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Review

FACTOR IX AND THROMBOSIS

Coagulation factor IX (FIX) plays an essential role in blood coagulation, as shown by the bleeding tendency associated with congenital FIX deficiency (haemophilia B, Christmas disease). Recent studies show that, after activation of FIX by the tissue factor:FVIIa complex, or by FXIa, FIX plays a key role in thrombin generation in the vicinity of platelets, and that FIXa is the thrombogenic trigger after infusion of prothrombin complex concentrates. Two recent case–control studies have shown that high FIX levels (activity or antigen) are associated with increased risk of venous thromboembolism. Although this may have a genetic basis, epidemiological studies in large random population samples have shown that FIX levels are associated with several thrombotic risk factors, including age, oral oestrogen use (oral contraceptive pill, hormone replacement therapy), menopause, obesity, cholesterol, triglycerides, smoking, blood pressure and low social class. FIX levels are also positively associated with coagulation activation markers, and strongly with levels of its cofactor, FVIII. Factor IXa is also increased in patients with acute coronary artery thrombosis. Anticoagulants that lower FIXa levels are effective in the prevention of venous and arterial thrombosis. This review examines these data and suggests that FIX may play a key role in thrombus formation, that high FIX levels may be risk predictors, and that selective FIXa inhibition merits evaluation in the prophylaxis and treatment of thrombosis.

FACTOR IX AND BLOOD COAGULATION

Coagulation factor IX is a vitamin K-dependent blood coagulation protein with a molecular weight of 65 000. FIX plays a key role in the intrinsic pathway of blood coagulation and is activated in the presence of calcium ions by the tissue factor:factor VIIa complex or by Factor XIa. In turn, FIXa activates factor X in the presence of factor VIIIa, calcium ions and platelets (or phospholipid). The gene structure, protein structure and biochemistry of FIX have been reviewed recently (Limentani *et al*, 1994; Reiner & Davie, 1994; Giannelli, 1997).

In recent studies of a model system of *in vitro* coagulation, Hoffman *et al* (1995) showed that FIXa and FXa play distinct roles in tissue factor-dependent initiation of coagulation. The main role of FXa appeared to be activation of platelets by generating an initial, small amount of thrombin in the vicinity of platelets. In contrast, FIXa enhanced thrombin generation by providing FXa on the platelet surface, leading to prothrombinase formation. Initiation of coagulation was highly dependent on activation of small amounts of FIXa and FXa in proximity to platelet surfaces.

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HAEMOPHILIA B

About 20% of patients with X-linked recessive congenital haemophilias have FIX deficiency (haemophilia B, Christmas disease), described by several groups in 1952 (Aggeler *et al*, 1952; Biggs *et al*, 1952; Soulier & Larrieu, 1953). Its differentiation from factor VIII (FVIII) deficiency (haemophilia A), which is present in the remaining 80% of such patients, had been heralded by the observation of Pavlovsky (1947) that mixing blood from different haemophilic individuals *in vitro* sometimes resulted in correction of the clotting time, suggesting two types of haemophilia. Although the clinical features of haemophilias A and B are similar, a recent epidemiological study of bleeding episodes and hospital admissions confirmed the clinical suspicion (Rizza, 1997) that haemophilia B is less clinically severe than haemophilia A (Ludlam *et al*, 2000).

An interesting variant of haemophilia B is the Leyden subphenotype (Briet *et al*, 1982), in which there are mutations in the factor IX promoter at −20 (T to A) (Reitsma *et al*, 1988) or at 12 other sites, all clustered in the −21 to +13 region (Giannelli, 1997). Patients with this subtype have low plasma FIXc levels at birth (< 1 IU/dl) with a severe bleeding phenotype; however, FIXc levels increase into the reference range (≈60 IU/dl) after puberty, with resolution of the phenotype (Briet *et al*, 1982). Like these Leyden mutations, a 26 (G to C) mutation (haemophilia B Brandenburg) also disrupts the binding site for the liver-enriched transcription factor LF-A1/HNF4; however, it also disrupts an androgen-responsive element, which overlaps the LF-A1/HNF4 site and, hence, FIXc levels do not rise with age, in contrast to the −20 mutation (Crossley *et al*, 1992). This androgen-responsive element may explain the increase in FIXc with age seen for the mutations associated with the Leyden phenotype.

FACTOR IXa AND ANIMAL MODELS OF THROMBOSIS

Experimental studies suggest that FIXa may have a critical role in thrombosis, as well as haemostasis. Infusion of purified FIXa into rabbits induces local or disseminated thrombosis (Gitel *et al*, 1977; Gurewich *et al*, 1979). In contrast, active site-blocked FIXa prevented clot formation *in vitro* (Tijburg *et al*, 1991) and reduced intra-arterial coronary thrombus formation *in vivo* (Benedict *et al*, 1991).

THROMBOSIS AND COAGULATION ACTIVATION AFTER INFUSION OF PROTHROMBIN COMPLEX CONCENTRATES OR FACTOR IX CONCENTRATES IN FACTOR IX-DEFICIENT HUMANS

After the initiation in the 1970s of treatment and prophylaxis of bleeding episodes with prothrombin complex concentrates (PCCs) in haemophilia B, liver disease, or reversal of oral anticoagulant therapy, an excess of thrombotic episodes was observed (Blatt *et al*, 1974; Aledort, 1977; Lusher, 1991, 1993; Watson & Ludlam, 1997). Such episodes included disseminated intravascular coagulation (DIC), venous thromboembolism and myocardial infarction with transmural haemorrhage. PCCs contain factors II, IX and X, with highly variable amounts of FVII. Thrombosis is thought to be related to the presence in PCC of activated coagulation factors, formed during the manufacture of PCCs. In liver disease, additional factors promoting thrombus formation include decreased clearance of activated factors and low circulating levels of endogenous coagulation inhibitors (antithrombin, protein C and protein S). PCCs are therefore best avoided in the correction of the coagulopathy in liver disease (Mannucci & Giangrande, 1994; British Committee for Standardization in Haematology, 1998; Scottish Intercollegiate Guidelines Network, 1999).

In haemophilia B, the risk of thromboembolism appears to have been reduced after the replacement of PCC with human single factor IX concentrates, whose manufacture involves additional purification steps that may remove activated coagulation factors and, hence, may reduce thrombogenicity (Menache, 1990; Watson & Ludlam, 1997). Several animal studies support this hypothesis (Menache *et al*, 1984; Harrison *et al*, 1985; Smith, 1988; MacGregor *et al*, 1991; Herring *et al*, 1993). Furthermore, comparisons of plasma levels of end-stage coagulation activation markers (fibrinopeptide A, FpA; prothrombin fragment F1+2; thrombin–antithrombin (TAT) complexes) in patients with haemophilia B have shown elevations after infusion of clinical doses of PCC, but not of high-purity human FIX concentrates (Mannucci *et al*, 1990, 1991; Hampton *et al*, 1991, 1993; Kim *et al*, 1991, 1992; Berntorp *et al*, 1993; Goudemand *et al*, 1993; Santagostino *et al*, 1994; Thomas *et al*, 1994).

Which activated coagulation factors in PCC cause such increased end-stage coagulation activation? Phillippou *et al* (1996) assayed activation peptides of FIX, FVII, FX and prothrombin in a cross-over study of PCC and high-purity human FIX concentrate in haemophilia B patients. As well as increases in prothrombin fragment F1+2 and TAT complexes after PCC infusion, significant elevations were observed in activation peptides for FIX and FX, but not for FVIIa. It was therefore concluded that FIXa (which in turn activated FX) was the likely trigger of end-stage coagulation activation in the PCC (Phillippou *et al*, 1996). In support of this conclusion, Gray *et al* (1995) correlated FIXa levels in high-purity FIX concentrates with their thrombogenicity using the same animal model in which Gitel *et al* (1977) had shown that FIXa was a more potent thrombogenic agent than thrombin or FXa.

As with high-purity human FIX concentrate, infusion of recombinant FIX concentrate in haemophilia B patients (which is becoming the treatment of choice because of probable freedom from transmissible human infectious agents) has not been associated with increases in end-stage coagulation activation markers, nor with increased risk of thrombotic events to date.

EPIDEMIOLOGY OF FACTOR IX AND COAGULATION ACTIVATION

Phillippou *et al* (1996) observed that mean baseline levels of prothrombin fragment F1+2, TAT complexes and FX activation peptides in haemophilia B patients appeared to be comparable with mean levels in non-haemophilic men. In contrast, mean baseline levels of FIX activation peptides were greatly reduced and rose gradually after infusion of either PCC or high-purity FIX concentrate (levels were similar 3 h after infusion, presumably as a response to increased circulating FIX in these FIX-deficient patients).

We hypothesized that increasing plasma levels of FIX (as well as FVII and FVIII) activity within the population range might be associated with increasing coagulation activation, as detected by increasing plasma levels of end-stage coagulation activation markers (prothrombin fragment F1+2 and TAT complexes). We also hypothesized that decreasing levels of coagulation factor inhibitor (antithrombin, protein C, protein S) activity within the population range might also be associated with increasing coagulation activation, and that risk factors for venous and arterial thromboembolism might also be associated with coagulation factors, inhibitors and activation markers. We therefore measured these variables in 747 men and 817 women aged 25–74 years, randomly sampled from the north Glasgow population in the third WHO-MONICA Survey (Lowe *et al*, 1997; 1999a; Woodward *et al*, 1997).

Factor IXc levels were significantly associated with prothrombin fragment F1+2 and TAT levels, as were increasing levels of FVIIc and FVIIIc and activated protein C resistance and decreasing levels of protein C activity (Lowe *et al*, 1997, 1999a). The most striking correlation of plasma factor IXc was with its cofactor, plasma VIIIc (Spearman $r = 0{\cdot}63$). We have recently confirmed this strong association in a study of 4000 men aged 60–79 years (20 year follow-up of the British Regional Heart Study, unpublished observations). The reasons for this strong association are unclear, but may include activation of VIII by FIXa (Rick, 1982).

We confirmed that factor IXc levels increase with age (Simpson & Biggs, 1962; Dodd *et al*, 1975; Sweeney & Hoernig, 1993) and with the use of oral contraceptives (Briet *et al*, 1978; Kluft & Lansink, 1997). In addition, we have shown that levels increased with female menopause, body mass index, total cholesterol, triglycerides, blood pressure, smoking habit (in men) and low social class (Lowe *et al*, 1997; Woodward *et al*, 1997). Although no significant association with the use of hormone replacement therapy (HRT) was noted in this study (Lowe *et al*, 1997), a further, larger study of 1000 women aged 40–59 years in

the Glasgow area showed that women taking oral HRT had significantly higher FIXc levels than women taking transdermal HRT, or women taking no HRT (Lowe *et al*, 2001). Hence, plasma FIXc levels are associated with most risk factors for venous or arterial thrombosis.

Recently, two age-responsive elements, AE5 and AE3, have been identified in the human factor IX gene (Kurachi *et al*, 1999; Kurachi & Kurachi, 2000). These appear to be distinct from the age- and androgen-responsive elements involved in the rise in factor IXc levels in the FIX Leyden mutations (Crossley *et al*, 1992). Transgenic mice expressing high levels of human FIX were observed to die much earlier than control animals or those producing lower levels of human FIX, suggesting that substantially elevated levels of FIX may be a risk factor for thrombosis (Kurachi & Kurachi, 2000).

FACTOR IX AND VENOUS THROMBOSIS

Increases in plasma levels of several coagulation factors have recently been associated with risk of deep venous thrombosis (DVT) in case–control studies. These include FVIIIc (Rosendaal, 2000), fibrinogen (Koster *et al*, 1994), prothrombin (Poort *et al*, 1996), FXIc (Meijers *et al*, 2000) and FXIII (inverse association; Franco *et al*, 1999).

Two recent case–control studies have associated increased levels of FIX with venous thrombosis. Lowe *et al* (2000a) restudied 66 women with idiopathic venous thromboembolism (VTE) and 163 controls from a previous case–control study of 103 cases and 178 controls among women aged 45–64 years in the Oxford area of the UK (Daly *et al*, 1996). Current oral anticoagulant users were excluded. High plasma levels of FIXc ($\geqslant$ 150 IU/dl) were associated with increased risk of VTE (OR 2·34; 95% CI 1·26, 4·35; $P = 0{\cdot}007$) after adjustment for HRT use. On multivariate analysis, independent associations of VTE were high FIXc, low antithrombin ($\leqslant$ 90 IU/dl), activated protein C resistance (APC ratio $\leqslant 2{\cdot}0$) and HRT use. Interactions between these variables for increasing risk of VTE were examined in a statistical model. As expected, the combination of APC resistance with low antithrombin is predicted to increase the risk of VTE significantly; however, the combination of high FIXc with either low antithrombin (e.g. PCC infusion in patients with liver disease) or APC resistance is also predicted to increase risk significantly. At any level of coagulation abnormalities, HRT use is predicted to increase the risk of VTE about threefold. This may be explained by the combined effects of oral HRT use on several thrombotic mechanisms: decreased antithrombin (Meade, 1997) and increases in FIXc, APC resistance and C-reactive protein (Lowe *et al*, 2000b; 2001).

van Hylckama Vlieg *et al* (2000) also studied the relationship of FIX (in this case FIX antigen) to DVT in the Leiden Thrombophilia Study (LETS). Persons with plasma IX antigen above the 90th percentile ($\geqslant$ 129 IU/dl) had a 2·5 (95% CI 1·6, 3·9) increased risk of DVT. The risk appeared to be higher in women than in men, and higher in premenopausal women not using oral contraceptives.

The results of these two studies therefore suggest that high FIX (activity or antigen) may be a mechanism for venous thrombogenesis. High FIX levels might be genetic or reflect environmental (or gene-environmental) effects of risk factors such as age, oestrogens (including pregnancy; Beller & Ebert, 1982), obesity or blood lipids. Further studies are required to establish with confidence the relationships between FIX, risk factors and venous thromboembolism.

If FIXc is strongly associated in the general population with its cofactor, FVIIIc ($r = 0{\cdot}6$; Lowe *et al*, 1997), it will require large, comparative studies to determine which of FIXc, FVIIIc [or von Willebrand factor (VWF), which is also highly correlated with FVIIIc; Rumley *et al*, 1999] shows the strongest association with venous or arterial thromboembolism. Given the importance of both FVIIIc and activated platelets (whose adhesion to the vessel wall and local aggregation are strongly promoted by VWF) in FIX activation (Hoffman *et al*, 1995), it may be the combination of these three factors that is important in the generation of thrombin and in thrombogenesis. This possibility is supported by the observation of van Hylckama Vlieg *et al* (2000) that the risk of DVT in the LETS study was highest when both FVIIIc and FIX Ag were above the 90th percentile (OR 8·0; 95% CI 3·6, 18·4).

In the prospective European Concerted Action against Thrombosis (ECAT)-DVT study, a shortened activated partial thromboplastin time (APTT) was an independent risk factor for venographic DVT after elective hip surgery (Lowe *et al*, 1999b). This was not explained by factor VIII and might be explained by other intrinsic factors, such as IX, XI or XII (which were not measured in this study).

FACTOR IX AND ARTERIAL THROMBOSIS

Patients with haemophilia A or B have a lower risk of coronary heart disease (CHD) than the general male population (Rosendaal *et al*, 1989, 1990). Increases in the FVIII:VWF complex are associated with increased risk of CHD in prospective cohort studies (Meade *et al*, 1994; Rumley *et al*, 1999). Plasma levels of FIXc are associated not only with FVIIIc, but also with several CHD risk factors in the general population, including age, oestrogen use, obesity, cholesterol and triglycerides, blood pressure, smoking and low social class (Lowe *et al*, 1997; Woodward *et al*, 1997). The associations of FIXc with CHD risk factors were highest in both men and women for triglycerides (Woodward *et al*, 1997), which may be of significance given the ability of triglyceride-rich very-low-density lipoprotein (VLDL) and other lipoproteins to bind vitamin K-dependent coagulation proteins (including FIX) and to support procoagulant enzymatic complexes in thrombin formation (Moyer *et al*, 1998; Xu *et al*, 1998). As yet, however, there are no reported prospective cohort studies of FIX and risk of CHD or stroke.

Myocardial infarction has occasionally followed infusion of PCC (Aledort, 1977; Lusher, 1993). The importance of FIXa (perhaps binding to lipid) in PCC for thrombosis has been noted (Phillippou *et al*, 1996). In acute coronary syndromes (acute myocardial infarction or acute unstable angina pectoris), plaque rupture exposes circulating blood to tissue factor:FVIIa complexes, which can activate factors

IX and XI on local platelet/lipid surfaces. Recently, Minnema *et al* (2000) reported increased plasma levels of FIX activation peptides in patients with acute myocardial infarction or acute unstable angina, compared with patients with stable angina. Levels of FXIc–C1 inhibitor complexes (reflecting acute FXI activation) and fibrinopeptide A levels were also higher in patients with acute myocardial infarction, but not levels of FX activation peptide or prothrombin F1+2 (Minnema *et al*, 2000). This study provides the first evidence for FIX (and FXI) activation in acute coronary syndromes. Such activation may play a role in coronary thrombogenesis through continuous generation of thrombin and fibrin formation, as well as through inhibition of endogenous fibrinolysis via activation of thrombin-activatable fibrinolysis inhibitor (TAFI) (van der Borne *et al*, 1997) The FIX activation peptide has recently been shown to predict acute coronary syndromes (Rosenberg, 2001).

FACTOR IX AND ANTITHROMBOTIC THERAPY

Oral anticoagulants reduce the activity of vitamin K-dependent clotting factors including prothrombin, FVII, FIX and FX. There has been recent interest in the increased sensitivity of some patients to oral anticoagulant-induced decrease in FIXc, which is not detected by the prothrombin time or its international normalized ratio (INR), used routinely for monitoring of oral anticoagulant effect. Such patients may have anti-phospholipid antibodies (Harbrecht *et al*, 1998) or mutations in the propeptide of FIX (Ala-10Thr or Ala-10Val) causing a reduced affinity of the carboxylase for factor IX precursor (Chu *et al*, 1996; Oldenburg *et al*, 1997). Patients with these mutations have normal baseline FIXc levels, but these fall to very low levels (< 1 IU/dl) with therapeutic doses of oral anticoagulants, leading to a markedly prolonged APTT and severe bleeding (Oldenburg *et al*, 1997). Although APPT and FIXc measurement may be indicated in patients with unexpected severe bleeding during treatment, at present baseline screening for APTT, FIXc or FIX mutations does not appear to be justified (Peters *et al*, 1997; van der Meer *et al*, 1999; Legnani *et al*, 2000). In a recent large study, Legnani *et al* (2000) observed that FIX levels varied greatly despite similar achieved anticoagulation intensity, making identification of those with very low FIXc levels from the APTT difficult: they provided a table of ranges for FIXc and APTT for INR classes that may help such identification.

Heparins also lower FIXc levels, which contributes to their anti-thrombotic effects (Beguin *et al*, 1989).

Given the potential importance of FIXa in thrombogenesis *in vitro* (Tijburg *et al*, 1991), in animal models (Gitel *et al*, 1977; Gurewich *et al*, 1979), in PCC-induced thrombosis (Phillippou *et al*, 1996), in idiopathic venous thrombosis (Lowe *et al*, 2000, van Hylckama Vlieg *et al*, 2000) and in coronary thrombosis (Minnema *et al*, 2000), it appears appropriate to study selective FIX inhibitors as anti-thrombotic therapy. FIXa can be inhibited chemically (Lollar & Fass, 1984), by blocking of the active site (Benedict *et al*, 1991; Spanier *et al*, 1997) or by an antibody (BC2) against an epitope in the FIX Gla epitope, which is not an active site inhibitor (Feuerstein *et al*, 1999a,b). Factor IXa inhibitors may have a higher ratio of anti-thrombotic activity to bleeding risk than heparins in animal studies (Benedict *et al*, 1991; Spanier *et al*, 1997; Feuerstein *et al*, 1999a,b). Future clinical trials of FIXa inhibitors in prophylaxis and treatment of thrombosis could test the hypothesis that increased FIXa levels play a role in venous and arterial thrombogenesis.

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