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## The effect of argatroban on the function of vascular endothelial cells stimulated by bound thrombin

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### Background

Stimulation of procoagulant and coagulant activity after thrombolytic therapy had been assumed to be a negative feedback mechanism based on homeostasis of a living body. The substance, which bears this negative feedback, had not been identified. However, a post-clotting thrombin was found to exist in the circulating blood on the researches for the development of the vascular restenosis after percutaneous transluminal coronary angioplasty (PTCA) and percutaneous transluminal coronary recanalization (PTCR). The post-clotting thrombin is the residual thrombin in clot, which is incorporated into fibrin clot by binding to fibrin(ogen) during the formation of fibrin clot. In addition, it is known that the post-clotting thrombin retains its clotting activity. We classified any post-clotting thrombins into "intact thrombin" and "bound thrombin" based on the concept we have proposed. Bound thrombin refers to thrombin, which binds to fibrinogen to convert into fibrin and is liberated from clot by mechanical crush and by clot-lysis. Thrombin that transiently binds to fibrinogen but not cleave fibrinogen into fibrin is named intact thrombin. In addition, native thrombin is defined as thrombin, which has not yet interacted with fibrinogen. On the other hand, if bound thrombin is directly related to the development of vascular restenosis following the proliferation and phenotype conversion of vascular smooth muscle (VSM) cell, it will be shown that bound thrombin can pass through the endothelial cell monolayer. Accordingly, it was thought that inhibition of passage of bound thrombin was an important strategy to prevent the restenosis.

In this seminar, we will present 1) chemical properties of bound thrombin and 2) the effect of argatroban on the function of vascular endothelial cells stimulated by bound thrombin.

### Structural characteristics of bound thrombin

To clarify the structural characteristics of bound thrombin, we isolated the bound thrombin from clot lysate. Rabbit fibrinogen was clotted with bovine thrombin, and clot lysate was prepared with urokinase. The bound thrombin was isolated from clot lysate by serial chromatography using a Sepharose 4B column immobilizing anti-bovine thrombin antibody and a Sepharose 4B column immobilizing anti-rabbit fibrinogen antibody.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under unreduced conditions demonstrated that there were two different protein bands in the isolated bound thrombin. On a C4 reverse-phase high performance liquid chromatography (HPLC), the bound thrombin from clot lysate was resolved by 4 M urea into  $\alpha$ -thrombin and a fibrin fragment, the N-terminal regions of which were identified as  $\alpha$ -,  $\beta$ - and  $\gamma$ -chains. Thus, in the bound thrombin, thrombin molecule would bind to rabbit fibrin fragment consisting of N-terminal central domain<sup>1</sup>.

We also aimed at clarifying the structural characteristics of the bound thrombin that is liberated by mechanical crush of fibrin clots. Fibrin clots were prepared with bovine thrombin and rabbit fibrinogen, and were crushed mechanically with a glass rod. The supernatant of the crushed clots was subjected to immunoaffinity chromatography to

isolate the bound thrombin. Western blotting analysis revealed that the bound thrombin could be reacted with both anti-thrombin and anti-fibrinogen under unreduced conditions. SDS-PAGE under reduced conditions revealed that there were three bands, two of which were found to be the N-terminal fragments of the  $\alpha$ - and  $\gamma$ -chains of fibrinogen. The bound thrombin could be dissociated into three distinct fibrin fragments and bovine  $\alpha$ -thrombin when denatured by 8 M urea. Thus, the bound thrombin liberated from crushed clots is a stable complex between bovine  $\alpha$ -thrombin and fibrin fragments of the N-terminal regions of rabbit  $\alpha$ - and  $\gamma$ -chains <sup>2</sup>.

#### **Generation of bound thrombin in the circulating blood**

After coronary interventions, levels of fibrinopeptide A (FPA), thrombin-antithrombin III (TAT) complex and prothrombin fragment 1+2 were significantly increased in many patients, indicating rethrombosis in the vessel. It has been reported that bound thrombin is liberated from fibrin clots by the action of fibrinolytic enzymes and that the liberated thrombin forms a complex with fibrin fragment E or (DD)E. Thus, bound thrombin could be generated by thrombolytic therapy or mechanical medical interventions such as PTCA. The liberated bound thrombin might be pathophysiologically involved in rethrombosis and reocclusion.

#### **Activation of coagulant and platelet by bound thrombin**

It was reported that bound thrombin activated Factor V, Factor VIII, and platelet aggregation as native thrombin. We also demonstrated that post-clotting thrombins (intact thrombin and bound thrombin), which was prepared from the clots of rabbit fibrinogen and bovine thrombin, increased cytosolic free  $Ca^{2+}$  concentration in platelet with the resultant aggregation of washed rabbit platelet <sup>3</sup>.

#### **Cellular actions of bound thrombin on vascular endothelial cells and vascular smooth muscle cells**

We investigated whether bound thrombin

had cellular actions on vascular cells such as endothelial cells and smooth muscle cells. Bound thrombin prepared from crushed clots stimulated the release of plasminogen activator (PA) from cultured rat aortic endothelial cells <sup>4</sup>. Fibrin enzymography revealed that the released PA was tissue-type PA (t-PA). Therefore, bound thrombin releases t-PA as native thrombin.

The proliferation of VSM cells plays a major role in vascular restenosis following coronary interventions. The highly proliferative VSM cells consist of synthetic type, which can produce growth factors and extracellular matrices thereby increasing the risk of intimal hyperplasia. It was demonstrated that SMemb, a myosin heavy chain (MHC) isoform, was highly expressed in synthetic cell type, while other MHC isoforms (SM1 and SM2) were expressed in contractile type. Coagulation factors including thrombin and tissue factor are involved in the development of vascular restenosis after interventional treatment. We hypothesized that bound thrombin in the thrombus plays an important role in the development of restenosis. Therefore, we investigated whether the bound thrombin can modulate phenotype conversion of VSM cells. We tested if the bound thrombin had an effect on the expression of SMemb in cultured VSM cells using reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization. Consequently, bound thrombin significantly upregulated the expression of SMemb mRNA, indicating that the bound thrombin could induce the phenotype conversion of VSM cells <sup>5</sup>.

#### **Argatroban inhibition of bound thrombin activity**

It was known that the rethrombosis was resistant to the treatment by heparin. In addition, in vitro study demonstrated that thrombin activity in the presence of fibrin clot was not inhibited by heparin. This result was interpreted by the following possibilities. 1) Heparin binding site on molecule of bound thrombin might be occupied by fibrin fragment of bound thrombin. 2) Conformation of the heparin binding site might be changed so that

heparin no longer binds to. However, a potent and selective low molecular synthetic thrombin inhibitor, such as argatroban, could inhibit the bound thrombin even when the heparin site was occupied by fibrin fragment. We tested the inhibitory action of argatroban on the bound thrombin derived from crushed clots in order to determine the type of inhibition. Collagen-thrombin was used as control for bound thrombin. Consequently, argatroban competitively inhibited collagen-thrombin, whereas argatroban uncompetitively inhibited bound thrombin. In addition, bound thrombin-induced platelet aggregation was dose-dependently inhibited by argatroban.

#### **The effect of argatroban on the function of vascular endothelial cells stimulated by bound thrombin.**

t-PA was released from the cultured aortic vascular endothelial cells of rats by native and bound thrombin. Argatroban inhibits the release of t-PA from rat vascular endothelial cells stimulated by native and bound thrombin. In addition, in an experiment using the dual chamber system, argatroban inhibits the passage of thrombin through the rat aortic endothelial cell monolayer.

#### **Summary**

Although the exact structure of bound thrombin has not yet been fully understood, it is assumed that bound thrombin exists as various types of structure, which depends on the combination of  $\alpha$ -thrombin with fragments of fibrin(ogen). Bound thrombin that could be generated by both thrombolytic therapy and mechanical angioplastic procedures was involved in many pathophysiological conditions such as rethrombosis, reocclusion, and vascular restenosis. Many studies including ours on bound thrombin demonstrated that argatroban was available to inhibit the biological activity of bound thrombin. In addition, from the structural analysis of bound thrombin, the inhibitory effect of argatroban on amyolytic activity and activity of platelet aggregation of bound thrombin was stronger than that of heparin.

#### **References**

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