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CHAPTER 9

GENETICALLY MODIFIED MICE IN THROMBOSIS AND HEMOSTASIS: A REVIEW

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Summary

This review is a systematic review of published experiments performed in genetically modified mice used in thrombosis and hemostasis research. Over the last five years the interest into experimental research in thrombosis and hemostasis has considerably shifted from evaluating antithrombotic effects of new anticoagulant or antiplatelet compounds to questions of molecular functions of specific coagulation proteins. These models offer the opportunity not only to study the pathophysiological process of thrombosis and hemostasis but also open the way to interdisciplinary research. This is illustrated by demonstration of the crosstalk between coagulation and vascular development, coagulation and inflammation, coagulation and infection, and coagulation with underlying internal diseases such as diabetes mellitus, collagen disease, and sepsis, provided by transgene mouse models. However, this enthusiasm should not blind investigators to the fact, that explanations gained from successful transgenic mutations in mice mimicking human disease, usually cannot directly be translated to the human organism. The chance of obtaining phenotypes that are possibly due to gene interference or compensation rather than purely to the desired targeted mutation is an important pitfall. However, we will undoubtedly obtain clue information helping us to better understand a pathological process, or the network of relationships with other proteins into which a protein is imbedded. The more for example thrombus formation, has been investigated and compared in different mouse strains under comparable circulation conditions, the more will strain-related differences regarding susceptibilities to the applied model be ruled out. One way of avoiding such misinterpretation might be to obtain the same results in more than just one mutagen strain. In addition, an international definition on required background qualities, such as the number of back-crossings or of littermate preconditions needed, might enable clearer communication through published articles between research groups.
The importance of animal models

Thrombus formation of the arterial system causes a number of diseases of high morbidity and mortality, such as myocardial infarction, stroke, and peripheral arterial occlusive disease (PAOD). In addition, the relatively frequent occurrence of venous thromboembolism, which is mostly related to abdominal or orthopedic postoperative phases, to acquired or genetic prothrombotic states, immobility, cancer, trauma, or infections, adds further on the thrombosis-related morbidity. From these facts, it is obvious that considerable health gain may be achieved by better prophylactic and therapeutic antithrombotic strategies. To develop such strategies, there is a need for a better understanding of the pathophysiological process and the ethiological causes of atherosclerosis, thrombosis and venous thromboembolism.

What are the optimal conditions to investigate the mechanisms involved in thrombus formation? Cells in culture or artificial hemodynamic models cannot provide a system mimicking the complex situation of the vascular circulation including changes of flow, dynamic coagulant and physiological anticoagulant responses, and the effect of intact or injured endothelial cells \textit{in vitro}. Thus, suitable animal models are called for. Use of large mammalian animals, such as primates, pigs, dogs and cats has decreased in the course of the last 15 years, whereas smaller animals like rabbits, guinea pigs, rats and mice were favoured. Indeed, there has been a strong tendency towards using smaller animal models over the last decade for reasons of ethical considerations, cost saving, direct availability, technical feasibility, and uncomplicated and rapid breeding. In a recent review summarising murine models of focal arterial and venous thrombosis models, it was shown that the number of annual publications reporting on murine thrombosis experiments has increased more than 10fold since 1990 and doubled since 1996. A similar development has been reported in cardiovascular and stroke research, with a clear trend towards using transgenic mouse models.

In a literature search on MEDLINE-database, an increasing number of publications reporting on mouse models in thrombosis and hemostasis research was noted with a shift towards genetically modified mice of 25% since 1996, as shown in Figure 1.
Figure 1. From 1991 to 1995 a total of 84 publications reporting on experiments using mice in thrombosis and coagulation research was found; among these there 28 using transgenically modified mice, whereas in the period between 1996 and 2000, the total number of murine experimental reports increased to 118, of which 69 were related to transgenic mice. These figures show a shift towards the use of transgenic mice in coagulation and thrombosis models of 25% within 5 years.

Why mice in thrombosis research?

For many researchers using mouse models, the most attractive challenge is the potential for creation of mice with targeted deletions (knock-out) or targeted mutations (knock-in) in their genome. These modifications enable the study of specific alterations in the coagulation system of a living organism. However, the small body size of a mouse represents a major disadvantage for any manipulation and instrumentation performed in these animals. All instruments and equipment have to be adapted to the miniature size, which is costly and sometimes not easily feasible. The only prominent advantage of the body small size is that tiny amounts of a new antithrombotic or antiplatelet agent are needed to test its in vivo efficacy in a statistically large enough group of animals. The increasing number of publications reporting on murine thrombosis models over the last five years shows that despite of the mentioned disadvantages of these small animals, considerable progress has been made on the field of elaborate instrumental handling.
Genetically modified mice in thrombosis research

What are the options to use a genetically modified mouse model for research in thrombosis and hemostasis? The easiest way in terms of time and expenses is to get a well-defined mouse strain from a collaborating laboratory or to purchase them from a large core laboratory supplier, for example Jackson Laboratories, Maine. Homozygous transgenic mice, backcrossed 6 times to one of their background strains, present with an over 90% homogeneous background and may therefore be compared to their background strain. Offspring of heterozygous transgenic mice are genotyped by either a PCR test or Southern blotting method. According to the Mendelian rule of genetics the F2 generation offspring will consist of 50% heterozygous (+/−), 25% homozygous (+/+ ) and 25% null (−/−) littermates, the latter (wildtype) serving as controls.

The second option is to develop a new transgenic mouse in the own laboratory, which is much more time-consuming and expensive and calls for specialized personnel, experience and technical know-how. In principle, mice are modified genetically by three different techniques. The first is direct injection of foreign DNA into blastocytes, i.e. into embryos at the one-cell stage, with resulting unknown copy numbers and unpredictable phenotype, also called random chromosomal integration (15). This method is mainly applied to achieve either increased production of a protein ("overexpression"), or to study the stronger or weaker effect of a mutant protein, and is not used frequently in coagulation research. One exception is the generation of rescued tissue factor (TF) mice, where a human DNA fragment containing the TF minigene was injected into fertilized, non-viable TF null mouse embryos, furnishing rescued founder offspring16.

The second technique is a targeted mutation for the creation of knock-outs and, as a less common variant, targeted point mutation creating mutant mice. This method, so-called homologous recombination, is used to modify genome by gene targeting, i.e. by modification of a specific chromosomal locus. Hereby, a specific DNA sequence is introduced into cultured embryonic stem (ES) cells, followed by injection of identified ES cell clones with the mutation into blastocytes, or aggregation of mutated ES cell clones with developing ES cells. The growing mice represent a genetic mosaic, with a mutated germ line (sperm or egg) carrying the mutation and passing it to their offspring. Carriers of the mutation will be heterozygous and mated with other heterozygous animals for homozygous (25%), heterozygous (50%) and wildtype (25%) offspring. However, in various cases, homozygous knockouts are not viable, as the deleted protein plays an essential role in embryogenesis or because major bleeding results in perinatal death. Such non-viable knock-out mice have been generated in thrombosis research for example for thrombomodulin-/-, Protein C -/-, tissue factor (TF)-/-, tissue factor pathway inhibitor (TFPI)-/-, prothrombin -/-, and factor X17.22. These models are of limited value, as they may only contribute to elucidate the role of a specific protein in embryogenesis. They cannot function as a model for further investigation of the altered coagulation state caused by the protein deficiency.
An alternative strategy is a targeted point mutation with only partial or functional deficiency of the protein, as for example in thrombomodulin, with creation of viable mice. Viable murine knock-out models in the coagulation field have been generated for the plasminogen activator inhibitor-1 (PAI-I-deficiency), plasminogen, fibrinogen, factor VIII, factor IX, factor XI. There are no murine models for deficiencies of factors XII, and XIII.

The third and most promising method for the future, however, seems to be the Hprt targeting of X-chromosomes, that does not yet play a major role in coagulation research. In contrast to standard transgenic assays, where multiple copies of heterologous DNA cassettes are randomly integrated into the mouse genome, this method uses a single copy of a transgene with chosen-site integration. The method has recently been applied for the human eNOS promoter targeted to the X-linked hypoxanthine phosphoribosyltransferase (Hprt) locus of mice by homologous recombination, resulting in the expression of tissue-restricted transgenes.

From genotype to phenotype: thrombus formation

Hypoxia induced thrombosis and prethrombotic state

Successful transgenic manipulations lead to different phenotypes, visible for example as fibrin deposition and clot formation in the small vessels of pulmonary, liver or kidney tissue, when a procoagulant factor has been overexpressed or an anticoagulant factor knocked-out or diminished. The new phenotype, i.e. clot formation in tissues, is often not recognizable spontaneously, but only following exposition to a thrombotic stimulus such as hypoxemia as shown in tissue factor-overexpressing or thrombomodulin-mutant mice. Presenting a prethrombotic state by deletion or overexpression of a specific protein, these models are also used to study the direct impact of a specific protein on thrombus formation. The altered phenotype is usually detectable either in lowered levels of the deleted protein or in its impaired anticoagulant activity in plasma at rest or under hypoxic stimulation and is visualized in histology as fibrin deposition in various tissue vasculatures. The viable mice with a prothrombotic state caused by targeted point mutation in the thrombomodulin gene may serve as an example. Thereby, fibrin deposition, evaluated both by histological staining and Western blot analyses, showed that these mice had documented fibrin deposition at all levels of the vascular system. After exposure to hypoxia they produced an additional 10-fold fibrin deposition in the same organs compared to a 4-5 fold increase in wildtype mice. In another study exposing CD-1 mice to hypoxia under normobaric conditions, thrombosis was also demonstrated in pulmonary vasculature and fibrin presence shown by antifibrin-antibody staining, immunoblottings and radio-labelled fibrin/fibrinogen deposition. In the same mice, isolated monocytes demonstrated increased TF immunostaining when exposed to hypoxia.

It is not completely clear, why hypoxemia induces fibrin deposition in the tissue microvasculature, but the following mechanisms may play a role: Mice exposed to a hypoxic
chamber will respond to this stimulus by activation of transcription factor early growth response-1 (Egr-1) leading to de novo transcription and translation of tissue factor in mononuclear phagocytes and smooth muscle cells, resulting in vascular fibrin deposition\(^1\). At the same time, hypoxia-mediated upregulation of plasminogen activator inhibitor-1 amplifies the prothrombotic response. On another level, hypoxia activates the transcription of hypoxia-inducible factor1 (HIF-1), which is responsible for the enhanced expression of a number of metabolic proteins or hormones such as erythropoietin, glycolytic enzymes, non-insulin-dependent glucose transporters, vascular endothelial growth factor, nitric oxide and heme oxygenase type 1. Thus, HIF-1 knock-out mice developed neural tube and cardiovascular defects with subsequent embryonic lethality on day 11, underlining a key role in vasculogenesis of HIF-1\(^1\). Taken together, hypoxia seems to induce activation of Egr-1 with subsequent transcription and cell surface expression of TF in vascular smooth muscle cells and macrophages, thus initiating the local procoagulant response.

**Lipopolysaccharide induced thrombosis**

Similar to hypoxia, the injection of lipopolysaccharides (LPS) from Gram-negative microorganisms may serve as a thrombotic stimulus. Mice presenting with a prethrombotic state due to a functional TM-deficiency, who were submitted to sublethal LPS injection, were found to have pulmonary clot formation, as shown in the TMpro/pro mice (own unpublished results). Thereby, TF is probably triggered by a strong cytokine response to LPS, i.e. by early peak responses of tumor necrosis factor (TNF\(\alpha\)) and interleukin 6 (IL-6), activating monocytic and endothelial TF-expression. Consequently, the equilibration between the coagulation and fibrinolysis system in an already prethrombotic state, is disturbed favouring the coagulation side with eventually highly increased thrombin generation. Resulting thrombus formation mainly consisting of fibrin clots in the pulmonary vasculature, provides a useful tool for histological visualisation and quantitation of a modified phenotype. These mice served also as a model to investigate the crosstalk between the coagulation and inflammation system referring to thrombin as a coagulation product with pro-and anti-inflammatory properties (own unpublished results).

**Pulmonary thromboembolism**

Intravenous administration of strong coagulation triggers such as thrombin\(^42\), thromboplastin\(^45\), collagen combined with epinephrine\(^46\), or ADP\(^44\), result in pulmonary clot formation in most inbred mouse strains. Mice injected with these substances will develop systemic thromboembolism within a few minutes, mainly in the pulmonary vasculature, due to blood sequestration and platelet activation with resulting thrombus formation. The endpoints of the method are mortality (many mice do not survive the respiratory distress), time elapsed between injection of the coagulation trigger and death, and histological screening for fibrin deposition and
clot detection in the pulmonary vascular bed. This model has been widely used to test antiplatelet or anticoagulant agents, to study physiologically present molecules having antithrombotic properties, and also to elucidate (patho)physiological mechanisms of platelet activation in context of thrombus formation in the arterial system.

**Thrombus induction in focal arterial and venous models**

In the macrocirculation, techniques have been applied to induce local arterial thrombosis in isolated segments of mostly the carotid or femoral artery in mice. Thrombus growth was usually evaluated by measurement of time-to-occlusion (TTO), i.e. time elapsing between the damaging stimulus set to the vascular endothelium and full vessel occlusion, when mean flow measurements were performed with a sonographic flow probe. Some investigators used computer-assisted histological quantification on cross-sectional slides or weight assessment of the extracted thrombus. Fluorescent labeling of platelets has been used to visualise thrombus formation, however, this method was mainly applied in the microvascular bed of the ear in hairless mice, in the mesenterium, or in the brain microvasculature.

Recanalisation of a segmental thrombus or time-to-recanalisation (TTR), i.e. time elapsing between injection of an antithrombotic agent after full vessel occlusion and reflow measured sonographically, has been shown to be a useful parameter, when the effect of fibrinolytic agents is tested. Standard recanalisation is usually achieved by the administration of rtPA, heparin and aspirin injected through a tail vein or a jugular vein catheter.

**Endothelial lesion** was induced by different types of stimuli, such as mechanical (forceps, guide wire), electrical, chemical (iron chloride), or photochemical (filtered green light combined with a fluorescent dye, as reviewed in detail. All applied methods have the same basic principle, i.e. an endothelial lesion is induced followed by platelet activation and triggering of the local coagulation system, reflecting the pathophysiological triad of Virchow.

**Optimising factors**

Most of the described thrombosis models can be performed without intubation and mechanical ventilation of the animals. However, maintenance of stable anesthesia, respiration rates, and body temperature seem to have a direct impact on heart rates, blood flow and mean aortic pressure (MAP). These parameters should be available for comparison of interindividual differences in thrombosis formation or thrombolysis and between treated and untreated groups. Again, strain-specific differences may be important as well. Local inflammatory responses for example, might be more or less pronounced and display different cytokine response patterns between different inbred strains. In addition, the vasoconstrictive reaction to a stimulus might be much stronger pronounced in one strain than in another. Exclusion of (unconscious) subjective bias—in particular when no objective end-point is available—is sometimes not possible, as
certain transgenic strains can be distinguished from their wild-type controls by appearance, which does not allow blinded experiments.

**Multifunctional roles of coagulation proteins**

Many transgenic mice deficient of one of the coagulation or fibrinolyis proteins have been generated in order to study the role of these factors in thrombosis and hemostasis. Such approaches sometimes resulted in non-viable mice. Death occurred either during the embryonic development or shortly after birth, suggesting that the protein of interest plays more than the one role in the coagulation system. For example, a thrombomodulin knock-out mouse\(^1\) died on embryonic day 8.5 with anomalies of the cardiovascular system. In contrast, another model designed by targeted point mutation of the thrombomodulin gene, which only inhibits binding of thrombin to its receptor thrombomodulin, without deletion of the entire thrombomodulin-gene, is viable, presenting with a prethrombotic state due to impairment of the anticoagulant protein C pathway\(^2\). This example shows, that deletion of a partial function of a coagulation protein may be in accordance with viability, whereas the deletion of the whole gene is not compatible with life because of the multifunctional role of the protein at target. Tissue factor, the primary cellular initiator of blood coagulation, is another example of a coagulation factor playing a multifunctional role. Generation of mice with complete disruption of their tissue factor gene resulted in midgestational lethality by exsanguination due to either failure in hemostasis or/and vasculature development\(^2\). However, when the missing murine tissue factor was replaced by a human tissue factor minigene in the tissue factor null mice, the animals were rescued from embryonic lethality.\(^16\) Thus, tissue factor is not only the initiator of the coagulation cascade, but also influences embryogenesis and plays a role in tumor metastasis.\(^19\)

Alternatively, a central role in the coagulation mechanism may be a sufficient cause of perinatal mortality. Protein C null mice presented with normal macroscopic anatomy at birth but with lethal perinatal consumptive coagulopathy, suggesting that complete PC deficiency is inconsistent with short-term survival.\(^19\) Similar patterns are seen with factor VII and factor X deficient mice that die from a bleeding diathesis shortly after birth.\(^5^\) Other murine models to be named here for having been used to study coagulation dysfunctions are the fibrinogen-, FVIII-, FV-, PAR-1-deficient mice and the PAI-1-, and plasminogen-deficient mice on the fibrinolytic side. Due to the fact, that some of the coagulation proteins, e.g. thrombin, have pro- and antiinflammatory properties, some of the models have been used to elucidate specific aspects of the cross-talk between the coagulation and inflammation system or in bacterial defense mechanisms.\(^5^2\)
Genetical backgrounds and strain related differences in phenotype

Over the last few years, with an increasing number of institutes and research centers starting to work with transgene animals, the consciousness of the importance of clearly defined genetical back-grounds has grown simultaneously. Thus, mice that were designed by homologous recombination, where frequently two different strains have been involved in the genome must have been back-crossed with one of the background strains several times, in order to avoid purely strain-related differences in the phenotype. After 6 generations of mating to for example C57BL/6, approximately 99% of the genetic background will be C57BL/6. Alternatively, chimeras can be mated with 129/SV+c+pmice, which have the same background as the embryonic stem cells. This will produce mice with the targeted gene mutation on the 129/SV+c+pm background for comparison with the mutation on the C57BL/6 background. Only when background genotypes have been „diluted“ by backcrossing procedures reaching an approximately 100% homogeneity in their offspring, can the mutation of interest be considered as the cause for resulting differences in their phenotype. Another possibility to provide a comparable background genome is to use littermates instead of the original background strain as controls, i.e. null brothers and sisters of the same generation and family. Depending on how many times the parents have been back-crossed, there might still be considerable individual differences with consequently wider ranges in the results and necessitating a larger amount of animals to be investigated. Even when mice are designed and bred with complete awareness and care of background interference, a risk for not achieving the phenotype aimed at will remain, as the function of one deleted gene can be compensated by another. Thus, relying on a transgenic mouse model always requires caution in interpreting the results, no matter how convenient they might appear.

Transgenic mice to study platelet function

Before the 'transgenic era', platelet function was mainly studied in vitro. The creation of mice presenting with genetic platelet dysfunctions as they appear in humans, provides a new possibility of studying platelets in an in vivo system. Thus, the role of CD39 - or vascular adenosine triphosphate diphosphohydrolase - on the inhibition of platelet activation, was investigated in a CD39-deficient mouse. After photochemical induction of an endothelial lesion in mesenteric small vessels, CD39-deficient mice had prolonged bleeding times with considerably reduced platelet interactions with the injured mesenteric vasculature. Additionally, purified mutant platelets failed to aggregate to standard agonists in vitro. This platelet hypofunction was reversible and associated with purinergic type P2Y1 receptor desensitization. The role of the purinoreceptor P2Y1 in ADP-induced platelet activation was evaluated in a P2Y1-deficient mouse model. These animals were protected from fatal pulmonary thromboembolism after injection of collagen and ADP and presented prolonged bleeding times compared to their wild-
compared to their wild-type controls, suggesting, that the ATP-receptor P2Y1 mediates ADP-induced platelet processes.

In similar experiments performed in mice lacking platelet-type12-lipoxygenase (P-12LO), which is a key enzyme in an alternative pathway of the arachidonic acid metabolism to 12-hydro(pero)xyeicosa-tetraenoic acid, the animals were submitted to systemic ADP-injection, resulting in a quadrupled mortality rate compared to their wild-type mice. This transgenic thrombosis model yielded evidence for the hypothesis that enhanced ADP sensitivity of P-12LO-/-- mice reveal a mechanism by which a product of the P-12LO pathway suppresses platelet activation by ADP\(^{41}\). Ware et al, studying platelet glycoprotein receptors, were able to show that transgenic engineering of a platelet adhesion receptor under control of the human glycoprotein Ib alpha (GP Ib) promoter Ib alpha was possible, thus, illustrating a new approach to manipulate platelet receptors and study structure-function relationships in hemostasis and thrombosis\(^{62}\). Von Willebrand disease is a frequently occurring congenital deficiency of von Willebrand factor, which is the most important ligand for platelet adhesion to (sub)endothelial structures. This disorder could be more extensively studied through the creation of a murine model presenting with severe vWF-deficiency and consequent defects in thrombus formation, using the ferric chloride induced thrombosis model in the exteriorized mesentery. These mice exhibited highly prolonged bleeding time and spontaneous bleeding events with strongly reduced factor VIII levels, and fluorescently labeled platelets did hardly interact nor lead to occlusion in the ferric chloride superfused arterioles\(^{41}\).

**Transgenic mouse models of thrombosis in coronary heart disease**

Genetically modified mice used in research of coronary heart disease rather focus on investigations of myocardial ischemia and reperfusion than on coronary thrombosis. In contrast to larger animals or humans, the murine coronaries are imbedded in the myocardium and are therefore not easily accessible for isolation and manipulation. Although it is possible to isolate and ligate, for example, the left anterior descending branch (LAD) of the left coronary artery, the surrounding myocardial tissue will always be damaged, which frequently induces ventricular arrhythmia\(^{64}\). This might be one reason for the fact, that there are no published models of coronary thrombosis in genetically modified mice. Studies of the pathophysiology of arterial thrombosis are preferably performed under the more favourable conditions of an easily accessible isolated arterial segment than in the coronaries. Even if a model of coronary thrombosis followed by recanalization would mimic the natural pathophysiology of humans more precisely, several additional problems remain to be solved such as the development and quantification of a standard infarction size caused by a reproducible vessel occlusion. The latter is, however, difficult to achieve due to interindividual patterns of circulation and collateral
formation. Moreover, because murine coronaries are small sized and completely embedded in
the myocard, oxygen will always be able to diffuse into the surrounding tissue even into regions
of infarction, further hampering reproducibility.

Local cardiac expression or deficiency of coagulation proteins was an interesting approach in
a transgene model of myocardial microvascular thrombosis in mice deficient of thrombomodulin
(TM), tissue-type plasminogen activator (tPA), or urokinase-type plasminogen activator (uPA).
Thereby, the impact of the locally expressed endothelium-derived gene products (tPA, uPA, TM)
on myocardial formation and degradation of fibrin was studied. These mice, presenting a
prethrombotic state, were used as a thrombosis model per se and demonstrated that tPA played
the most important role in local regulation of fibrin deposition in the heart17.

For further information on experimental coronary thrombosis or stenosis models we refer to
other reviews65,66,89.

Conclusion

Considering the presented development in transgenic murine thrombosis research, we conclude,
that over the last four years the interest into experimental research in thrombosis and hemostasis
has considerably shifted from evaluating antithrombotic effects of new anticoagulant or
antiplatelet compounds to questions of molecular functions of specific coagulation proteins, thus
opening the field to new therapeutic strategies. The presently worldwide enthusiasm about the
creation of new transgenic mouse models is certainly justified, as these models offer the
opportunity not only to study the pathophysiological process of thrombosis and hemostasis but
also open the way to interdisciplinary research. This is may be illustrated by demonstration of the
crosstalk between coagulation and vascular development, coagulation and inflammation,
coagulation and infection, and coagulation with underlying internal diseases such as diabetes
mellitus, collagen disease, and sepsis, provided by transgene mouse models. However, this
enthusiasm should not blind investigators to the fact, that explanations gained from successful
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