

# Thrombus Formation and Propagation in the Onset of Cardiovascular Events

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Ischemic cardiovascular disease is a major cause of morbidity and mortality worldwide and thrombus formation on disrupted atherosclerotic plaques is considered to trigger its onset. Although the activation of platelets and coagulation pathways has been investigated intensively, the mechanisms of thrombus formation on disrupted plaques have not been understood in detail. Platelets are thought to play a central role in the formation of arterial thrombus because of rapid flow conditions; however, thrombus that develops on disrupted plaques consistently includes large amounts of fibrin in addition to aggregated platelets. While, thrombus does not always become large enough to completely occlude the vascular lumen, indicating that the propagation of thrombus is also critical for the onset of cardiovascular events. Various factors, such as vascular wall thrombogenicity, altered blood flow and imbalanced blood hemostasis, modulate thrombus formation and propagation on disrupted plaques. Pathological findings derived from humans and experimental animal models of atherothrombosis have identified important factors that affect thrombus formation and propagation, namely platelets, extrinsic and intrinsic coagulation factors, proinflammatory factors, plaque hypoxia and blood flow alteration. These findings might provide insight into the mechanisms of thrombus formation and propagation on disrupted plaques that lead to the onset of cardiovascular events.

**Key words:** Atherothrombosis, Platelet, Tissue factor, Blood flow, Factor XI

## Introduction

Thrombus formation on disrupted atherosclerotic plaques is generally considered as being a trigger for atherothrombosis, namely the onset of cardiovascular events. Since thrombus does not always become large enough to completely occlude vessels with subsequent acute symptomatic events, thrombus propagation is also critical to the onset of clinical events<sup>1)</sup>. Thrombus formation is modulated by factors, including the thrombogenicity of plaques and blood, local hemorheology and proinflammatory factors. Although the activation of platelets and coagulation pathways has been intensively investigated, the mechanisms involved in thrombogenesis or thrombus propagation at plaque disruption sites remain obscure.

Many studies of experimental animals have determined the molecular mechanisms of thrombus forma-

tion. However, the mechanisms of thrombus formation and propagation in atherosclerotic vessels remain vague because thrombi are induced in the “normal” arteries of many experimental animal models via chemical or physical damage. Since atherothrombosis develops on disrupted atherosclerotic plaque, vascular wall components and associated blood flow alterations are essential factors for thrombogenesis.

This article examines the mechanisms of thrombus formation and propagation on disrupted atherosclerotic plaques from pathological aspects, including recently identified factors.

## Pathology of Plaque Disruption

The underlying mechanisms of the onset of cardiovascular events comprise plaque rupture and plaque erosion (**Fig. 1**).

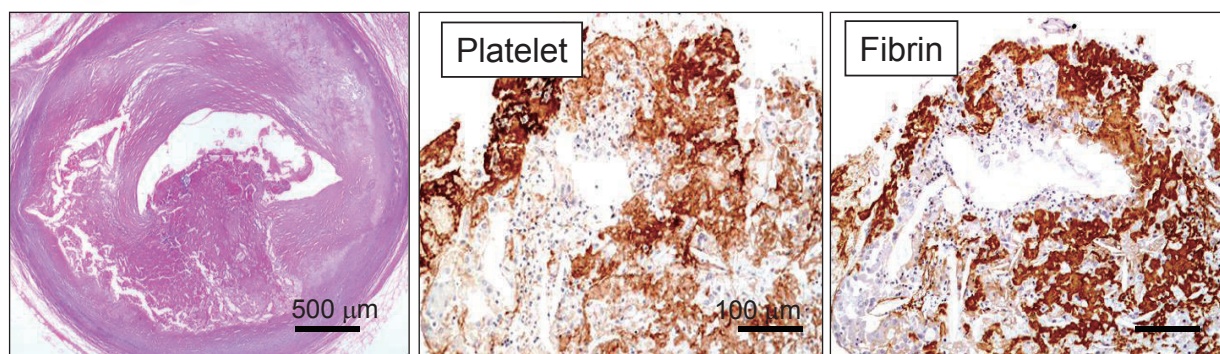
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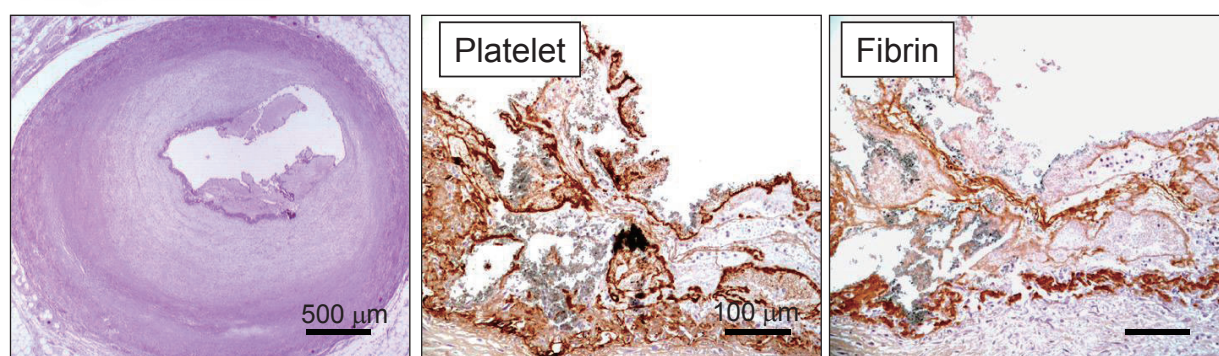
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## Plaque rupture



## Plaque erosion



**Fig. 1.** Microphotographs of coronary plaque rupture and erosion with thrombi

Ruptured plaque comprises large necrotic core and disrupted thin fibrous cap accompanied by thrombus formation. Eroded plaque is fibrous and rich in smooth muscle cells, without visible atheromatous component. Both types of thrombi comprise platelets and fibrin (Ref. 8, with permission).

Plaque rupture is the most common cause of acute coronary syndrome (ACS), and it is induced by fibrous cap disruption that allows blood to contact a highly thrombogenic necrotized core, resulting in thrombus formation. It is likely rupture occurs in plaques with a large necrotic core, fibrous caps that are usually <65  $\mu\text{m}$  thick, and the massive infiltration of macrophages, T lymphocytes and occasional smooth muscle cells (SMC)<sup>2,3</sup>. Accumulating evidence supports the notion that inflammation plays a key role in the pathogenesis of plaque rupture. Macrophages mainly with the M1 phenotype<sup>4</sup> overexpress proteolytic enzymes in plaques. In addition, activated T lymphocytes and macrophages secrete interferon  $\gamma$ , which inhibits collagen synthesis and induces SMC apoptosis<sup>5</sup>. These findings show that inflammatory pathways significantly participate in plaque destabilization.

Plaque erosion is responsible for 22%–44% of ACS<sup>6</sup>, which is characterized by an absence of endothelium or intimal injury without reaching lipid core. The morphological characteristics include an abundance of SMC and proteoglycan matrix, especially versican

and hyaluronan, whereas the necrotic core is small or absent and macrophages and T cells are scant<sup>2,3</sup>. Patients with plaque erosion are younger, and unlike those with plaque rupture, males do not predominate<sup>6</sup>. Mechanisms that might contribute to erosion include endothelial dysfunction, hemodynamic forces, hyper vasoconstriction and sub-endothelial matrix modification; however, details remain unclear<sup>6</sup>. A contribution of Toll-like receptor 2 and neutrophil activation has recently been reported<sup>7</sup>.

### Pathology of Thrombus on Disrupted Plaques

Arterial thrombi are thought to mainly comprise aggregated platelets because of rapid flow conditions. However, thrombi that develop on disrupted plaques consist of not only aggregated platelets, but also large amounts of fibrin in addition to other types of blood cells (Fig. 1)<sup>8-10</sup>. In addition, the proportions of fibrin and platelets differs between coronary thrombi on ruptured and eroded plaques, which are fibrin- and rather

platelet-rich, respectively<sup>8</sup>). These morphological features indicate that the coagulation pathway is activated as well as the platelets at the sites of plaque disruption even under rapid flow conditions, and that thrombogenic mechanisms differ between plaque rupture and erosion.

### Platelet Activation on Plaques

Platelet activation at the sites of disrupted plaques is an initial step in the formation of thrombus. Activated platelets release adenosine diphosphate (ADP), serotonin and thromboxane A<sub>2</sub>, which promote platelet activation further. This self-amplifying process results in thrombus propagation. In addition, released adenosine triphosphate (ATP) from erythrocytes and leukocytes can also activate platelets. Because ADP and ATP play key roles in platelet aggregation<sup>11</sup>, their metabolites in the bloodstream are important for the regulation of platelet activation and recruitment.

#### 1) Ecto-Nucleoside Triphosphate Diphosphohydrolase

Ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase, CD39) is a membrane-bound enzyme that rapidly hydrolyzes both ADP and ATP to AMP and thereby inhibits platelet activation and thrombus formation<sup>12, 13</sup>. Endothelial E-NTPDase expression is down-regulated under conditions of inflammation and disrupted blood flow<sup>14, 15</sup>. Hatakeyama *et al.*<sup>16</sup> showed that E-NTPDase was also expressed in vascular SMC and down-regulated in atherosclerotic lesions, being more significantly decreased in patients with unstable, than stable angina. These findings suggest that E-NTPDase expression by SMC is also reduced by inflammatory and/or oxidative stress. The local expression of E-NTPDase by gene transfer in the injured arterial walls of rats significantly suppresses platelet aggregation and occlusive thrombus formation<sup>17</sup>. These lines of evidence suggest that reduced E-NTPDase activity in atherosclerotic lesions largely promotes thrombus formation on disrupted plaques.

#### 2) C-Type Lectin-Like Receptor 2

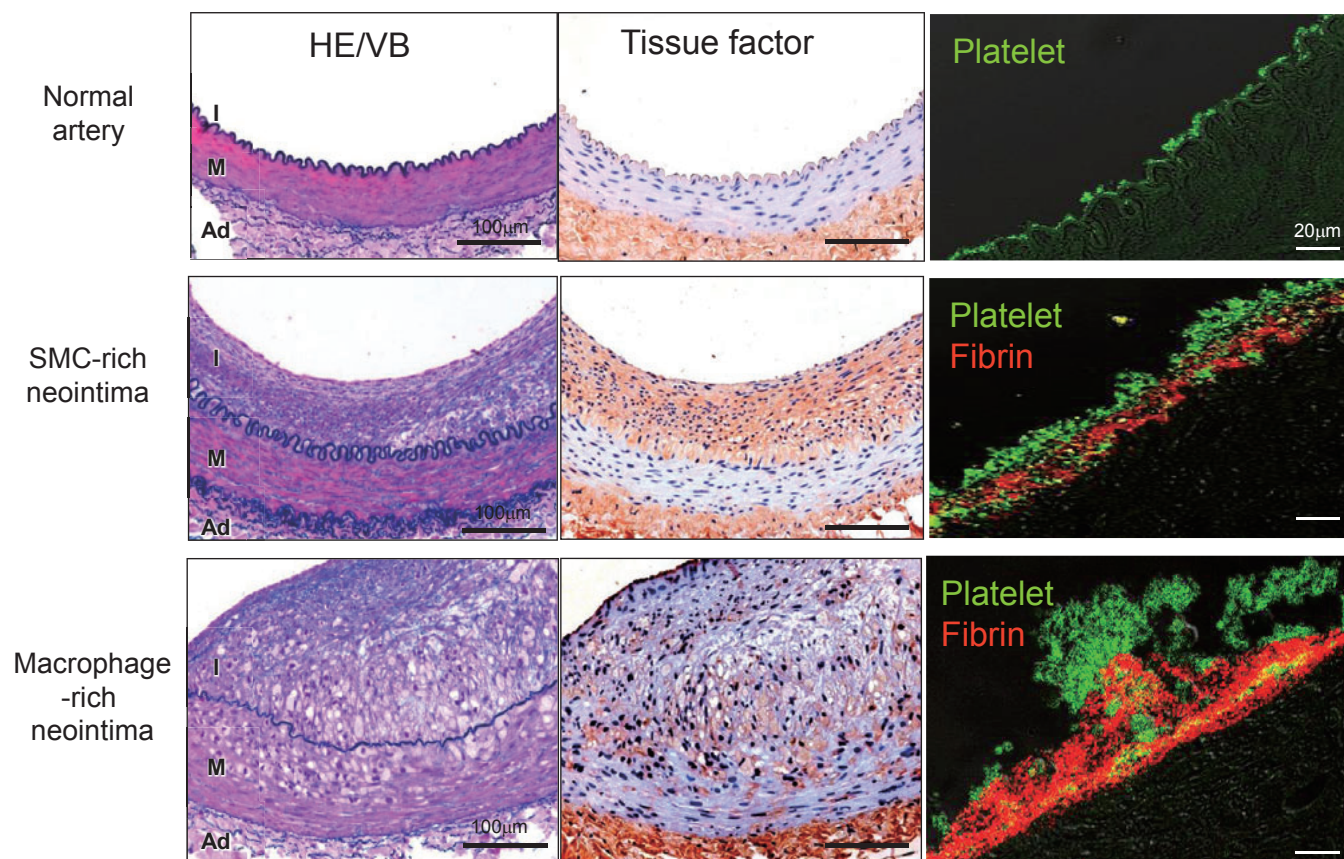
Platelet activation is mediated by platelet-specific receptors including those for glycoprotein (GP) Ib, GPIIb/IIIa, protease activated receptors (PAR) 1, PAR4, and P2Y.<sup>18</sup> C-type lectin-like receptor 2 (CLEC-2) is a novel receptor for the platelet-activating, snake-venom protein, rhodocytin. CLEC-2 elicits powerful platelet activation signals in conjunction with Src, Syk kinases, and phospholipase C<sub>2</sub>, like the collagen receptor GPIIb/IIIa-FcR $\gamma$ -chain complex<sup>19</sup>. Therefore, endogenous ligands for CLEC-2 in vessel walls

contribute to thrombosis and hemostasis. Podoplanin has recently become recognized as an endogenous ligand for CLEC-2<sup>19</sup>. Hatakeyama *et al.*<sup>20</sup> showed that podoplanin was expressed in human advanced, but not early atherosclerotic lesions (diffuse intimal thickening), that primarily consist of SMC. Inoue *et al.*<sup>21</sup> showed that SMC stimulate platelets through binding CLEC-2 and identified S100A13 as one of its ligands. S100A13 is a Ca<sup>2+</sup>-binding protein that belongs to the S100 protein family, and it is implicated in inflammation, angiogenesis and tumor development<sup>22, 23</sup>. S100A13 is expressed on the surface of SMC under oxidative stress, but not on that of normal SMC<sup>21</sup>. Increased amounts of CLEC-2 ligands, podoplanin and S100A13 in advanced atherosclerotic plaques access platelet activation and promote thrombus formation.

### Coagulation Factors on Thrombus Formation

The coagulation pathway also plays a critical role in thrombus formation. The binding of plasma factor VII/VIIa (FVII/FVIIa) to tissue factor (TF) initiates this pathway, and TF/FVIIa complexes of the extrinsic pathway initiate blood coagulation by activating both FX and FIX. The FVIIIa/FIXa complexes of the intrinsic pathway provide an alternative route to the generation of FXa, which is part of the prothrombinase complex (FVa/FXa) that activates prothrombin to thrombin that subsequently plays a central role in the coagulation protease cascade<sup>24</sup>. In addition, activated platelets enhance the coagulation cascade by providing a negatively charged phospholipid surface for the assembly of prothrombinase complexes and by binding FXI via the GPIIb receptor<sup>25</sup>.

TF triggers the extrinsic pathway, and it is widely distributed in non-uniform tissues of the brain, lungs, heart, kidneys and other organs<sup>26</sup>. Adventitial fibroblasts in normal arteries, as well as macrophages and SMC in atherosclerotic lesions express TF, large amounts of which locate in the extracellular matrix of advanced lesions in humans<sup>27, 28</sup>. These pathological findings indicate that TF expression in atherosclerotic plaques plays a major procoagulant role in thrombus formation after plaque disruption. Mallat *et al.*<sup>29</sup> reported that 97% of the total procoagulant activity extracted from atherosclerotic plaques was due to TF. In addition, TF circulates in a blood microparticle (MP)-associated form, and clinical studies have identified increased amounts of TF antigen circulating in the plasma of patients with cardiovascular disease (CAD)<sup>30, 31</sup>. However, Hisada *et al.*<sup>32</sup> reported that the activity of the MP-associated form of TF did not significantly increase in the cardiovascular disease patients; therefore, plaques



**Fig. 2.** Immunohistochemical microphotographs of tissue factor and thrombus in rabbit normal and atherosclerotic femoral arteries (Left column) Representative microphotographs of normal femoral artery and of femoral arteries at 3 weeks after balloon injury of conventional (SMC-rich neointima) or 0.5% cholesterol diet (Macrophage-rich neointima). (Middle column) Tissue factor is expressed in SMC- and macrophage-rich neointima, and in adventitia. (Right column) Thrombus 15 minutes after balloon injury on normal artery comprises only small aggregated platelets, whereas that on neointima comprises a mixture of platelets and fibrin. Thrombus on macrophage-rich neointima is much larger. Abbreviations: Ad, adventitia; HE/VB, hematoxylin and eosin/Victoria blue; I, intima; M, media (modified from Ref. 35, with permission).

would be the major source of TF that drives atherothrombosis.

### Rabbit Model of Atherothrombosis

We developed an animal model that expressed TF in vascular lesions to understand the role of TF in plaques on thrombus formation<sup>33-35</sup>.

Plaques were created in rabbit arteries injured using a balloon catheter and, then, the rabbits were fed with either a conventional or a cholesterol diet to developing SMC- or macrophage-rich plaques, respectively. The expression and activities of TF increased in both types of plaques, with more being localized in the macrophage-rich types<sup>35</sup>; this mimics the situation in human atherosclerotic plaques.

Large thrombi produced on injured plaques in this model comprised a mixture of platelets and fibrin,

whereas small thrombi that developed on the injured normal artery were rich in platelets (Fig. 2)<sup>35</sup>. The intravenous injection of a recombinant TF pathway inhibitor (TFPI) or TFPI gene transfection into injured vessel walls significantly reduced thrombus size, suggesting that thrombus formation on arterial lesion largely depends on the TF-dependent coagulation pathway<sup>34, 36, 37</sup>.

### Tissue Factor Expression in Plaques

The expression of TF in vascular cells is induced by many factors, such as proinflammatory cytokines (interleukin-1, tumor necrosis factor- $\alpha$ ), bacterial endotoxin and modified low density lipoprotein<sup>38</sup>.

#### 1) C-Reactive Protein

C-reactive protein (CRP) is an inflammatory acute-

phase reactant that has emerged as a powerful predictor of CAD<sup>39-41</sup>). This protein is present in all stages of atherosclerotic plaques from the early to advanced lesions, and more prevalent in thrombotic, than non-thrombotic plaques<sup>42, 43</sup>), implicating CRP in atherothrombogenesis. Because CRP functions in rabbits but not in mice as an acute-phase reactant during inflammation in the same way that it does in humans<sup>44</sup>), Koike *et al.*<sup>45</sup>) established a line of transgenic (Tg) rabbits that overexpressed human CRP (hCRP). Matsuda *et al.*<sup>46</sup>) found that hCRP enhanced vascular wall thrombogenicity via increased TF expression in SMC and promoted thrombus formation after plaque disruption in this model.

## 2) Plaque Hypoxia

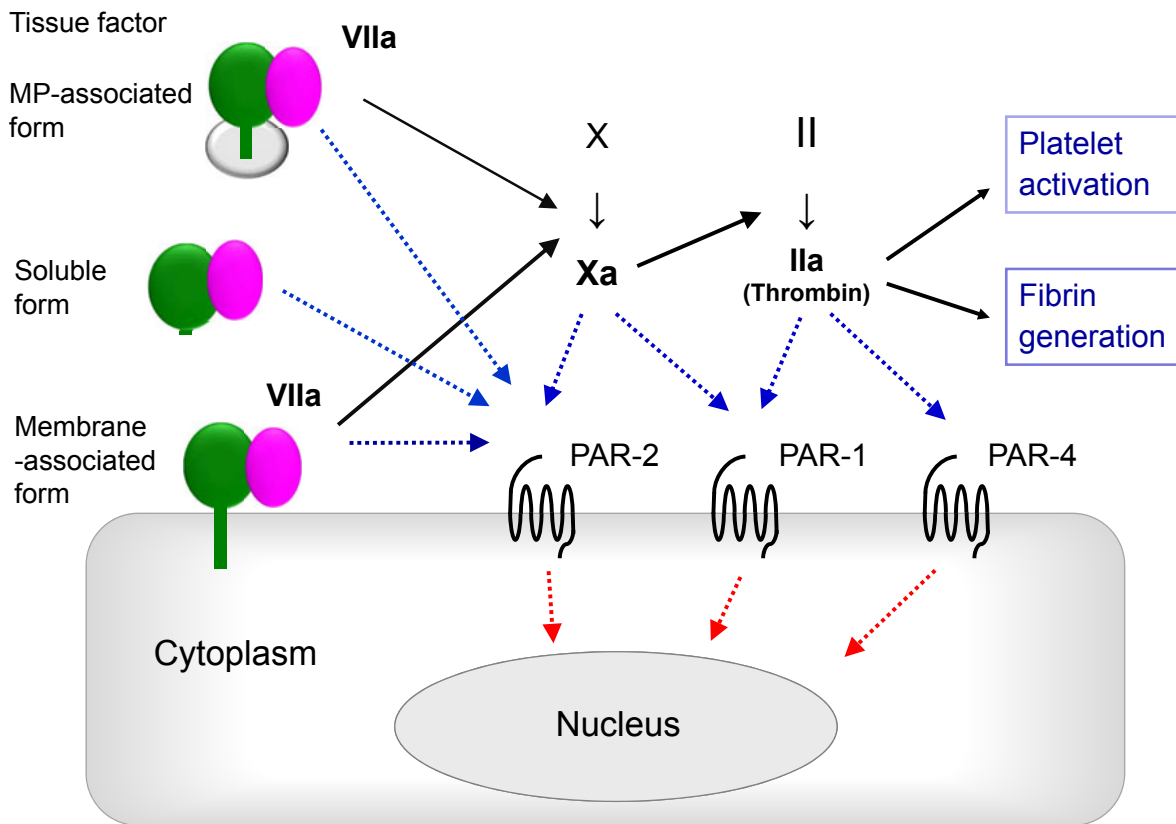
Hypoxia affects the biological functions of vascular cells via the regulation of metabolism, inflammation, angiogenesis and several other processes<sup>47</sup>). Hypoxia signaling can modulate tissue remodeling or the severity of cardiovascular disorders. Hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and HIF-2 $\alpha$  belong to a group of transcription factors that mediate most of the cellular responses to hypoxia at the transcriptional level<sup>48</sup>). Sluimer *et al.*<sup>49</sup>) demonstrated that hypoxia was present in the center of an advanced human carotid plaque by pimonidazole hydrochloride, a hypoxic marker, and also that the hypoxic area, HIF and macrophages were correlated with intra-plaque angiogenesis. Plaque hypoxia has been detected in animal models of atherosclerosis<sup>50, 51</sup>). Leppänen *et al.*<sup>52</sup>) revealed that even though the rabbit normal aorta was not hypoxic, the cores of plaques >500  $\mu$ m thick were hypoxic, and they were characterized by ATP depletion, low glucose, low glycogen and high lactate values. These findings suggest that limited oxygen diffusion capacity due to increased plaque thickness is a potent determinant of the onset of hypoxia within atherosclerotic lesions. Matsuura *et al.*<sup>50</sup>) also detected hypoxic areas located predominantly within lipid-laden macrophage-rich plaque in a rabbit model of atherosclerosis. These hypoxic areas positively correlated with the number of HIF-1 $\alpha$  and NF- $\kappa$ B p65-immunopositive nuclei and TF-immunopositivity. They also found hypoxic conditions increased TF and plasminogen activator inhibitor (PAI)-1 expression and procoagulant activity in atherosclerotic plaque tissues and cultured macrophages. These results suggest that hypoxia itself is an important determinant of TF expression. O'Rourke *et al.*<sup>53</sup>) reported that hypoxia can induce TF mRNA in HIF-1 $\alpha$ -deficient HeLa cells, suggesting that hypoxia could also increase TF independently of HIF-1 $\alpha$ .

## Non-Hemostatic Functions for TF

Accumulating evidence shows that TF/FVIIa complexes not only initiate the coagulation pathway, but also affect various non-hemostatic biological agents by activating proteinase-activated receptors (PARs)<sup>54, 55</sup>). Many cell types express these receptors, which play important roles in cardiovascular physiology and pathophysiology, and in the systems of other many organs<sup>55</sup>). The TF/VIIa complex, FXa and thrombin are potent PAR activators<sup>54</sup>). Therefore, TF/VIIa complex directly or indirectly activates PARs (Fig. 3), which mediate contraction, migration, proliferation, hypertrophy, and production of the extracellular matrix of SMC, thereby contributing to the development of vascular lesions and the pathophysiology of vascular diseases such as atherosclerosis<sup>56-59</sup>). Furthermore, the cytoplasmic domain of TF serves as a regulator for angiogenesis and SMC migration/proliferation<sup>60, 61</sup>). Therefore, TF expressed in plaques contributes to not only thrombus formation after plaque disruption, but also contributes to the development of plaque.

## Factors That Contribute to Thrombus Propagation

Thrombus formation on disrupted plaques is a fundamental process in the onset of acute cardiovascular events. However, it does not always lead to complete thrombotic occlusion with subsequent acute symptomatic events<sup>1</sup>). Autopsy studies of patients who died of non-cardiac causes have found a 4%–10% incidence of coronary plaque disruption with fresh non-occlusive thrombi<sup>62-64</sup>). Clinical studies using invasive vascular imaging techniques have revealed a much higher incidence of asymptomatic coronary plaque disruption, and multiple plaque disruption is a frequent complication in patients with ACS<sup>65-67</sup>). Disrupted plaques at various stages of healing are also occasionally found post-mortem in patients with or without ACS<sup>64, 68</sup>). Furthermore, several pathologic studies of aspirated coronary material have shown that plaque disruption and thrombus formation occur significantly earlier than symptom onset, coronary thrombi are days or weeks old in many patients, and the presence of an older thrombus is an independent predictor of mid or long-term mortality in patients with acute myocardial infarction (MI)<sup>69-71</sup>). This evidence indicates that coronary plaque disruption with thrombosis is rather prevalent and that many lesions might not be associated with acute symptomatic events. Therefore, other factors affecting thrombus propagation must contribute to the onset of acute coronary events.



**Fig. 3.** Tissue factor-dependent coagulation pathway and PARs

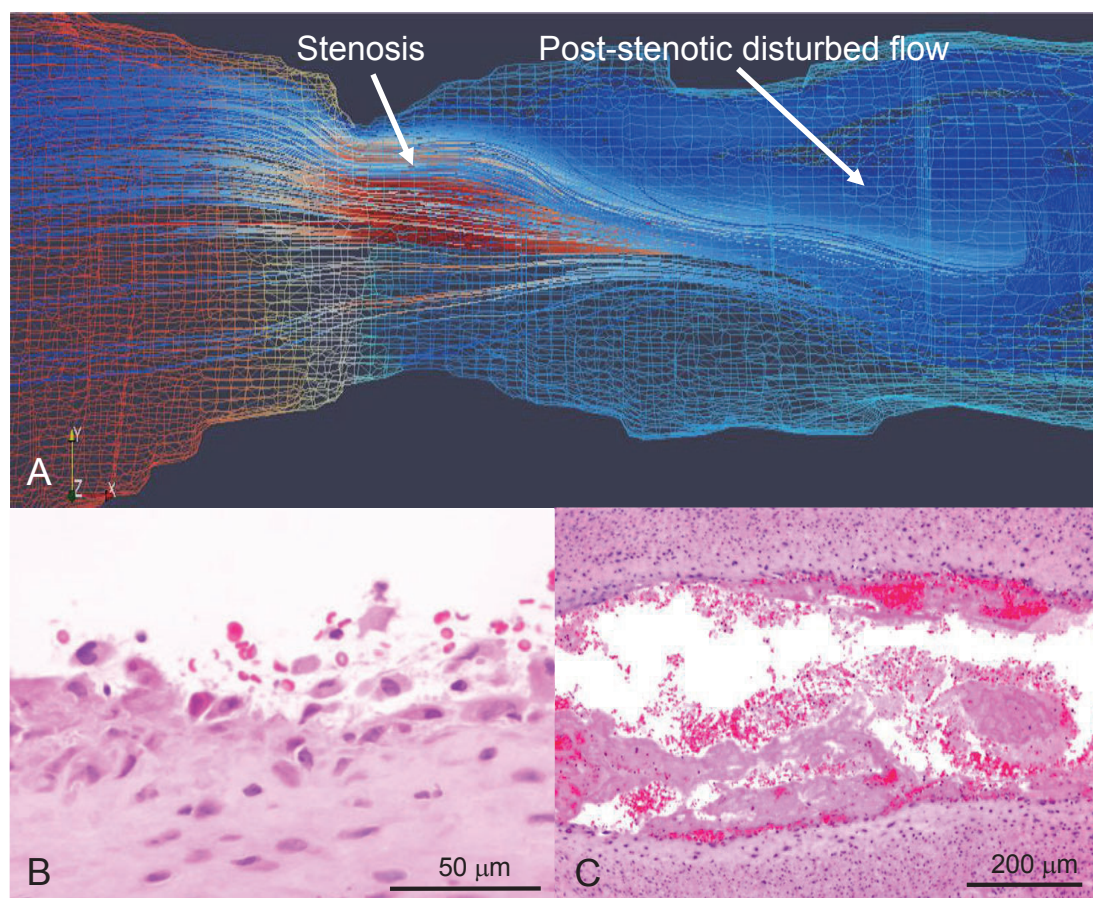
Membrane- and microparticle (MP)-associated TF binding to factor VIIa triggers coagulation pathway, whereas soluble form of TF with factor VIIa does not. Downstream coagulation factors activate proteinase-activated receptors (PARs) that play other non-coagulative biological roles.

### 1) Blood Flow Changes and VWF-ADAMTS13 Axis

Blood flow is a key modulator of thrombosis. Stenosis or irregular vessel geometries in advanced atherosclerotic arteries might induce disturbed blood flow (i.e., flow separation, recirculation, and reattachment of forward flow) that are thought to favor thrombus propagation<sup>72, 73</sup>. In addition, plaque disruption and coronary intervention can induce distal embolisms and vasoconstriction, which reduce or disrupt coronary blood flow at sites of plaque disruption<sup>74-76</sup>. Regions with low wall shear stress, particularly oscillatory or reversed shear stress that can occur at bifurcations or at sites distal to stenotic lesions, are likely associated with the recruitment of platelets and coagulation factors<sup>73</sup>. Reduced blood flow promoted fibrin-rich mural thrombi on atherosclerotic lesions in our animal model<sup>77</sup>. Disrupted blood flow induced by luminal stenosis can induce plaque disruption (erosion), and computational flow simulation suggests that increased wall shear stress, turbulence kinetic energy and blood pressure significantly contribute to the onset of plaque erosion<sup>78</sup>. The combination of changes in blood flow and the

increased thrombogenicity of vascular walls is crucial for thrombus formation and propagation<sup>79</sup> (**Fig. 4**).

Blood flow velocity significantly affects the function of von Willebrand factor (VWF), a large multimeric glycoprotein that is synthesized and stored by endothelial cells and megakaryocytes. The VWF multimers released upon stimulation are rich in ultra-large (UL) forms that are hyperactive in binding platelet GP Ib and can induce platelet aggregation<sup>80, 81</sup>. Released UL-VWF multimers are rapidly cleaved by the plasma protease, a disintegrin and metalloprotease with a thrombospondin type 1 motif 13 (ADAMTS-13), to smaller and less active forms under flow conditions<sup>82</sup>. An extreme deficiency of ADAMTS-13 activity results in increased circulating levels of UL-VWF multimers that correlate with the onset of thrombotic thrombocytopenic purpura (TTP)<sup>83</sup>. Shida *et al.*<sup>84</sup> reported that ADAMTS-13 cleaved VWF and down-regulated mural thrombus growth at the site of ongoing thrombus generation under high shear rate conditions. This evidence suggests that ADAMTS-13 prevents thrombus propagation and thrombotic occlusion by regulating the size of



**Fig. 4.** Computational flow simulation and microphotographs of erosive injury of rabbit stenotic femoral artery with SMC-rich plaque

Rabbit femoral arteries 3 weeks after balloon injury are constricted using a vascular occluder. (A) Representative computational reconstructed image and flow simulation in Reynolds-Averaged Navier-Stokes model. Red and blue mesh indicates high and low pressure, respectively, on wall. Flow velocity in this model increases at stenosis and decreases at post-stenotic portion, resulting in disrupted flow. (B, C) Representative microphotographs of erosive injury and thrombus formation. Neointimal endothelial cells and SMCs are broadly detached at post-stenotic portion 15 minute after vascular stenosis (B) (Ref. 79, with permission). Large mural thrombi are formed at the portion 60 minute after vascular stenosis (C).

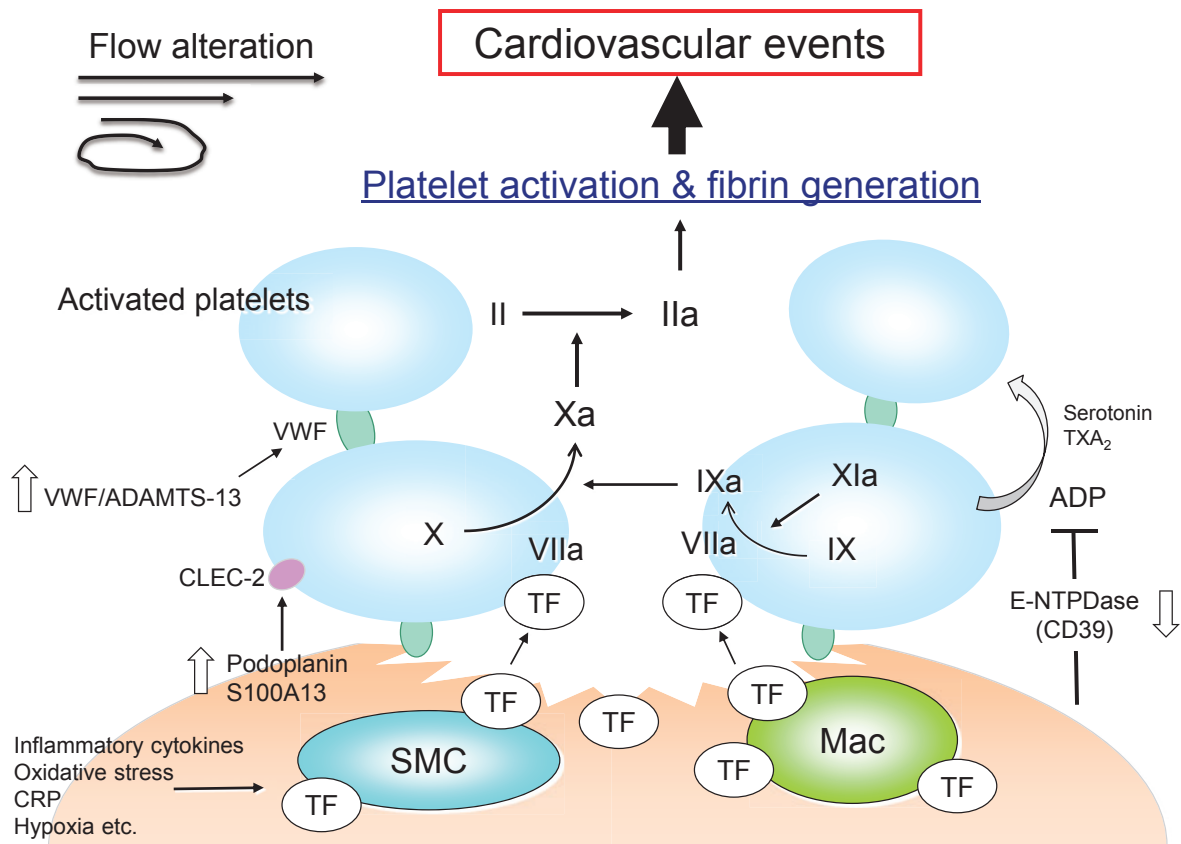
VWF multimers under high shear rate conditions. Clinical studies have found decreased ADAMTS-13 activity or a higher ratio of VWF/ADAMTS-13 in patients with acute MI<sup>85-87</sup>. We reported that ADAMTS-13 closely localized with VWF in coronary thrombi from patients with ACS, and that reducing ADAMTS-13 activity enhanced thrombus formation<sup>88</sup>. ADAMTS-13 activity is decreased during aging, in systemic inflammation, and in focal vascular inflammation<sup>89-91</sup>.

## 2) Factor XI in the Intrinsic Coagulation Pathway

The intrinsic coagulation pathway is essential for thrombus stabilization and propagation. This pathway is activated when blood contacts negatively charged substances or artificial surfaces and induces the conversion of FXII into activated FXII, which then cleaves

prekallikrein to generate active kallikrein, and activate FXI. Factor XI is also activated by thrombin and is essential for further thrombin generation during thrombus formation<sup>92</sup>. It also causes the inhibition of fibrinolysis that is dependent on thrombin activatable fibrinolysis inhibitor (TAFI)<sup>93</sup>. These lines of evidence indicate that FXI-mediated thrombin activation and inhibited fibrinolysis contribute to thrombus propagation.

On the other hand, FXI is generally considered to be less important in normal hemostasis, because patients with an FXI deficiency have a mild or absent bleeding tendency and a mild trauma-induced bleeding disorder<sup>94, 95</sup>. However, recent findings indicate that FXI is activated during blood coagulation, and that even small amounts of FXI can induce thrombus growth by generating thrombin and by protecting thrombi



**Fig. 5.** Activation of platelets and coagulation pathway at disrupted atherosclerotic plaque

ADAMTS-13, a disintegrin and metalloprotease with a thrombospondin type 1 motif 13; ADP, adenosine diphosphate; CLEC-2, c-type lectin-like receptor 2; CRP, c-reactive protein; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; Mac, macrophage; SMC, smooth muscle cell; TF, tissue factor; TXA<sub>2</sub>, thromboxane A<sub>2</sub>; VWF, von Willebrand factor.

from fibrinolysis via TAFI. Therefore, FXI apparently plays a critical role in thrombus stability and propagation. Clinical studies have associated high levels of FXI with the onset of venous thrombosis and ischemic stroke<sup>96-98</sup>, whereas patients with a congenital FXI deficiency appear to be protected from these diseases<sup>99-101</sup>. We showed that inhibiting FXI activity suppressed thrombus propagation without prolonging bleeding time in a rabbit model of arterial and venous thrombosis<sup>102-104</sup>. Other clinical and experimental animal studies have also shown that lowering plasma FXI activity reduces thrombus formation while minimally affecting bleeding time<sup>105, 106</sup>.

These findings together indicate that plasma FXI plays a pivotal role in thrombus propagation and that FXI is a potential therapeutic target for preventing thrombosis without the adverse effect of bleeding.

### Conclusion

Atherosclerotic plaques are in a prothrombotic

state. The downregulation of E-NTPDase as well as nitric oxide and prostaglandin I<sub>2</sub> in plaque endothelial cells promotes platelet activation, and increased podoplanin and S100A13 expression also activates platelets via CLEC-2. Furthermore, high levels of TF are expressed in plaques under conditions of inflammation, oxidative stress and hypoxia that together largely promote thrombus formation at sites of plaque disruption. Intrinsic coagulation factors and changes in blood flow also play critical roles in thrombus propagation, leading to clinical manifestations (Fig. 5).

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## Conflict of Interest

None declared

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