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学位論文題目：Molecular mechanism of anticoagulant activity of Annexin A4

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【Background】

Blood coagulation plays an important role in biodefense to prevent bleeding, while thrombus formed in blood vessels causes serious medical conditions such as stroke and myocardial infarction. The coagulation reaction initiates through two pathways: extrinsic pathway and intrinsic pathway. The intrinsic pathway is initiated by the contact between a negatively charged surface and coagulation factor XII (FXII), and the extrinsic pathway is activated by damaged tissue. In both pathways, thrombin is generated through the chain reactions and the bleeding is stopped by fibrin clot. FXII is a serine protease of the intrinsic pathway which is activated by interaction with anionic surfaces. As genetic defects in FXII show no apparent bleedings, the physiological significance of the intrinsic pathway had not been clarified for a long time. However, in recent years, FXII and coagulation factor XI (FXI) have been proposed to be involved in thrombi formation because FXII and FXI deficiency in mice protects them against thrombosis without and with little affecting their tendency to bleed, respectively. Moreover, endogenous molecules such as extracellular RNA, polyphosphate, and sulfatide (3-*O*-sulfated galactosylceramide) have been identified as negatively charged surfaces able to trigger the intrinsic pathway *in vivo*. Therefore, these studies suggest that the intrinsic pathway is highly involved in thrombosis.

The annexin (ANX) family consists of members characterized by four highly conserved C-terminal repeat fold structures and a unique N-terminal region. ANXA4, a Ca^{2+}-binding protein, was revealed to be one of the members with the highest anticoagulant activity. ANXA4 is highly expressed in the placenta of pregnant women with Pregnancy-Induced Hypertension (PIH) compared with normal placenta, and the plasma level of ANXA4 suddenly increases after delivery. From these backgrounds, we hypothesized that ANXA4 has an anticoagulant activity *in vivo* and inhibits thrombus formation by inhibiting the intrinsic pathway. In this study, we aimed to clarify the molecular mechanism of ANXA4 anticoagulant activity.

【Results and Discussion】

(1) ANXA4 inhibits the intrinsic coagulation pathway

Firstly, we investigated the anticoagulant activity of ANXA4 in both extrinsic and intrinsic pathways and revealed that ANXA4 inhibits both pathways. Furthermore, we elucidated on the anticoagulant mechanism of ANXA4 in the intrinsic pathway. As the coagulation time in FXII-deficient plasma was largely extended, we concluded that sulfatide initiates coagulation by activating FXII. Next, the anticoagulant activity of ANXA4 was investigated by using synthetic substrates for activated FXII (FXIIa) or activated FXI (FXIa) and detecting

the FXIIa or FXIa fragmentation by SDS-PAGE. ANXA4 was found to inhibit the auto-activation of FXII and the activation of FXI, but had no effect on the serine protease activity of FXIIa and FXIa themselves. In addition, sulfatide seemed to be a cofactor for FXI activation as it was found to promote FXI activation with FXIIa. To clarify the inhibitory mechanism of ANXA4 in FXII auto-activation and FXI activation, we performed a pull-down assay and a binding assay of sulfatide liposome. Our results revealed that ANXA4 does not bind directly to FXII, FXI, FXIIa, nor FXIa but prevents FXII from binding to sulfatide. These results suggest that ANXA4 interaction with sulfatide inhibits the FXII auto-activation.

(2) Search of the active site using ANXA4 mutants

ANXA4 mutants were generated to elucidate the anticoagulant site of ANXA4. Because the anticoagulant activity of ANXA4 is believed to originate from its ability to bind sulfatide, the expected sulfatide-binding sites of ANXA4 were mutated. Solid-phase binding assay revealed that ANXA4 mutants exhibit a decreased binding ability to sulfatide. In the coagulation test initiated by sulfatide, the mutants showed similar activity to the wild-type. This result indicated that the sulfatide binding site present in repeat 1 is not the anticoagulant domain. Furthermore, since ANXA4 interacts with sulfatide in a Ca^{2+}-dependent manner, Ca^{2+}-binding sites present in repeat 1, 2, and 4 were mutated. The Ca^{2+}-binding site mutant of repeat 4 showed low anticoagulant activity. Moreover, repeat 4 deletion mutant (ΔR4) and repeat 3 deletion mutant (ΔR3) were generated and we analyzed the hydrophobic surface and the three-dimensional structure by bis-ANS, a probe on the hydrophobic cavity, and NMR, respectively. The ΔR4 structure seemed to be destabilized. Besides this, although ΔR4 and ΔR3 are both exposed to the hydrophobic surface, only ΔR4 significantly decreased the anticoagulant activity and the FXII and FXI inhibitory activity, indicating that the active site of ANXA4 is located in repeat 4.

(3) ANXA4 and sulfatide in megakaryocytes and platelets

Whether ANXA4 and ANXA5, which have the highest anticoagulant activity among the annexin family, work as inhibitors of thrombi formation *in vivo* is still unknown. Then, ANXA4, ANXA5, and sulfatide expressions were investigated in megakaryoblast, megakaryocyte, and platelets. RT-PCR revealed that ANXA4 and ANXA5 transcripts are expressed in MEG-01, imMKCL, and platelets more than other annexin family proteins. Furthermore, the proteins ANXA4 and ANXA5 were also detected by western blotting in platelets. Immunostaining experiments showed that ANXA4 and ANXA5 are localized in the cytoplasm of megakaryocytes and platelets. Furthermore, immunostaining and flow cytometry experiments revealed that ANXA4 is translocated to the activated platelet membrane. The expression of sulfatide in megakaryocytes was determined by investigating the genetic expression of galactosyltransferase, CGT and sulfotransferase, CST. The absence of detection of the sulfatide synthases transcript by RT-PCR suggested that megakaryocytes and platelets do not produce sulfatide. However, since sulfatide was found on the cell surface of megakaryocytes by immunostaining, it was suggested that sulfatide originates from the other cells or culture medium and is re-localized on the surface of platelets.

【Outline of the paper】

The results of the above research are summarized in this dissertation in the following composition. **Chapter 1.** Introduction. The background of this research: the current status of anticoagulants agents, the activation mechanisms of the intrinsic coagulation pathway, and the research to date on the anticoagulant activity of annexin family proteins. **Chapter 2.** Experimental methods used in this study. **Chapters 3 and 4.** The results and discussion of the molecular mechanism of the anticoagulant activity of ANXA4 by investigating the inhibitory activity of FXII and FXI activation and ANXA4 mutagenesis. **Chapter 5.** The results and discussion of the expression and localization of the annexin family proteins and sulfatide synthase in megakaryocytes and platelets for the exploration of the possibility of ANXA4 as an anticoagulant protein *in vivo*. **Chapter 6.** Comprehensive consideration based on the findings of this study: applicability of ANXA4, the significance of the expression of ANXA4 and sulfatide in the body.