. 1998 Sun et al. 1998
Fibrinolytic			
u-Pa & t-PA	Yes	Normal Growth retardation	Carmeliet et al. 1994
uPAR	Yes	Normal	Dewerchin et al. 1996 Bugge et al. 1995
Plasminogen	Yes	Normal Growth retardation	Bugge et al. 1995 Ploplis et al. 1995
PA-I	Yes	Normal	Carmeliet et al. 1993
Thrombomodulin	No	Lethal	Healy et al. 1995
Platelet			
β_3	Yes	Normal Partial embryonic loss	Hodivala-Dilke et al. 1999
β_3-DiYF	Yes	Normal	Law et al. 1999
P-Selectin	Yes	Normal	Subramaniam et al. 1996
PAR-1	Yes	Normal	Connolly et al. 1996
PAR-3	Yes	Normal	Kahn et al. 1998
$G_{\alpha a}$	Yes	Normal Perinatal death	Offermans et al. 1997
TXA_2 receptor	Yes	Normal	Thomas et al. 1998
P2Y1	Yes	Normal	Leon et al. 1999

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B.7
Critical Issues in Experimental Models

B.7.1
The Use of Positive Control

Clearly, there are many antithrombotic agents that can be used to compare and contrast the antithrombotic efficacy and safety of novel agents. The classic antithrombotic agents are heparin, warfarin, and aspirin. However, new, more selective agents such as hirudin, low molecular weight heparins, and clopidogrel are commercially available that will either replace or augment these older treatments. Novel antithrombotic agents should certainly be demanded to demonstrate better efficacy than currently available therapy in animal models of thrombosis. This should be demonstrated by performing dose-response experiments that include maximally effective doses of each compound in the model. At the maximally effective dose, parameters such as APTT, PT, template bleeding time, or other, more sensitive measurements of systemic hypocoagulability or bleeding should be compared. A good example of this approach is a study by Schumacher et al. (1996), who compared the antithrombotic efficacy of argatroban and dalteparin in arterial and venous models of thrombosis. Consideration of potency and safety compared to other agents should be taken into account when advancing a drug through the testing funnel.

The early *in vivo* evaluation of compounds that demonstrate acceptable *in vitro* potency and selectivity requires evaluation of each compound alone in order to demonstrate antithrombotic efficacy. The antithrombotic landscape is becoming complicated by so many agents from which to choose that it will become increasingly difficult to design preclinical experiments that mimic the clinical setting in which polyantithrombotic therapy is required for optimal efficacy and safety. Consequently, secondary and tertiary preclinical experiments will need to be carefully designed in order to answer these specific, important questions.

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B.7.2
Evaluation of Bleeding Tendency

Although the clinical relevance of animal models of thrombosis has been well-established in terms of efficacy, the preclinical tests for evaluating safety, i.e, bleeding tendency, have not been as predictable. The difficulty in predicting major bleeding, such as intracranial hemorrhage, resulting from antithrombotic or thrombolytic therapy stems from the complexity and lack of understanding of the mechanisms involved in this disorder. Predictors of anticoagulant-related intracranial hemorrhages are advanced age, hypertension, intensity and duration of treatment, head trauma, and prior neurologic disease (Stieg and Kase 1998, Sloan and Gore 1992). These risk factors are clearly difficult, if not impossible, to simulate in laboratory animals. Consequently, more general tests of anticoagulation and primary hemostasis have been employed.

Coagulation assays provide an index of the systemic hypocoagulability of the blood after administration of antithrombotic agents; however, as indicated earlier, the sensitivity and specificity of these assays varies from compound to compound, so these assays do not provide a consistent safety measure across all mechanisms of inhibition. Consequently, many laboratories have attempted to develop procedures that provide an indication of bleeding risk by evaluating primary hemostasis after generating controlled incisions in anesthetized animals. Some of the tests used in evaluating FXa inhibitors include template bleeding time, tail transection bleeding time, cuticle bleeding time, and evaluation of clinical parameters such as hemoglobin and hematocrit. Unfortunately, template bleeding tests, even when performed in humans, have not been good predictors of major bleeding events in clinical trials (Bernardi et al. 1993, Bick 1995, Rodgers and Levin 1990). However, these tests have been able to demonstrate relative advantages of certain mechanisms and agents over others. For example, hirudin, a direct thrombin inhibitor, appears to have a narrow therapeutic window when used as an adjunct to thrombolysis in clinical trials, producing unacceptable major bleeding when administered at 0.6 mg/kg, i.v. bolus, plus 0.2 mg/kg/hr (Antman et al. 1996, GUSTO Investigators 1996). When the dose of hirudin was adjusted to avoid major bleeding (0.1 mg/kg and 0.1 mg/kg/hr), no significant therapeutic advantage over heparin was observed. If the relative improvement in the ratio between efficacy and bleeding observed preclinically with Xa inhibitors compared to thrombin inhibitors such as hirudin is supported in future clinical trials, this will establish an important safety advantage for FXa inhibitors and provide valuable information for evaluating the safety of new antithrombotic agents in preclinical experiments.

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B.7.3
Selection of Models Based on Species-Dependent Pharmacology/Physiology

As alluded to earlier, species selection for animal models of disease is often limited by the unique physiology of a particular disease target in different species or by the species specificity of the pharmacological agent for the target. For example, it was discovered relatively early in the development of platelet GPIIb/IIIa antagonists that these compounds were of limited use in rats (Cox et al. 1992) and that there was a dramatic species-dependent variation in the response of platelets to GPIIb/IIIa antagonists (Bostwick et al. 1996, Cook et al. 1993, Panzer-Knodle et al. 1993). This discovery lead to the widespread use of larger animals (particularly in dogs, whose platelet response to GPIIb/IIIa antagonists resembles humans) in the evaluation of GPIIb/IIIa antagonists. Of course the larger animals required more compound for evaluation, which created a resource problem for medicinal chemists. This was especially problematic for companies that generated compounds by combinatorial parallel synthetic chemistry in which many compounds can be made, but usually in very small quantities. However, some pharmacologists devised clever experiments that partially overcame this problem. Cook et al. (1996) administered a GPIIb/IIIa antagonist orally and intravenously to rats, and then mixed the platelet-rich plasma from the treated rats with platelet-rich plasma from untreated dogs. The mixture was then evaluated in an agonist-induced platelet aggregation assay and the resulting inhibition of canine platelet aggregation (rat platelets were relatively unresponsive to this GPIIb/IIIa antagonist) was due to the drug present in the plasma obtained from the rat. Using this method, only a small amount of drug is required to determine the relative bioavailability in rats. However, the animal models chosen for efficacy in that report (guinea pigs and dogs) were selected based on their favorable platelet response to the GPIIb/IIIa antagonist.

Similarly for inhibitors of FXa, there are significant variations in the activity of certain compounds against FXa purified from plasma of different species and in plasma-based clotting assays using plasma from different species. DX-9065 is much more potent against human FXa (Ki = 78 nM) than against rabbit (Ki = 102 nM) and rat (Ki = 1980 nM) FXa. Likewise, in the PT assay, DX-9065a was very potent in human plasma (concentration required to double PT, PTx2, was 0.52 μM) and in squirrel monkey plasma ($PT \times 2 = 0.46\,\mu M$), but was much less potent in rabbit, dog, and rat plasma ($PT \times 2 = 1.5$, 6.5, and 22.2 μM, respectively). Other FXa inhibitors have also demonstrated these species-dependent differences in activity (Tidwell et al. 1980, Nutt et al. 1991, Taniuchi et al. 1998). Regardless, the investigator must be aware of these differences so that appropriate human doses can be extrapolated from the laboratory animal studies.

Although in many cases the exact mechanism for the species-dependent differences in response to certain therapeutic agents remains unclear, these differences must be examined to determine the appropriate species to be used for preclinical pharmacological evaluation of each agent. This evaluation can routinely be performed by *in vitro* coagulation or platelet aggregation tests prior to evaluation in animal models.

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B.7.4 Selection of Models Based on Pharmacokinetics

Much debate surrounds the issue as to which species most resembles humans in terms of gastrointestinal absorption, clearance, and metabolism of therapeutic agents. Differences in gastrointestinal anatomy, physiology, and biochemistry between humans and commonly used laboratory animals suggest that no single animal can precisely mimic the gastrointestinal characteristics of humans (Kararli 1995). Due to resource issues (mainly compound availability) and animal care and use considerations, small rodents, such as rats, are usually considered for primary *in vivo* evaluation of pharmacokinetics for novel agents. However, there is great reservation about moving a compound into clinical trials based on oral bioavailability data derived from rat experiments alone. Usually, larger animals such as dogs or non-human primates, which have similar gastrointestinal morphology compared to humans, are the next step in the evaluation of pharmacokinetics of new agents. The pharmacokinetic characteristics of FXa inhibitor, YM-60828, have been studied extensively in a variety of laboratory animals. YM-60828 demonstrated species-dependent pharmacokinetics, with oral bioavailability estimates of approximately 4%, 33%, 7%, and 20% in rats, guinea pigs, beagle dogs, and squirrel monkeys, respectively**.** Although these results suggest that YM-60828 has somewhat limited bioavailability, evaluating the pharmacokinetic profile of novel agents in a number of species (Sanderson et al. 1998) is a well-established approach used to aid in identifying compounds for advancement to human testing. That is, acceptable bioavailability in a number of species suggests that a compound will be bioavailable in humans. Which of the laboratory species adequately represents the bioavailability of a specific compound in humans can only be determined after appropriate pharmacokinetic evaluation in humans. Nevertheless, preclinical pharmacokinetic data are important in selecting the appropriate animal model for testing the antithrombotic efficacy of compounds because the ultimate proof-of-concept experiment is to demonstrate efficacy by the intended route of administration.

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B.7.5 Clinical Relevance of Data Derived from Experimental Models

Animal models of thrombosis have played a crucial role in the discovery and development of a number of compounds that are now successfully being used for the treatment and prevention of thrombotic diseases. The influential preclinical results using novel antithrombotics in a variety of laboratory animal experiments are listed in Table 7 below, along with the early clinical trials and results for each compound. This table intentionally omits many compounds that were tested in animal models of thrombosis, but failed to be successful in clinical trials or, for other reasons, did not become approved drugs. However, these negative outcomes would not have been predicted by animal models of thrombosis because the failures were generally due to other shortcomings of the drugs (e.g, toxicity, narrow therapeutic window, or undesirable pharmacokinetics or pharmacodynamics) that are not always clearly presented in the scientific literature due to proprietary restrictions in this highly competitive field.

Nonetheless, it is clear that animal models have supplied valuable information for investigators responsible for evaluating these drugs in humans, providing pharmacodynamic, pharmacokinetic, and safety data that can be used to design safe and efficient clinical trials. For detailed applications see the following references: Bugge et al. (1995), Bugge et al. (1996), Carmeliet et al. (1993), Carmeliet et al. (1994), Carmeliet et al. (1996), Christie et al. (1999), Connolly et al. (1996), Cui et al. (1996), Evans et al. (1989), Healy et al. (1995), Herzog et al. (1999), Hodivala-Dilke et al. (1999), Huang et al. (1997), Kahn et al. (1998), Kay et al. (2000), Kung et al. (1998), Law et al. (1999), Leon et al. (1999), Lin et al. (1997), Offermanns et al. (1997), Ploplis et al. (1995), Subramaniam et al. (1996), Thomas et al. (1998), Toomey et al.

Table 7 Animal models of thrombosis and their clinical correlates

Compound	Preclinical Animal Model	Preclinical Results	Ref	Clinical Indication	Clinical Result	Ref
Recombinant tissue plasminogen activator (Activase)	Rabbit pulmonary artery thrombosis	Lysis of preformed pulmonary thrombus	Matsuo et al. 1981	Acute myocardial infarction—thrombolysis	Improved recanalization	Collen et al. (1984)
Abciximab (ReoPro)	Canine coronary cyclic flow reduction	Significant inhibition of platelet-dependent thrombosis	Coller et al. 1986; Coller et al. 1989	High-risk coronary angioplasty	Reduction in death, myocardial infarction, refractory ischemia, or unplanned revascularization	EPIC Investigators (1994)
Tirofiban (Aggrestat)	Canine coronary cyclic flow reduction	Significant inhibition of platelet-dependent thrombosis	Lynch et al. 1995;	Unstable angina	Reduction in death, myocardial infarction, refractory ischemia	PRISM Investigators (1998)
Eptifibitide (Integrilin)	TPA-induced coronary thrombolysis	Significant improvement in lysis of occlusive thrombus	Nicolini et al. 1994	Acute myocardial infarction—thrombolysis with tPA	Improvement in incidence and speed of reperfusion	Ohman et al. 1997
Enoxaparin (Lovenox)	Canine coronary cyclic flow reduction	Significant inhibition of platelet-dependent thrombosis	Lynch et al. 1995	Unstable angina	Significant decrease in death, myocardial infarction, and need for revascularization at 30 days	Cohen et al. 1998
Hirudin (Refludan)	Rabbit jugular vein thrombus growth	Inhibition of thrombus growth compared to standard heparin	Agnelli et al., 1990	Deep vein thrombosis after total hip replacement	Significantly decreased rate of DVT	Eriksson et al. 1997
Argatroban	Canine coronary artery electrolytic-injury (TPA-induced thrombolysis)	Accelerated reperfusion and prevented reocclusion	Fitzgerald et al. 1989	Unstable angina	No episodes of MI during drug infusion	Gold et al. 1993

(1996), Vassalli et al. (1997), Waugh et al. (1999), and Zoldhelyi et al. (2000).

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B.8 Safety Assays in Thrombosis and Haemostasis

See Mousa (2006).

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