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Mouse models of focal arterial and venous thrombosis

Abstract Mouse models of arterial and venous thrombosis have gained increasing interest over the last 15 years, due to direct availability of a growing number of genetically modified mice, improved technical feasibility, standardization of new models of local thrombosis, and low maintenance costs. In order to provide an overview of suitable models for the study of arterial and venous thrombosis in mice, we have systematically searched MEDLINE electronic databases for publications reporting on murine thrombo-embolic models from 1966–1999. We found that the variety of murine thromboembolic models through 1995 was rather limited, as most methods used intravenous injections of strong coagulation triggers such as thrombin, thromboplastin and collagen, causing lethal thromboembolism. Between 1996 and the end of 1999, a number of more sophisticated murine models of local acute or chronic thrombosis have been established. They seem to be more suitable for mimicking the natural scenario of thrombosis and, therefore, are preferable models for pathophysiological or drug evaluation studies. In this paper various models are described and their advantages and limitations discussed.

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Introduction

Arterial thrombosis is mostly associated with arteriosclerosis, clinically manifested as myocardial infarction, stroke, and peripheral arterial occlusion causing a high mortality and substantial morbidity (6, 59). In addition, the incidence of venous thromboembolism due to either acquired or genetic prothrombotic states, to immobility, cancer, trauma, and infections adds considerably to the burden of vascular disease (55, 62). To improve the clinical management of these disorders, thrombosis researchers have amplified their efforts over the last 15 years in establishing suitable animal models of thrombosis (3). This is reflected by the increasing number of articles publisher between 1966 and 1999, reporting on murine thrombosis models in an electronic database (MEDLINE). Different murine models of focal venous and arterial thrombosis are summarized and their methodological strengths and weaknesses as well as their relevance to improve the understanding and management of thrombosis discussed.

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Why thrombosis in mice?

Research in thrombosis can basically be divided into two categories: research into 1) the (patho)physiology of thrombus growth and lysis and 2) the development of a specific new anticoagulant, antiplatelet, or fibrinolytic agent which preserves its antithrombotic effect in *in vivo* thrombosis models. In fact, many agents displaying anticoagulant or profibrinolytic properties *in vitro* do not preserve these qualities *in vivo*, i.e., are not or only partly effective as antithrombotic agents. Thus, before using a drug in a large animal model or in humans, small rodent models, for example in mice, are often used to explore initially the *in vivo* characteristics of a new agent. The MEDLINE search performed from 1966 to 1999 clearly showed a tendency towards more frequent use of murine models in the last 4 years. Of a total of 430 references dealing with "mice and thrombosis" within 31 years, we found 118 of these (i.e., about 25 %) to have been published since 1996. Figure 1 gives an overview of the number of publications per year for the various time periods since 1966 that have reported on murine thrombosis models. As can be seen, the number of murine models used in thrombosis research has increased over the last decade, probably for reasons of ethical considerations, cost saving, direct availability, technical feasibility, and uncomplicated breeding. However, the most prominent reason for research in mouse models is without doubt the potential for creation of mice with targeted deletions (knock-out) or targeted mutations (knock-in) in their genome (9, 36, 50, 51). These modifications enable the study of highly specific alterations in the coagulation system of living organisms. The small body size of a mouse can be regarded as an advantage as well as a limitation: it is an advantage that only small amounts of a new antithrombotic or antiplatelet agent are needed to test their *in vivo* efficacy in a statistically sufficiently large group of animals. On the other hand, manipulating an isolated vessel segment of the tiny vascular system, such as surgical isolation and ligation, or the insertion of venous, arterial, peritoneal or other catheters, as well as intubating the animal may cause major challenges to the investigator. Nevertheless, enormous progress has been made on this field in recent years.

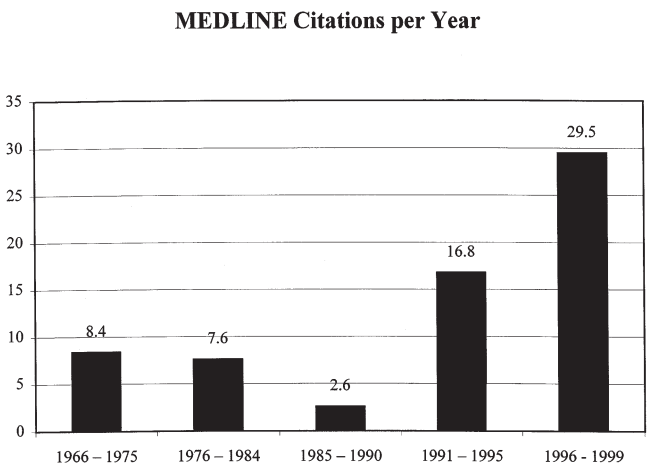

Fig. 1 Number of publications per year reporting on murine thrombosis models, as recruited in MEDLINE database, showing a 4-fold output in the 1990s compared to the decades from 1966 to 1990.

Methods of inducing thrombosis

According to the triad of Virchow, there are basically three possible mechanisms to evoke thrombus formation: 1) by inducing vessel wall injury, 2) by producing stasis, and 3) by creating a prothrombotic state or directly activate the coagulation system. In reality, of course, more than one of these pathomechanisms is involved in thrombosis formation with one stimulus dragging along the other. Thus, endothelial injury usually causes platelet activation which in turn can trigger the coagulation system or cause indirect coagulation activation by, for example, interleukin-6 expression mediated by tissue factor (45, 46, 49). In the arterial system, the interrelationship between endothelial injury and platelet activation is thought to play a major role in thrombus formation, whereas in the venous system stasis and coagulation have a more dominant position (25, 58, 69).

In the following section the models of thrombosis are described according to the kind of stimulus set to the vascular system. The earliest method of inducing thrombosis in mice consisted of systematic administration of strong coagulation triggers such as thrombin (34, 44), thromboplastin (8), collagen combined with epinephrine (5, 23), or ADP (30, 61), resulting in pulmonary clot formation. It is beyond the scope of this paper to go into the details regarding this model of pulmonary thromboembolism, since the pathomechanism of this type of thrombus is very far from the one occurring in a localized deep vein or arterial thrombosis. The model has, however, been used frequently to test antithrombotic agents and accounts for about 60 % of the recruited references in MEDLINE. Instead, this review will focus on models using endothelial injuries caused by various kinds of physical or chemical stimuli. Interestingly enough, the number of arterial models of thrombosis is clearly larger than that of venous models, although in other rodents, quite a number of venous models have been established (2, 37, 38).

Focal arterial thrombosis

Thrombus growth

Induction of local arterial thrombosis has been achieved in isolated segments of either the carotid or femoral arteries in

mice in various studies. Investigations have been performed to establish new standardized thrombosis models, to study pathogenetic aspects of thrombus formation, or to test new anticoagulant or antiplatelet agents. As an outcome parameter the time-to-occlusion (TTO) is a favored end-point, most of the time indirectly evaluated by means of blood flow measurements using a sonographic flow probe, or by using a thermal probe measuring the temperature decline at the moment of clot formation and flow stop. In carotid and coronary arteries the interest is often focused on cerebral (14, 19, 20, 28, 32, 70) and myocardial (31, 39, 64) infarcts or transient ischemia following thrombotic arterial occlusion

Recanalization

Agents enhancing fibrinolysis are frequently evaluated in recanalization models, where standardized thrombus growth is first induced by methods described below, followed by injection or infusion of a test agent. Time-to-recanalization (TTR) is then compared between control and treatment groups. Standard recanalization is usually achieved by administration of rtPA alone or combined with heparin and aspirin. The agents can either be injected through a tail vein or a jugular vein catheter (71).

Mechanical injury

In a model of mechanical injury, described by Cheung et al. (13), the common carotid artery is injured by the induction of a flexible guidewire, which is rotated several times at the intravascular site of injury. Due to the consequent extensive endothelial damage, platelet adherence, mural thrombosis, medial smooth muscle cell loss and necrosis already become apparent on the same day. Within the following 14 days, thrombosis and platelet deposition declines from 100 % on day 1 to 25 %. This model was applied in protease-activated receptor-1 deficient (PAR-1) mice to study cell proliferation and media thickening. Since thrombus formation occurs in the initial phase, the model can also be used as a purely acute phase thrombotic model, although standardization of the endothelial lesion size must be carefully evaluated for this purpose.

Electric injury

Arterial vessel wall injury is achieved in this model described by Carmeliet et al. (11,12) by application of an electric current through the arterial vessel wall, using an electromicrocoagulator. Hereupon, immediately a mixed thrombus consisting mainly of platelets and fibrin occurs. This method was originally used to create a model of neointima formation. The electric stimulus causes severe injury with cell necrosis in all layers of the vascular wall and induces vascular wound healing processes with smooth-muscle cell migration from the media leading to neointima formation. Media thickening in restenosis research is also of interest in this model. Because of limited possibilities to standardize the initial clot formation and because of the vast necrosis throughout the vessel wall, the model is less suitable to serve as a thrombosis model for efficacy evaluation of new agents.

Photochemical injury

Thrombus induction using the photochemical reaction of combined light irradiation of the vessel wall and intravenous infusion of rose bengal was first performed in the cerebral circulation of rats by Watson (65–67) and Dietrich (19–21) with argon laser light and filtered green light (540 nm). Matsuno et al. induced focal thrombosis on an isolated segment of the rat femoral artery and the carotid artery of the hamster, and has recently modified the method for the mouse femoral artery (40–42). The mechanism of thrombus formation is triggered by direct irradiation of the vessel wall with filtered green light (540 nm wavelength), followed by intravenous injection of rose bengal, which is an efficient photodynamic generator of oxygen radicals. The latter probably react with structural proteins and lipids in cellular membranes to induce peroxidation reactions which leads to endothelial damage (26, 65). Endothelial lesion is followed by platelet adhesion, aggregation, and thrombus formation. Histological and TEM analysis demonstrate mixed thrombi consisting of granulated and degranulated platelets and fibrin strands (42). In a later phase, following spontaneous thrombolysis, intima thickening occurs (33, 40). The method has found various modifications, when applied in the murine microvasculature (see below). This model seems to be easily reproducible and can be applied in most small rodent animals, either in the macro- or in the microvasculature. The administration of the photosensitizing dye as well as the irradiation dose and duration are well defined. Thrombus formation starts within an acceptable observation time and can be surveyed in real time. Moreover, no mechanical injury is needed to induce thrombosis.

Chemical injury: ferric chloride

The model, whereby ferric chloride is applied either to the femoral or carotid artery, was first described in guinea pigs (1) and has meanwhile been modified for rats (7, 35, 63) and mice (23, 24, 71). Usually, the carotid artery is bluntly isolated and a miniature sonographic flow probe positioned distal of the segment where the thrombus is to be induced. After reaching

steady-state blood flow, a 2 x 2 mm piece of filter paper soaked in a 10 % ferric chloride ($FeCl_3$) solution is placed on the vessel, left for 3 min to incubate, and then removed. At this moment, blood flow is usually still high and will often even increase, before declining and exhausting completely because of the growing thrombus. At the moment of total vessel occlusion and complete flow stop, the averaged flow is on the zero level and no pulsations can be detected either on the flowmeter scale or visually *in situ*. The time from removal of the filter paper until total occlusion is usually referred to as time-to-occlusion (TTO). It can happen that, prior to full occlusion, the thrombus is spontaneously recanalized and a pattern of no-flow-reflow is seen on the registered flow curve. This condition can last for several minutes, until either full occlusion or patency is reached. Therefore, the time period chosen for observation must be defined in the protocol in order to gain a reliable end-point parameter such as TTO or histological thrombus quantitation. In some cases a thermal probe measuring temperature decline at the occlusion has been applied as an additional endpoint parameter (35). But usually, TTO and histological quantitation of transsectional luminal patency or of thrombus masses are two parameters providing sufficiently reliable endpoint data. A few considerations, however, should be taken into account when using this model. First, in order to reach satisfactory reproducibility, care must be taken when manipulating the artery to avoid vessel distortion or twisting, so that no unwanted endothelial lesion and flow impairment can occur. Additionally, the vagal nerve must not be brought into contact with the $FeCl_3$ solution, since this can stimulate the vagal system leading to lethal bradycardia. When applied to the adventitia, $FeCl_3$ diffuses through the media to the intima and endothelium, where it causes cell damage by oxidation and inflammation and inducing thrombus. As mentioned above, the platelet-rich, fibrin and erythrocyte containing clots can be recanalized using rtPA alone or in combination with aspirin and heparin. Figure 2 shows a flow curve of a C57BL/6 mouse that was submitted in our own laboratory to clot formation using 15 % of $FeCl_3$ and to fibrinolysis treatment with rtPA and heparin. A curve pattern of no-flow-reflow is typically seen before full recanalization.

The models described above have the advantage that they can be performed without intubation and mechanical ventilation of the animal; hence no thoracotomy is needed and the experiments can be performed within less than two hours. Basal mean blood flow, however, should be comparable inter- and intraindividually and also between compared mouse strains. Blood pressure measurements might be useful but are not absolutely necessary and often not possible, as one carotid artery is used for thrombus induction and the contralateral artery as a control. Non-invasive blood pressure measurements by, for example, tail cuff method could be used in this regard. Moreover, occluding both carotid arteries in an acute experiment would lead to death within a few hours or less,

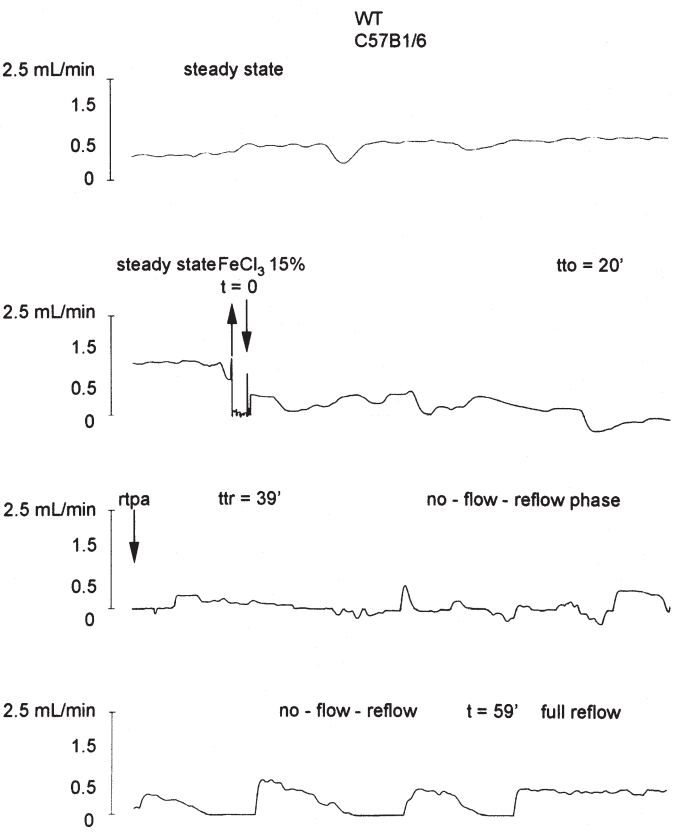

Fig. 2 Averaged blood flow curve as registered in the carotid artery of a C57BL/6 mouse before (steady state) and after thrombus growth (application of $FeCl_3$) and total occlusion, followed by intravenous administration of rtPA and consequent recanalization. The typical pattern of no-flow-reflow was observed, before full patency occurred.

whereas one carotid artery occlusion is compensated by the remaining patent neck arteries, provided that the circle of Willis is complete (4). When using a flowmeter, registration can be chosen as 'averaged' or 'pulsatile'. Usually it is most convenient to register the averaged flow; however, changing to the 'pulsatile' mode at time intervals allows simultaneous registration of the heart rate, which is useful for comparisons between hemodynamic parameters of individual animals and different groups. It is essential that central body temperature is kept constant, by use of a heated base and a rectal thermal probe, and that the influence of anesthetics on vasotonus and heart rate is taken into consideration.

Models of venous focal thrombosis

Not many reports on murine focal venous thrombosis models can be found in the literature. Most of them are described for small rodents other than mice, where preformed blood clots are inserted in defined venous segments and thrombus growth

is, for example, measured by assessment of ^{125}I-fibrinogen accretion (37). In mice, this technique has not been widely applied, probably due to the small sized venous caliber of this rodent. Nevertheless, one model was found that seems to be suitable for thrombotic studies on the venous system due to its good technical feasibility, established standardization and reproducibility (47, 48). This method uses the femoral vein of CD-1 mice, which is dissected free between the inguinal ligament and the superficial epigastric branch. On the 1 cm long isolated segment, a standardized pinch is exerted with a pressure of 1500 g/mm^2 using a circular pinching forceps. Two small thrombogenic injuries corresponding to the contact surfaces are produced. Thrombus growth on these injuries is visualized with a fiberoptic transilluminating device, placed beneath the vessel. The growing thrombus appears as a bright yellow spot, whereas the flowing blood is dark. Apart from the spot extension, its brightness indicates thrombus size and is analyzed by computer-assisted gray level analysis. The resulting thrombus consists mainly of aggregated platelets; it typically grows rapidly within the first 5 min after injury application and then grows slowly over the following 30 min. This is a well-standardized, quantitative and continuous mural thrombosis model, originally used to study antiphospholipid-syndrome associated thrombosis. Finally, our own preliminary but promising experience, using the ferric chloride technique in C57Bl/6 mice on the external jugular vein, as described above for the carotid artery, inducing now a venous thrombosis, deserves a mention. The method is in its standardization phase, presently and shall be reported in the near future.

Microvascular thrombosis

Although microvascular thrombosis has a different hemodynamic effect than focal thrombosis in the macrocirculation, it is appropriate to briefly describe these well-established methods in this review, especially since they provide a possibility of studying real time thrombus formation in specific vessels.

Photochemistry and intravital fluorescent microscopy

As described above, the vessel wall is transluminated with either argon laser light or green light (540 nm) and a photosensitizing agent, e.g., rose bengal or FITC is injected intravenously thus inducing endothelial damage. Platelets activated by endothelial injury start to deposit on the injured vessel segment and to form a clot. Visualization is usually reached by intravenous injection of a fluorescent dye and by monitoring blood flow and clot formation under transillumination of a halogen lamp under a fluorescence microscope. The model was first described by Sato (56, 57) in the rat mesenteric arterioles and venules, using sodium fluorescein. It has since been modified for application in mesenteric vessels of ICR (International Cancer Research) mice by Sheu et al. (60) and in the ear of the hairless mouse by Roesken et al. (52, 53), who used FITC-dextran. Rapid thrombus growth is usually observed, first in venules with platelet deposition starting about 30 s after irradiation, followed by complete vessel occlusion after 6 to 8 min. After 24 h, 75 % of the thrombosed venules are recanalized spontaneously. In arterioles thrombus onset starts markedly later, at 2–2.5 min following irradiation, and total occlusion occurs within less than 30 min with only 1 out of 10 cases presenting spontaneous recanalization within 24 h. Denis et al. (17, 18) studied defects in hemostasis and thrombosis in a transgenic mouse presenting severe von Willebrand (vWF) disease. In this model, the exteriorized mesentery was superfused with ferric chloride and the accumulation of fluorescently labeled platelets was observed by intravital microscopy. Microthrombosis induced in the ear of mice, needs no surgical treatment. This mesenteric approach also seems to be easily reproducible, and thrombosis can be observed in acute, subacute, as well as in chronic phases, both in the venous and arterial system. These models are suitable to study the effect of antithrombotic agents, although limited to the microvascular bed.

Conclusion

The revolution of gene technology in medicine has left its traces on the field of thrombosis and hemostasis by the creation of transgenic mouse strains, lacking or over-expressing single proteins of the coagulation and fibrinolysis system or presenting with arteriosclerotic lesions (10, 13, 15–17, 27, 29, 30, 54, 68). These mice will provide new models and contribute to the unraveling of the pathophysiology of thromboembolic diseases. Models of focal arterial thrombosis that could be applied in these new mouse strains in the future are available, well-standardized and feasible, due to the progress made mainly within the last five years. Experience in a wide range of various transgenic mouse strains is, however, still lacking presently. Thus it remains uncertain whether inflammatory mediator responses to thrombus induction are different in mutagenic mice compared to wild-type mice. However, one experiment should be mentioned in this context using the ferric chloride method in IP3-receptor KO mice, demonstrating the importance of prostacyclin as an inhibitor of clot formation (43). The choice among venous models, however, is still small, and especially those models allowing thrombosis observation in the macrocirculation, not only in the acute but also subacute and chronic phase, still need to be developed. Irrespective of the method used to achieve thrombus in mice, it is important to be aware of inter- and intraindividual as well as strain-related differences. New methods must be carefully evaluated

and compared before the model can be relied on. Such an evaluation includes influences of anesthetical drugs on heart rate, blood pressure, blood flow and vasomotion. Care must also be taken to provide optimal conditions with respect to stable body temperature, fluid balance, and respiration. With the much needed improvement in the understanding and management of thrombosis, murine models of arterial and venous thrombosis will remain valuable tools providing much needed molecular insight into the pathogenesis and treatment of thrombosis.

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