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Factor IX binding to collagen

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At a site of vascular injury, numerous cellular and protein constituents of blood bind to components of extracellular matrices in the vessel wall. These interactions are critical for normal clot formation. In this issue of the *Journal of Thrombosis and Haemostasis*, Gui *et al.* [1] present an analysis of mice expressing a variant of factor (F)IX with a defect in binding to collagen. Their results suggest that FIX should be added to the list of proteins that bind to collagen during hemostasis. The work is the most recent chapter in a story that began more than 25 years ago. In 1983, Heimark and Schwartz [2] and Stern *et al.* [3] reported that FIX and FIXa bound to vascular endothelial cells with K_{ds} of < 10 nmol. Other vitamin-K dependent coagulation proteins were not competitive inhibitors of FIX/IXa binding, suggesting that the binding site contained a protein, and was not simply the cell phospholipid membrane. While the phospholipid-binding Gla-domain of FIX was required for endothelial cell binding, replacing it with the FVII Gla-domain resulted in loss of binding [4]. Cheung *et al.* [5] localized the endothelial cell binding site to the N-terminal Ω -loop of the Gla-domain, and noted that binding was abolished by replacing Lys5 with alanine. Lys5 is conserved in FIX across vertebrate species, and there is evidence that it interacts with the phosphate head groups of phosphatidylserine when the Ω -loop inserts into a lipid layer. However, a basic residue is not present at this position in the Gla-domains of other proteins (Fig. 1), suggesting it may not be required for phospholipid binding. Consistent with this, alanine replacement of Lys5 in FIX does not affect coagulant activity *in vitro* [5].

In 1987, Rimon *et al.* [6] reported that FIX could be cross-linked to a 140-kDa endothelial cell protein. Attempts to characterize the unknown molecule met with mixed results. It was noted that FIX could be cross-linked to a degradation product of type IV collagen, a ubiquitous component of epithelial and endothelial cell basement membranes. The importance of this observation was not immediately clear, but Cheung *et al.* [7] subsequently presented evidence indicating that type IV collagen and the endothelial cell binding site for FIX were likely one and the same. As with FIX binding to endothelial cells, an alanine substitution for Lys5 in FIX disrupted binding to collagen [7]. The six α -chains of the type IV collagen family form three distinct heterotrimers ($\alpha1\alpha1\alpha2$, $\alpha3\alpha4\alpha5$ and $\alpha5\alpha5\alpha6$) [8]. The $\alpha1$ and $\alpha2$ chains are present in basement membranes of all tissues, whereas the others have more restricted distributions. Each heterotrimer has three domains (Fig. 2). The N-terminal 7S domain is rich in cysteine and lysine and forms disulfide bonds with other heterotrimers. The collagenous domain is primarily a triple helical structure with numerous Gly-Xaa-Yaa repeats. The C-terminal non-collagenous (NCI) domain interacts with the NCI domain of an adjacent heterotrimer. Wolberg *et al.* determined that each $\alpha1\alpha1\alpha2$ heterotrimer contains two FIX binding sites within the collagenous domain located 50 and

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98 nm from the C-terminus of the domain (Fig. 2) [9]. The measurements place the FIX sites at a distance from known locations for interactions with collagen-binding integrins.

In 2002, Gui *et al.* [10] described the effects of the Ala5 substitution on FIX infused into FIX-deficient mice. Approximately 80% of wild-type FIX was rapidly cleared from plasma, with the remainder circulating with a half-life of ~7 h. In contrast, only ~60% of FIX-Ala5 was removed rapidly, and the half-life of protein remaining in the circulation was reduced compared with the wild-type protein. The results suggested that a portion of FIX resides in a collagen-bound pool that equilibrates with the circulating pool. This interpretation is consistent with observations that recovery of infused FIX tends to be greater in patients with cross-reactive material (CRM) positive hemophilia B compared with those who are CRM-. FIX binding sites on collagen may be occupied by endogenous FIX in CRM+ patients, limiting binding of infused FIX. While these data indicated that binding to collagen influences vascular distribution of FIX, they did not show that binding is important for FIX function during coagulation.

To address this issue, Gui *et al.* [1] generated mice expressing FIX-Ala5 using a knock-in strategy. The animals have plasma FIX antigen levels that are ~120% of wild-type levels, consistent with some degree of redistribution of the normal pools of FIX. A chromogenic assay for FIX activity based on activation by FXIa matched the antigen assay results. The FIX Gla-domain is required for binding to factor XIa [11], so it appears that the Lys5Ala substitution does not interfere with this interaction. Given the location of the substitution in the phospholipid binding Ω -loop, it was imperative to demonstrate normal activity in phospholipid-dependent assays. This was done using a tissue factor-initiated thrombin generation assay. FIX-Ala5 performed similarly to wild-type FIX, indicating the Ala5 substitution has a minimal effect on FIX activation by FVIIa-tissue factor, or FIXa activation of FX.

Mice lacking FIX have a significant bleeding diathesis [12]. Most animals will exsanguinate when challenged with surgical removal of the tail tip. The primary activator of FIX *in vivo* is likely the FVIIa/tissue factor complex. FIX activation by FXIa may be important in certain situations, however, mice lacking FXI do not bleed excessively after transection of the tail [13], indicating FIX is activated primarily by FVIIa/tissue factor with this type of injury. Interestingly, FIX-Ala5 mice have excessive bleeding after tail transection compared with wild-type mice, indicating that FIX binding to collagen is required for hemostasis. However, bleeding is significantly less severe than in FIX null mice, possibly because FIX activation on phospholipid surfaces remains intact.

Application of concentrated ferric chloride (FeCl_3) to the exterior of blood vessels results in denudation of the endothelial layer, exposure of collagen to flowing blood and rapid vessel occlusion by platelet-rich thrombi [12,13]. FIX-Ala5 mice demonstrate a significant delay, compared with wild-type mice, in time to occlusion of mesenteric arterioles exposed to FeCl_3 , with a pronounced prolongation of the time required for thrombi occluding 50% of the vessel lumen to progress to complete occlusion. This instability of larger thrombi is reminiscent of results with FXI- and FXII-deficient mice in the same model [13]. FeCl_3 -induced collagen exposure probably results in FXII and FXI activation, which contribute to thrombin generation through activation of FIX. It is important to note that FIX-deficient mice, like FXI- and FXII-deficient mice, do not experience mesenteric vessel occlusion in response to FeCl_3 , whereas occlusion is only delayed in FIX-Ala5 mice. So, as in the tail bleeding model, the defect in FIX-Ala5 does not completely prevent FIX activation, or FIXa contributions to thrombin generation. Again, it seems reasonable to postulate that FVIIa/tissue factor, or perhaps FXIa, is contributing to FIX activation in a collagen-independent manner. These data, while suggestive, do not rule out the possibility that the phenotype of

FIX-Ala5 mice is caused by an abnormality unrelated to FIX binding to collagen. Perhaps the most compelling piece of data supporting the hypothesis that a defect in FIX binding to collagen is responsible for the results in the FeCl₃ model comes from experiments with a laser-induced thrombosis model. In contrast to FeCl₃-induced injury, the vascular endothelium remains largely intact after laser injury, and the amount of collagen exposure to blood is likely much lower than with FeCl₃. In previous work, FIX null mice were noted to be resistant to laser-induced thrombus formation [14]. In the current study, thrombus formation was similar in FIX-Ala5 and wild-type mice after laser injury.

While the phenotype of FIX-Ala5 mice may well be related to poor FIX binding to collagen, it remains to be determined how this process actually contributes to coagulation. FIX binds to extracellular matrix isolated from vascular endothelial cells, but this interaction does not support assembly of the intrinsic FXase complex in the absence of cells [7]. Binding to collagen could serve to establish a pool of FIX at sites where it is most needed, such as in the synovium of joints. Gui *et al.* propose that collagen may facilitate formation of the intrinsic FXase complex by bringing FIX, FVIII bound to von Willebrand factor and platelets into proximity at a wound site. Along similar lines, collagen-bound FIX may be in a position to be activated by FXIa at sites where collagen exposure to blood induces contact activation. The FIX-Ala5 mice will be an important tool in future work to identify roles for this novel property of FIX in hemostasis and during disease processes.

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Prothrombin	A N T F - L γ γ V R K G N L γ
Factor VII	A N A F - L γ γ L P R G S L γ
Factor IX	Y N S G K L γ γ F V Q G N L γ
Factor X	A N S F - L γ γ M K K G H L γ
Protein C	A N S F - L γ γ L R H S S L γ
Protein S	A N S L - L γ γ T K Q G N L γ
Protein Z	A G S Y L L γ γ L F γ G N L γ

Fig. 1.

Amino acid sequence of the N-termini of human plasma proteins containing a Gla-domain. The position of Lys5 is indicated by the black oval. The symbol γ indicates the positions of glutamic acid residues that have undergone γ -carboxylation.

Fig. 2. Schematic diagram of the domain structure of the $\alpha 1\alpha 1\alpha 2$ heterotrimer of type IV collagen. The positions of integrin binding sites (long arrows) and the two putative factor IX binding sites (arrow heads labeled FIX) in the collagenous domain are shown. Cyanogen Bromide 3 (CB3) is a region in the triple helical collagenous domain that contains several integrin binding sites.