Inhibitors of the initiation of coagulation

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Initiation of blood coagulation occurs mainly through tissue factor (TF) that becomes exposed to blood following vascular injury. Cell-associated TF binds to the serine protease FVIIa and initiates a cascade of amplified zymogen activation reactions leading to thrombus formation. As TF-FVIIa directed inhibitors might achieve anticoagulant efficacy without significantly interfering with normal haemostasis, the TF-FVIIa complex is an interesting target in thrombosis-related disease. Various approaches have been used to inhibit the TF-FVIIa complex including active site-inhibited FVIIa, TF antibodies, tissue factor pathway inhibitor (TFPI), naturally occurring inhibitors, peptide exosite inhibitors and active site inhibitors. Several experimental studies using these inhibitors have displayed promise. However, none of these TF/FVIIa inhibitors has reached clinical testing. Further studies are required to evaluate the clinical efficacy of these novel inhibitors.

Activation of the coagulation pathway

After vessel wall injury membrane bound tissue factor (TF) is exposed to circulating blood and forms a complex with the zymogen FVIIa. The TF-FVII complex is converted to the enzymatically active TF-FVIIa complex by FXa or autocatalytically by TF-FVIIa [1]. In addition to vascular TF, TF may also originate from circulating blood in the form of encrypted TF carried by microparticles [2–4]. Also, TF has been identified in the open canicular system and alpha-granules of platelets [5]. The activity of the TF-FVIIa complex is influenced by the membrane composition as its proteolytic activity requires high concentrations of anionic phospholipids. The TF-FVII/FVIIa complex subsequently activates FX into FXa and FIX into FIXa (extrinsic pathway), a process that is enhanced within membrane areas with high phosphatidylserine content [6]. Generated FXa initially converts limited amounts of prothrombin into thrombin sufficient to activate FVIII, FV and FXI (Figure 1). The FXa activity is restricted to the surface of TF positive cells as any FXa that diffuses off the cell surface is immediately inhibited by tissue factor pathway inhibitor (TFPI) and anti-thrombin (AT). Thrombin generated by FXa then activates platelets, exposes negatively charged phospholipids and thereby induces a thrombin burst (Figure 1) [7]. FX can also be activated to FXa by a different pathway: the complex of

FIXa with its cofactor FVIIIa (intrinsic Xase complex) where FIX is activated to FIXa by FXIa via the intrinsic pathway (Figure 1). Thus, FIXa and FXa represent points of convergence for the intrinsic and extrinsic pathways. FXa in complex with its cofactor FVa (prothrombinase complex) activates prothrombin to thrombin ultimately resulting in the formation of a fibrin clot. Thrombin also serves to amplify coagulation further by activation of cofactors FV and FVIII and zymogen FXI in the intrinsic pathway (Figure 1). Moreover, thrombin activates platelets leading to platelet activation which is necessary for the formation of a haemostatic plug [1]. This results in a tightly regulated balance between normal clot formation and dissolution (haemostasis) and pathogenic clot formation (thrombosis). The TF-FVIIa complex is an interesting target in thrombosis-related disease because TF-FVIIa directed inhibitors might achieve anticoagulant efficacy without significantly interfering with normal haemostasis [8]. This may be because of the fact that intravascular TF is inhibited at lower concentrations than vascular TF which requires higher concentrations for inhibition and mainly contributes to haemostasis [2]. This is of particular importance as the challenge remains to achieve optimal anticoagulation without bleeding complications.

Various studies identified TF as an important player in atherosclerotic disease [9]. In particular, in acute coronary syndromes TF is found on smooth muscle cells

Figure 1

TF-dependent activation of the coagulation cascade: direct or indirect through FIXa/FVIIIa activation

and macrophages within the plaque [10–12] and in the circulating blood [13–15] and thus may be a novel target to inhibit thrombotic complications. Moreover, activation of the TF-FVIIa pathway may contribute to myocardial reperfusion injury [16], restenosis [17], venous thrombosis [18] and inflammatory diseases [19]. In addition to the activation of coagulation, TF-FVIIa may contribute to vascular remodelling, cancer progression and angiogenesis through activation of signalling pathways [20, 21].

The complex nature of the TF-FVIIa interaction and pro-coagulant activity offers a variety of approaches to inhibit TF-FVIIa function (Figure 2). These include the endogenous inhibitor tissue factor pathway inhibitor (TFPI), modified versions of FVIIa and TF, antibodies directed against TF and the FVII/FVIIa complex, naturally occurring inhibitors, exosite and active site inhibitors of FVIIa.

Tissue factor pathway inhibitor

Tissue factor pathway inhibitor is a Kunitz-type proteinase inhibitor and is primarily expressed in endothelial cells. The anticoagulant function of TFPI involves the FXa-dependent inhibition of TF-FVIIa, thereby directly inhibiting the initiation phase of coagulation. Although FXa is not required for inhibition of TF-FVIIa, TFPI inactivates the majority of FXa bound to TF-FVIIa. Therefore, TFPI evolves its anticoagulant function once the early stages of the pathway have been initiated. The first stage in the inhibitory function of TFPI involves the reversible inhibition of FXa via ionic binding of

Figure 2

Approaches for inhibition of the TF-FVIIa complex: TF-FVIIa complex is shown on a phospholipid membrane (grey) showing the FVIIa (green) and TF (blue) domains and potential sites for inhibition

FXa to full-length plasma or cell-associated TFPI involving the P1 residue (Arg107) in the second Kunitz-type domain (TFPI K2). This interaction is enhanced by the presence of first (K1), the third Kunitz-type domain (K3) or the basic carboxyl-region. Once FXa is assembled in the prothrom-

binase complex with FVa it is protected from TFPI inhibition, because of competition for the active site of FXa with prothrombin which is present at much higher concentrations than TFPI. The second stage in the inhibition of TF-dependent coagulation involves the binding of the TFPI-FXa complex to TF-FVIIa via the P1 residue in TFPI (Lys36) and the active site of FVIIa. Unlike the binding of FXa, this requires calcium [22]. Glycosyl phosphatidylinositol (GPI)-anchoring or proteoglycan association of TFPI and membrane binding through the Gla domain of FXa increase the inhibitory potential. The FXa-dependent inhibition of the TF-FVIIa complex by TFPI generates an inactive quarternary complex in the plasma membrane. Internalization of FVIIa results in degradation, yet a small portion of FVIIa is recycled.

Inhibition of TF-FVIIa induced coagulation utilizing recombinant TFPI was analysed in various models of disseminated intravascular coagulation. It reduced mortality and prevented thrombosis in a baboon model [23]. TFPI dose-dependently inhibited coagulation activation during endotoxaemia in humans [24]. In a phase II study the reduction in 28-day all-cause mortality in severe sepsis was observed in the rTFPI group compared with placebo [25]. However, in a randomized controlled study in patients with severe sepsis no effect on all-cause mortality was found [26].

FVIIa inhibitors

Active site-inhibited FVIIa (FFR-FVIIa) is a possible therapeutic inhibitor of the TF-FVIIa-dependent initiation of coagulation. FFR-FVIIa is generated from FVIIa by reaction with FFR (D-Phe-L-Phe-L-Arg-chloromethylketone). This tripeptide is covalently incorporated into the active site of FVIIa. FFR-FVIIa competes with endogenous FVIIa for binding to TF and, thereby limits the formation of functional TF-FVIIa complex [27]. The effectiveness of FFR-FVIIa in models of vessel injury, ischaemia/reperfusion injury, venous thrombosis and sepsis to reduce thrombus formation and inflammatory changes has been shown [28–30].

In a dose escalation trial using FFR-FVIIa as an anticoagulant therapy for percutaneous coronary interventions adjunctive heparin was required to prevent vascular thrombosis. At low doses of FFR-FVIIa, vascular thrombosis was observed suggesting that either there was incomplete blockade of the heparin generation pathway, or that the picomolar quantities of thrombin generated independent of the TF-VIIa pathway may be sufficient to cause periprocedural thrombosis although not to prevent access-site bleeding [31].

Because of the lack of natural inhibitors that specifically interfere with FVIIa activity, a number of artificial inhibitors have been developed. Recently, two classes of peptide exosite inhibitors were selected from phage display libraries for their ability to bind to TF-FVIIa. They bind to two

distinct exosites on the serine protease domain of FVIIa and exhibit steric and allosteric inhibition [32–34]. Although both peptide classes are potent and selective inhibitors of the TF-FVIIa complex they fail to inhibit 100% activity even at saturating concentrations. This may be overcome by fusion of the two peptides [35] or by using a protease switch with a substrate phage [36].

A variety of synthetic compounds have been designed as active site inhibitors of FVIIa and the TF-FVIIa complex [37–40]. A number of naphthylamidines have recently been reported to have FVIIa inhibitory activity [41]. These compounds were synthesized by coupling amidinobenzaldehyde analogues to a polystyrene resin. Some of these small molecule inhibitors have been tested *in vivo* and shown to be efficacious in inhibiting thrombus formation and reducing bleeding [40, 42–45]. However, a problem with these compounds may be a non-specific inhibition of other coagulation serine proteases aside from inhibition of FVIIa activity. Moreover, none of the small molecule active site inhibitors of TF-FVIIa has so far shown sufficient oral bioavailability to be considered as an oral anticoagulant.

In search of naturally occurring anticoagulants, two synergistically acting anticoagulant proteins, hemexetin A and B, that inhibit TF-FVIIa as a non-competitive inhibitor of TF FVIIa amidolytic activity were isolated from the venom of the African ringhals cobra [46]. Further studies are required to evaluate their anticoagulant potential *in vivo*.

TF inhibitors

Two TF residues Lys165 and Lys 166 are critical for substrate interaction of the TF-FVIIa complex. On the basis of this finding a soluble TF mutant (residue 1–219) with alanines substituted for these lysines (TFAA) was developed as an anticoagulant [47]. TFAA binds FVIIa and the resulting complex is unable to activate the substrate FX. Initial experiments in arterial thrombosis models showed anti-thrombotic activity with reduced bleeding [47, 48]. The potency of TFAA was further increased 20-fold by incorporating amino acid substitutions that increase the affinity for FVIIa [49].

Two different types of anti-TF antibodies have been developed as anti-thrombotic agents. One type interferes with the association of TF with FVIIa [8] and the other interferes only with substrate docking [50]. Although both types are effective in inhibiting TF function it seems that inhibition of substrate binding is more efficient [51]. These antibodies were found to attenuate both the coagulopathy induced by sepsis in baboons [52] and chimpanzees [53] as well as arterial thrombus formation after endarterectomy in chimpanzees [54]. Further work led to a chimeric monoclonal antibody to TF blocking the binding of FX to the TF-FVIIa. In an open-label dose-escalation study of this drug including 26 patients with stable coronary artery disease, no serious adverse events were observed [55].

Exogenous inhibitors of substrate binding on the TF-FVIIa complex have been isolated from hookworms. Nematode anticoagulant protein c2 (rNAPc2) is an 85-amino-acid serine protease inhibitor isolated from canine hookworms that directly inhibits the catalytic complex of TF-FVIIa by first binding to FXa. In contrast to TFPI NAPc2 binds at an exosite of FX/FXa at the active site of FXa. The anticoagulant effect of NAPc2 was investigated in an open-label sequential dose ranging study in patients undergoing orthopaedic surgery. The efficacy and safety profile was comparable with the prophylactic use of low molecular weight heparins [56]. Moreover, in patients undergoing coronary angioplasty NAPc2, in combination with unfractionated heparin, was found to be safe and effective in inhibiting thrombin generation [57]. In addition to inhibition of coagulation anti-TF antibodies and NAPc2 may reduce tumour growth and metastasis by interfering with signalling pathways [58, 59].

Various strategies have been used to generate inhibitors of the initiation pathway of blood coagulation. Several types of inhibitors of TF, FVII/FVIIa or the TF/FVIIa complex have been developed and evaluated on these targets as anticoagulants *in vitro* and *in vivo*. A number of studies have raised the possibility of improved mode of anticoagulation, yet further experimental and clinical studies are required before the promise inherent in these novel approaches can be fully evaluated.

Competing Interests

There are no competing interests to declare.

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