

Tissue factor and thrombosis: The clot starts here

A. Phillip Owens III; Nigel Mackman

Division of Hematology/Oncology, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

Summary

Thrombosis, or complications from thrombosis, currently occupies the top three positions in the cardiovascular causes of morbidity and mortality in the developed world. There are a limited number of safe and effective drugs to prevent and treat thrombosis. Animal models of thrombosis are necessary to better understand the complex components and interactions involved in the formation of a clot. Tissue factor (TF) is required for the initiation of blood coagulation and likely plays a key

role in both arterial and venous thrombosis. Understanding the role of TF in thrombosis may permit the development of new antithrombotic drugs. This review will focus on the role of TF in *in vivo* models of thrombosis.

Keywords

Tissue factor / factor VII, thrombosis, arterial thrombosis, venous thrombosis, animal models

Correspondence to:

Nigel Mackman, PhD
Division of Hematology/Oncology
Department of Medicine
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina, USA
Tel.: +1 919 843 3961, Fax: +1 919 966 7639
E-mail: nmackman@med.unc.edu

Received: November 13, 2009

Accepted after minor revision: March 29, 2010

Prepublished online: June 10, 2010

doi:10.1160/TH09-11-0771

Thromb Haemost 2010; 104: 432–439

Introduction

Tissue factor (TF) serves as the primary initiator of the coagulation cascade (1–2). It is the cellular receptor for the plasma factor VII/VIIa (FVII/VIIa). The TF:FVIIa complex activates both FIX and FX. Activated FX (FXa), along with its cofactor FVa, is referred to as the prothrombinase complex and cleaves prothrombin to thrombin, which then cleaves fibrinogen to fibrin. The transglutaminase FXIII then cross-links fibrin which acts to stabilise the platelet-rich thrombi. The TF:FVIIa complex is inhibited by a multivalent serine protease inhibitor, known as tissue factor pathway inhibitor (TFPI) (3).

Circulating blood is normally maintained in a fluidic state. The coagulation system is only activated at sites of vessel injury and this prevents excess blood loss. Following vessel injury, TF on adventitial cells, such as adventitial fibroblasts, pericytes and smooth muscle cells (SMCs), activate the clotting system (4–6). Haemostatic clots are localised to the vessel wall and do not greatly impair blood flow in the vessel. In contrast, thrombotic clots result in impairment of blood flow and even complete occlusion of the vessel. Thrombotic events commonly result from a pathologic response to vascular injury, such as atherosclerotic plaque rupture, the main cause of arterial thrombosis. Vessel wall-derived TF has been described to provide a “haemostatic envelope” around blood vessels in healthy individuals (5). In contrast, pathologic expression of TF within the vessel wall or in the blood may trigger thrombosis.

In addition to TF expression in the vessel wall, there are reports of TF within the blood, so-called “circulating TF”. Importantly, the level of circulating TF in the blood of healthy donors is extremely low and does not appear to contribute to haemostasis (3, 7). How-

ever, the level of circulating TF is elevated in various disease states. The relative contribution of vessel wall vs. circulating TF to thrombosis is currently a highly debated topic. Levels of TF in the vessel wall are reported to far exceed the amount in blood with an estimated ratio of 1,000:1 (~20 pM vessel wall TF vs. 20 fM circulating) (8–9). Other investigators have reported higher concentrations of TF in the blood of healthy individuals (10–12) with the highest levels (37 pM) found in a cohort of arthritis patients (13). This circulating TF is present at very low levels on monocytes and in the form of small membrane microparticles, also known as microvesicles (MVs) (14). MVs are derived from activated or apoptotic cells (15). TF expression can also be induced in blood monocytes and possibly endothelial cells during pathogenic states, such as sepsis (16–18). Levels of circulating TF are also increased in chronic pathologic conditions, such as cardiovascular disease, cancer, and sickle cell disease (19–24).

The role of TF in thrombosis was originally studied using inhibitory drugs in animal models of thrombosis. More recently, genetic mouse models have been used. However, deletion of the TF gene was found to be embryonic lethal. (25–27). In order to overcome this lethality, we generated a “low TF” mouse that express very low levels of human TF from a minigene (~1% normal levels) in the absence of mouse TF (28). These genetically modified mice, along with mice containing cell type-specific deletions of the TF gene, have allowed studies on the relative contribution of vessel wall vs. circulating TF to thrombosis. Additionally, TFPI heterozygous mice (TFPI^{+/-}) or transgenic overexpression are also a useful tool to analyse the effects of increasing TF activity (29). The goal of this review is to summarise the literature on TF inhibition via small molecule inhibitors, blocking antibodies and recombinant TFPI

(rTFPI) on thrombosis in animal models, as well as results from genetic mouse models.

Arterial thrombosis

Arterial thrombosis is the most common cause of death in the developed world. The primary cause of arterial thrombosis is either instability or rupture of an atherosclerotic plaque resulting in loca-

lised clot formation and blockage of blood flow with subsequent myocardial infarction or stroke (► Fig. 1A). Arterial thrombi are platelet-rich and are referred to as white clots. Thrombotic events are most devastating when they occur in the coronary and carotid arteries, which are prone to atherosclerotic disease. Various risk factors can increase the incidence of arterial thrombosis, such as smoking, hypertension, hyperlipidaemia, and diabetes mellitus. TF is present at high concentrations in atherosclerotic plaques in both cellular and acellular regions (30–32). Furthermore, levels of circulating TF are elevated in patients with cardiovascular disease,

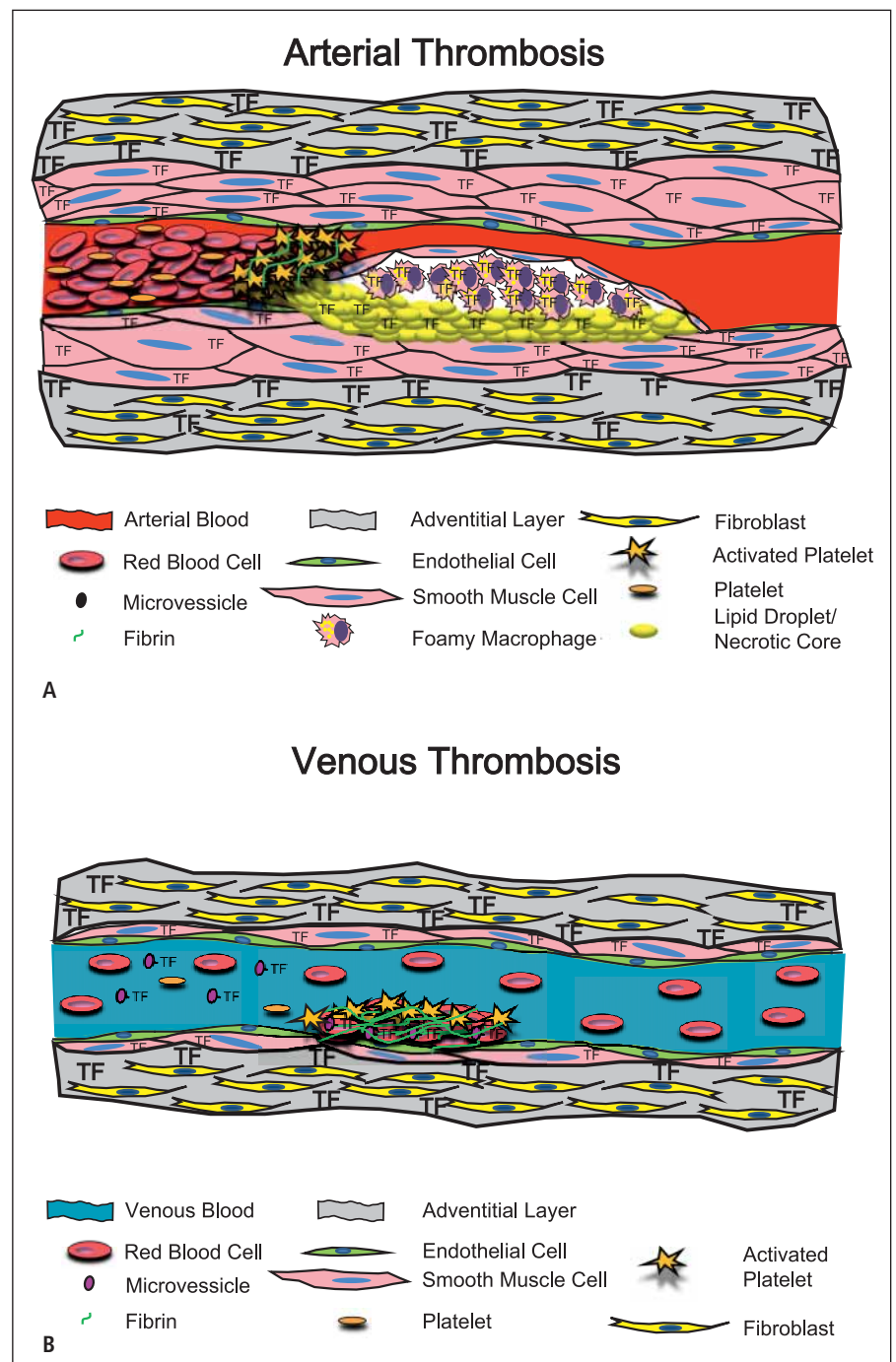


Figure 1: Models showing the cellular sources of TF that contributes to arterial and venous thrombosis. A) Arterial thrombosis. The primary trigger of arterial thrombosis in a diseased vessel is the rupture of an atherosclerotic plaque. This involves disruption of the fibrous SMC cap exposing blood to TF in the plaque. The resultant clot is mainly composed of platelets with lower levels of cross-linking fibrin, and is referred to as a “white clot”. B) Venous thrombosis. Venous thrombosis occurs on a relatively undisturbed endothelial layer. Venous thrombosis may be triggered by circulating TF associated with MVs. The clot is mainly composed of fibrin with platelets and trapped red blood cells and is referred to as a “red clot”.

suggesting this source of TF may also contribute to thrombosis (33–34). This has led to the concept that targeting TF may reduce arterial thrombosis.

Four types of injury are primarily utilised to initiate arterial thrombosis in animal models. These consist of the Folts electrical-induction model, the balloon catheter injury model, the ferric chloride model (FeCl₃), and the Rose Bengal laser-induced injury model. All of these models cause significant injury to the endothelium leading to exposure of blood to TF in the subendothelial media. The Folts electrical method can be used with or without arterial occlusion to 50% with a cuff or suture. The resulting positive electrical potential on the inner surface of the vessel leads to mechanical injury and endothelial cell disruption (35). As shown in ►Table 1, inhibition of TF utilising a monoclonal anti-rabbit TF antibody in a rabbit electrical model prevented thrombus formation in the abdominal aorta and the carotid artery (36–37). Similarly, an inhibitor of the TF:FVIIa complex (PHA-927) prevented electrical injury-induced femoral artery thrombosis (38). Furthermore, examination of fibrinolysis in electrical stimulation-induced coronary thrombosis demonstrated inhibitors of the TF:FVIIa complex or downstream inhibition of FXa prevented re-occlusion (39–40). Taken together, the results indicate that TF initiates thrombosis in the electrical stimulation model.

The balloon catheter injury model is used in larger animals to mimic atherosclerotic plaque disruption. Expansion of an intraluminal balloon results in disruption of the endothelium (41). Utilising this model in the coronary artery of pigs and the thoracic aorta of rabbits, two studies demonstrated that infusion of rTFPI reduced thrombosis (42–43). However, a TF:FVIIa inhibitor may

not be as effective at reducing thrombosis in diseased vasculature, which contains increased levels of TF. This question was eloquently answered when rabbits were fed a hyperlipidaemic diet to induce atherosclerotic plaques in the femoral artery. These plaques were found to contain three times more TF than normal vessels. Mechanical rupture of the plaques using the balloon catheter injury-induced model of thrombosis was reduced with a TF:FVIIa inhibitor (44). These results indicate that the balloon catheter injury and subsequent thrombosis is dependent on TF activity.

The FeCl₃ model induces a chemical injury when topically applied to the outer medial/adventitial artery wall, and is referred to as an outside-in model of injury. FeCl₃ reaches the arterial lumen via an endocytic-exocytic pathway resulting in complete denudation of the endothelium, the presence of highly reactive oxygen species (ROS), and exposure of the blood to medial TF resulting in localised thrombosis (45). Thrombosis is monitored by a reduction in blood flow and time to occlusion of the vessel is used as a quantitative measure of thrombosis. One of the benefits of this model is that different doses of FeCl₃ give different times of occlusion. This small animal model of thrombosis is thought to be comparable to arterial thrombosis in large animal models because there are cyclic flow variations induced by the presence of thrombi in arteries after FeCl₃ injury (46). The role of TF has been examined in this model using mice with either a conditional deletion of the TF gene in SMC or with increased expression of TFPI in SMCs (29, 47). Both studies demonstrated that a reduction of TF activity was associated with an increase in the time to occlusion, indicating that “vessel wall” TF mediates FeCl₃-induced thrombosis.

Table 1: Role of tissue factor (TF) in arterial thrombosis models.

Injury	Vascular location	Animal species	Inhibitor/ gene	TF dependent	Study description	Reference
Electrical stimulation	Carotid artery	Rabbit	mab rabbit TF	Yes	Thrombosis was significantly attenuated in all nine rabbits tested	37
Electrical stimulation	Abdominal aorta	Rabbit	mab rabbit TF	Yes	Prevented procoagulant activity in circulating blood	36
Electrical stimulation	Femoral artery	N-H primate	PHA-927	Yes	Prevented thrombus-induced vessel occlusion with little impact on bleeding	38
Balloon catheter injury	Thoracic aorta	Rabbit	rhTFPI	Yes	Bolus infusion of rTFPI inhibited fibrin formation and neointimal development	42
Balloon catheter injury	Coronary artery	Pig	rhTFPI	Yes	Reduced acute thrombosis and intimal hyperplasia after angioplasty	43
Balloon catheter injury	Femoral artery	Rabbit	FVIIai	Yes	Dose-dependently reduced thrombus formation	44
Ferric chloride	Carotid artery	Mouse	SMC Cre TFPI Flox deletion	Yes	Attenuation of arterial thrombosis without systemic side-effects	29
Ferric chloride	Carotid artery	Mouse	SMC Cre TF Flox deletion	Yes	Conditional deletion of SMC TF resulted in a marked reduction of thrombosis	47
Rose Bengal	Carotid artery	Mouse	TFPI+/-	Yes	TFPI+/- augmented thrombosis suggesting TFPI inhibition of TF attenuates coagulation	50
Rose Bengal	Carotid artery	Mouse	Low TF gene	Yes	Low TF mice were protected from carotid thrombosis	9

Abbreviations: N-H: non-human; mab: monoclonal antibody; PHA-927: TF:FVII complex inhibitor; rh: recombinant human.

The Rose Bengal (tetrachlorotetraiodofluorescein) model of thrombosis is another model of human arterial thrombosis created by the generation of ROS (48). The Rose Bengal model utilises a photochemical reaction to cause localised induction of endothelial injury via ROS formation. Rose Bengal can be injected into the blood (inside-out injury) or is topically applied (outside-in injury). Exposure of Rose Bengal to a laser (543 nm) induces singlet oxygen radicals (49). Most studies inject Rose Bengal into the blood via the tail vein. Thrombosis is then monitored with a flow probe that determines the time to occlusion. Utilising the low TF mouse model and bone marrow transplantation, Day et al. demonstrated that TF in the carotid artery vessel wall, and not haematopoietic cell-derived TF, is responsible for arterial thrombosis (9). This study, together with the aforementioned FeCl₃ SMC conditional deletion studies, strongly indicates that circulating TF does not contribute to arterial thrombosis in healthy mice. Another study with TFPI^{+/-} mice, which have increased levels of TF activity, demonstrated a faster time to occlusion compared to TFPI^{+/+} mice when carotid atherosclerotic plaques were disrupted utilising Rose Bengal (50).

Venous thrombosis

Venous thromboembolism (VTE), which is a collective term for both deep-vein thrombosis (DVT) and pulmonary embolism (PE), is the third leading cause of cardiovascular death in the developed world. Commonly formed in the large veins of the leg, these clots are primarily composed of red cells and fibrin (known as red clots) (Fig. 1B). A common complication of venous thrombosis is PE, which occurs when part of the thrombus breaks away and travels to the lung resulting in partial or complete cessation of blood flow in the pulmonary artery. As opposed to arterial thrombosis, which occurs due to arterial injury and exposure of the sub-endothelium, venous thrombosis mainly occurs due to changes in the composition of the blood, changes in blood flow and/or activation of the endothelium (51–52). Many different factors can increase the incidence of VTE, such as the presence of cancer, obesity, and major surgery (53–54). Thrombophilia is the propensity to develop thrombosis as a result of changes in the blood itself. This can result from an increase in levels of circulating clotting factors, a resistance of clotting factors to inactivation (i.e. factor V Leiden), or a decrease in levels of anticoagulants.

While arterial thrombosis is caused by TF-derived from the vessel wall or within a rupture plaque, venous thrombosis occurs in the absence of gross vein wall disruption (55). It has been suggested that increased levels of circulating TF may trigger venous thrombus formation (56–57). Furthermore, mononuclear cell-associated TF is elevated 24 and 48 hours postoperatively and precedes clinical occurrence of VTE, strongly suggesting a positive association between surgery-induced TF expression and VTE (58). Interestingly, monocytes from patients with VTE had increased levels of TF antigen and activity compared with controls (59–61). In addition, TF antigen has been detected in human deep-vein thrombi

(62). These data suggest that TF is present in venous thrombi and that it may play a key role in initiating venous thrombosis.

Two methods of inducing venous thrombosis are utilised in animal models (Table 2). These are the inferior vena cava (IVC) ligation/stasis model and the collagen-coated thread technique. Although several studies have also utilised Rose Bengal and FeCl₃ of veins, these are not widely accepted as good models due to their non-physiological ablation of the endothelium. The most commonly used model is ligation of the IVC, which results in stasis of the blood and formation of thrombi (63–64). A recent study by Zhou et al. using a rat IVC ligation model found TF staining in monocytes within the thrombi and endothelial cell adjacent to the thrombi (65). Szaloney et al. demonstrated that TF blockage with an inhibitor of the TF:FVIIa complex (PHA-796) reduced thrombosis in the IVC ligation model in non-human primates (66). In a translational study, Biro et al. demonstrated that MVs from cardiac surgery patients increased thrombosis in a rat model of IVC ligation (67). This effect was abolished when the MVs were preincubated with an anti-human TF antibody, demonstrating MVs promote venous thrombosis in a TF-dependent manner. Day and colleagues confirmed the role of TF in venous thrombosis by showing that low TF mice have smaller thrombi than controls in an IVC ligation model (9). However, they found that decreasing the level of TF expression in haematopoietic cells did not affect thrombosis in this model. Nevertheless, a reduction of TF activity either via use of an inhibitory drug, genetic manipulation, or inhibitory antibody reduced venous thrombosis in these animal models.

The collagen-coated thread technique has been utilised as a model of venous thrombosis in rabbits. The procedure consists of interrupting blood flow to the jugular vein, and either inserting a cotton thread pre-soaked in 1 mg/ml fibrillar collagen, and then restoring blood flow, or inserting a silicon catheter with the pre-soaked cotton thread attached to the lumen (68). Hember et al. demonstrated that a monoclonal antibody to rabbit TF inhibited fibrin accumulation and thrombus propagation in this model of venous thrombosis (68). Further, they demonstrated that leukocytes stained for TF within the thrombi. Further studies are needed to determine the cellular source(s) of TF responsible for initiating venous thrombosis.

While these models allow the analysis of pathways that contribute to venous thrombosis in animals, they both have limitations with regard to the formation of venous thrombi. The IVC ligation model has been criticised because this procedure may injure the vasculature during suture positioning, thus possibly releasing vessel wall TF into the blood. Further, the IVC ligation model stops all venous blood flow, which will reduce the delivery of MVs. In contrast, most venous thrombi occur in regions of disturbed or reduced flow. While the collagen-coated thread model has blood flow, the process of introducing the thread via clamping and insertion of a surgical graft, may also introduce vessel wall TF into the circulation. A new model of venous thrombosis is gaining popularity and is thought to be more physiologically relevant. Known as the St. Thomas' Model of venous thrombosis, a stenotic reduction of blood flow (~80–90%) in the IVC is produced by a silk ligation, and then a clamp is used to damage the endothelium (69).

Table 2: Role of tissue factor (TF) in venous and microvascular thrombosis models.

Thrombotic method	Vascular location	Animal species	Inhibitor / gene	TF dependent	Study description	Reference
Ligation	IVC	N-H Primate	PHA-796	Yes	Thrombosis was inhibited in a dose-dependent manner	66
Ligation	IVC	Rat	anti-TF Ab	Yes	Inhibition of TF abolished thrombosis from MVs collected from cardiac surgery patients	67
Ligation	IVC	Mouse	Low TF gene	Yes	Low TF mice were protected from venous thrombosis	9
Collagen-coated thread	Jugular Vein	Rabbit	anti-TF Ab	Yes	Inhibition of TF significantly downregulates fibrin accumulation	68
Laser injury	Cremaster arterioles	Mouse	Low TF gene	Yes	Inhibition of thrombus formation displaying small platelet thrombi lacking TF or fibrin	74
Laser injury	Cremaster arterioles	Mouse	Low TF gene	Yes	TF-bearing MVs derived from haematopoietic cells initiate thrombus formation	75

Abbreviations: N-H: non-human; Ab: antibody; PHA-927: TF:FVII complex inhibitor

This model of stenosis allows for delivery of leukocytes and MVs to the site of thrombosis to a greater extent than the ligation model. Kollnberger et al. demonstrated that mice lacking the TF gene in myeloid cells had a dramatic reduction in venous thrombus size when compared to controls when utilizing this stenosis model (70). This study demonstrates that IVC thrombosis is also dependent upon TF.

Microvascular thrombosis

Microvascular thrombosis examines clot formation in small cremaster or mesenteric arterioles of mice. Specifically, a laser is focused through an optical port of a microscope onto the target vessel. The laser beam is focused so that the resultant heat injury is only induced in a particular part of the vessel. Utilising this laser technology can physically injure a single or multiple endothelial cells with minimal damage to cells in the vessel wall. The thrombus is then observed by intravital microscopy to view the accumulation of TF, fibrin, and platelets. This model of thrombus formation has some advantages over the aforementioned arterial and venous models. The procedure is minimally invasive and requires minimal animal handling or perturbation. Furthermore, the injuries induced are small in size and multiple injuries can be produced in one mouse (71). However, the disadvantages of this model are the use of non-physiologic heat injury and the very small size of the vessels that may not mimic thrombosis in human coronary and carotid arteries.

Furie et al. have demonstrated that MVs from the blood rapidly accumulate into the thrombus. This is due to an interaction between P-selectin glycoprotein ligand 1 on the MV and P-selectin on the surface of the activated platelet. Accumulation of TF precedes fibrin formation within the thrombus (72–73). Recently, Chou et al. demonstrated that both vessel wall and haematopoietic cell-derived TF-positive MVs contributed to thrombosis in this model

(► Table 2) (74). Furthermore, additional studies demonstrated that circulating TF is specifically delivered via MVs (75). These data demonstrate that both vessel wall TF and haematopoietic cell-derived TF-positive MVs contribute to formation of a thrombus in the model of laser-injured microvasculature.

Disseminated intravascular coagulation

Disseminated intravascular coagulation (DIC) is characterised by microvascular thrombosis (76). Sepsis and endotoxaemia are the most common pathologic conditions leading to DIC (77–78). DIC can also be caused by severe trauma, such as surgery, resulting in the release of “tissue debris” into the circulation and subsequent activation of coagulation (79–81).

After introduction of endotoxin or live Gram-negative bacteria into experimental animals, thrombin generation is increased after a period of 3–5 hours (82). Originally it was thought that the contact system, comprised of FXI, FXII, and plasma kallikrein, mediated DIC-induced thrombosis. However, studies have demonstrated that these proteins were not activated in sepsis (83–84). Further experiments demonstrated that induction of TF expression and subsequent generation of thrombin was the mechanism of DIC-induced thrombosis. Most human patients diagnosed with DIC have detectable levels of TF antigen in their plasma (85). Moreover, sepsis induces the expression of TF by vascular cells, such as monocytes (86).

There are numerous studies that have examined the role of TF in DIC (► Table 3). A monoclonal TF antibody (87), rTFPI (88), and FVIIa inhibitor (89) were all shown to reduce DIC and death in a lethal baboon model of sepsis. Furthermore, chimpanzees pre-injected with a monoclonal anti-TF antibody (90) or a monoclonal antibody to FVII/FVIIa (91) were also protected from endotoxin-induced coagulation. Similar results were found in a rabbit model of DIC, where a polyclonal rabbit TF inhibited activation of

Table 3: Role of tissue factor (TF) in models of disseminated intravascular coagulation (DIC).

Thrombotic method	Vascular location	Animal species	Inhibitor / gene	TF dependent	Study description	Reference
Endotoxin or TF Injection	Blood analysis	Rabbit	Polyclonal rabbit TF Ab	Yes	DIC was attenuated, however, endotoxic shock remained unchanged	92
Endotoxin injection	Blood analysis	Baboons	mAb TF	Yes	Protected against DIC activation of coagulation	87
Endotoxin injection	Blood analysis	Baboons	rTFPI	Yes	Activation of coagulation, measured by TAT, inhibited and decreased IL-6	88
Endotoxin injection	Blood analysis	Baboons	FVIIai	Yes	Activation of coagulation inhibited, as well as IL-6 and IL-8, but not TNF- α	89
Endotoxin injection	Blood analysis	Chimps	mAb TF	Yes	Activation of coagulation inhibited, but no effect on IL-6 or TNF- α cytokines	90
Endotoxin injection	Blood analysis	Chimps	mAb FVII/FVIIa	Yes	DIC-induced coagulation inhibited	91
Endotoxin injection	Blood analysis	Mouse	Low TF Gene	Yes	Low TF mice are protected from endotoxaemia activation of coagulation	93
Endotoxin injection	Blood analysis	Mouse	LysM and Tie2 Cre TF Flox deletion	Yes	Conditional deletion of myeloid TF protects against endotoxaemia-induced coagulation	94

Abbreviations: mab: monoclonal antibody; FVIIai: active site inhibitor of FVII; r: recombinant

coagulation in animals injected with endotoxin (92). Recently, we found that low TF mice had reduced levels of thrombin-anti-thrombin (TAT) in a mouse model of endotoxaemia (93). Moreover, this study also demonstrated that haematopoietic-derived TF played a role in the activation of coagulation in endotoxaemic mice. Our recent studies indicate that TF expressed by both haematopoietic cells and non-haematopoietic cells contributes to activation of coagulation in endotoxaemic mice (94). Monocytes were the major haematopoietic cell that expressed TF. However, deletion of TF in endothelial cells did not reduce the activation of coagulation indicating that TF expression by other non-haematopoietic cells drives thrombosis in this model. In sum, these studies demonstrate TF plays a central role in DIC induced by endotoxaemia and sepsis.

Conclusion

In conclusion, all of the aforementioned models of arterial, venous, and microvascular thrombosis, as well as DIC, are all dependent on TF for initiating thrombosis. Models of arterial thrombosis induce vessel wall injury, similar to atherosclerotic plaque rupture, and are primarily dependent on vessel-wall derived TF for initiation of the coagulation cascade. Other studies indicate that circulating TF contributes to venous thrombosis and microvascular thrombosis. Finally, both haematopoietic and non-haematopoietic cell-derived TF activate coagulation in a mouse model of endotoxaemia. Further work is needed to elucidate the different cell types that express TF and their role in different models of thrombosis. Mice with cell type-specific deletion of the TF gene may be able to answer some of these questions.

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