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Pathology and Pathophysiology of Atherothrombosis: Virchow's Triad Revisited

Atsushi Yamashita and Yujiro Asada
*University of Miyazaki,
 Japan*

1. Introduction

In 1856, Rudolf Virchow published “Cellular pathology” based on macroscopic and microscopic observation of diseases, and described a triad of factors on thrombosis. The three components were vascular change, blood flow alteration, and abnormalities of blood constituents. Although Virchow originally referred to venous thrombosis, the theory can also be applied to arterial thrombosis, and it is considered that atherothrombus formation is regulated by the thrombogenicity of exposed plaque contents, local hemorheology, and blood factors. Thrombus formation on a disrupted atherosclerotic plaque is a critical event that leads to atherothrombosis. However, it does not always result in complete thrombotic occlusion with subsequent acute symptomatic events (Sato et al., 2009). Therefore, thrombus growth is also critical to the onset of clinical events. In spite of intensive investigation on the mechanisms of thrombus formation, little is known about the mechanisms involved in thrombogenesis or thrombus growth after plaque disruption, because thrombus is assessed with chemical or physical injury of “normal” arteries in most animal models of thrombosis.

Vascular change is an essential factor of atherothrombosis. Atherothrombosis is initiated by disruption of atherosclerotic plaque. The plaque disruption is morphologically characterized, however, the triggers of plaque disruption have not been completely understood. Tissue factor (TF) is an initiator of the coagulation cascade, is normally expressed in adventitia and variably in the media of normal artery (Drake et al., 1989). Because the atherosclerotic lesion expresses active TF, it is considered that TF in atherosclerotic lesion is a major determinant of vascular wall thrombogenicity (Owens & Mackman, 2010). Therefore, atherosclerotic lesions with TF expression are indispensable for studying atherothrombosis. To examine thrombus formation on TF-expressing atherosclerotic lesions, we established a rabbit model of atherothrombosis (Yamashita et al., 2003, 2009). This allowed us to investigate the “Virchow's triad” on atherothrombosis.

Blood flow is a key modulator of the development of atherosclerosis and thrombus formation. The areas of disturbed flow or low shear stress are susceptible for atherogenesis, whereas areas under steady flow and physiologically high shear stress are resistant to atherogenesis (Malek et al., 1999). The transcription of thrombogenic or anti-thrombogenic genes is also regulated by shear stress (Cunningham & Gotlieb, 2005). The blood flow can be altered by vascular stenosis, acute luminal change after plaque disruption, and microvascular constriction induced by distal embolism (Topol & Yadav, 2003). The blood flow alteration after plaque disruption may affect thrombus formation.

Blood circulates in the vessel as a liquid. This property suddenly changes after plaque disruption. The exposure of matrix proteins and TF induce platelet adhesion, aggregation and activation of coagulation cascade, resulted in platelet-fibrin thrombus formation. Clinical studies revealed increased platelet reactivity, coagulation factors, and reduced fibrinolytic activity in patients with atherothrombosis (Feinbloom & Bauer, 2005), and that risk factors for atherothrombosis can affect these blood factors (Lemkes et al., 2010, Rosito et al., 2004). In addition, recent evidences suggest that white blood cells can influence arterial thrombus formation. It seems that abnormalities on blood factors affect thrombus growth rather than initiation of thrombus formation.

This article focuses on pathology and pathophysiology of coronary atherothrombosis. Because mechanisms of atherothrombus formation are highly complicated, we separately discuss the “Virchow’s triad” on atherothrombogenesis and thrombus growth.

2. Pathology of atherothrombosis

Traditionally, it is considered that arterial thrombi are mainly composed of aggregated platelets because of rapid blood flow condition, and the development of platelet-rich thrombi has been regarded as a cause of atherothrombosis. However, recent evidences indicate that atherothrombi are composed of aggregated platelets and fibrin, along erythrocytes and white blood cells, and constitutively immunopositive for GPIIb/IIIa (a platelet integrin), fibrin, glycophorin A (a membrane protein expressed on erythrocytes), von Willbrand factor (VWF, a blood adhesion molecule). And neutrophils are major white blood cells in coronary atherothrombus (Nishihira et al., 2010, Yamashita et al., 2006a). GPIIb/IIIa colocalized with VWF. TF was closely associated with fibrin (Yamashita et al., 2006a). The findings suggest that VWF and/or TF contribute thrombus growth and obstructive thrombus formation on atherosclerotic lesions, and that the enhanced platelet aggregation and fibrin formation indicate excess thrombin generation mediated by TF.

Overexpression of TF and its procoagulant activity have been found in human atherosclerotic plaque, and TF-expressing cells are identified as macrophages and smooth muscle cells (SMC) in the intima (Wilcox et al., 1989). The TF activity is more prominent in fatty streaks and atheromatous plaque than in the diffuse intimal thickening in aorta (Hatakeyama et al., 1997). Thus, atherosclerotic plaque has a potential to initiate coagulation cascade after plaque disruption, and TF in the plaque is thought to play an important role in thrombus formation after plaque disruption. Interestingly, TF pathway inhibitor (TFPI), a major down regulator of TF-factor VIIa (FVIIa) complex, is also upregulated in atherosclerotic lesions (Crawley et al., 2000). In addition to endothelial cells, macrophages, medial and intimal SMCs express TFPI. These evidence suggest that imbalance between TF and TFPI contribute to vascular wall thrombogenicity.

Two major patterns of plaque disruption are plaque rupture and plaque erosion (Figure 1). Plaque rupture is caused by fibrous cap disruption, allowing blood to come in contact with the thrombogenic necrotized core, resulting in thrombus formation. Ruptured plaque is characterized by disruption of thin fibrous caps, usually less than 65 μm in thickness, rich in macrophages and lymphocytes, and poor in SMCs (Virmani et al., 2000). Thus, the thinning of the fibrous cap is thought to be a state vulnerable to rupture, the so-called thin-cap fibroatheroma (Kolodgie et al., 2001). However, the thin-cap fibroatheroma is not taken into

account in the current American Heart Association classification of atherosclerosis (Stary et al., 1995). Plaque erosion is characterized by a denuded plaque surface and thrombus formation, and defined by the lack of surface disruption of the fibrous cap. Compared with plaque rupture, patients with plaque erosion are younger, no male predominance. Angiographically, there is less narrowing and irregularity of the luminal surface in erosion. The morphologic characteristics include an abundance of SMCs and proteoglycan matrix, especially versican and hyaluronan, and disruption of surface endothelium. Necrotic core is often absent. Plaque erosion contains relatively few macrophages and T cells compared with plaque rupture (Virmani et al., 2000). Thrombotic occlusion is less common with plaque erosion than plaque rupture, whereas microembolization in distal small vessels is more common with plaque erosion than plaque rupture (Schwartz et al., 2009). The proportions of fibrin and platelets differ in coronary thrombi on ruptured and eroded plaques. Thrombi on ruptured plaque are fibrin-rich, but those on eroded plaque are platelet-rich. TF and C reactive protein (CRP) are abundantly present in ruptured plaque, compared with eroded plaques (Sato et al., 2005). These distinct morphologic features suggest the different mechanisms in plaque rupture and erosion.

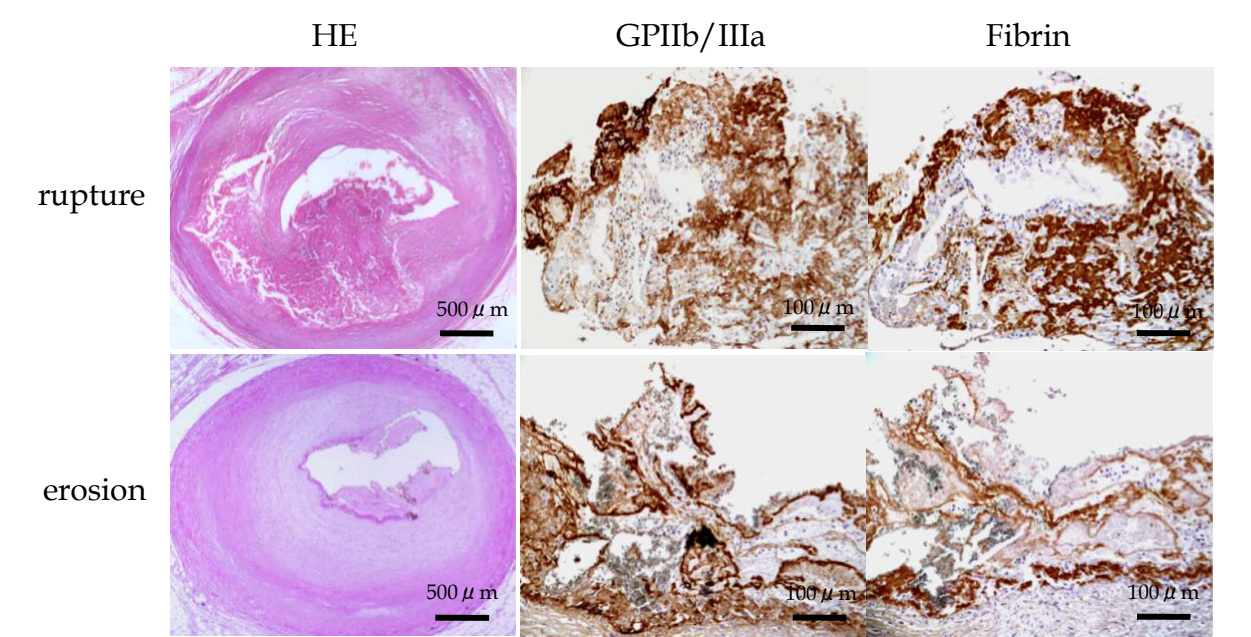


Fig. 1. Human coronary plaque rupture and erosion in patients with acute myocardial infarction.

Large necrotic core and disrupted thin fibrous cap is accompanied by thrombus formation in ruptured plaque. Eroded plaque has superficial injury of SMC-rich atherosclerotic lesion with thrombus formation. Both thrombi comprise platelets and fibrin. HE, Hematoxylin eosin stain (from Sato et al. 2005, with permission).

3. Pathology of asymptomatic atherothrombus

On the other hands, the disruption of atherosclerotic plaque does not always result in complete thrombotic occlusion with subsequent acute symptomatic events. The clinical studies using angioscopy have revealed that multiple plaque rupture is a frequent complication in patients with coronary atherothrombosis (Okada et al., 2011). Healed stages

of plaque disruption are also occasionally observed in autopsy cases with or without coronary atherothrombosis (Burke et al., 2001). To evaluate the incidence and morphological characteristics of thrombi and plaque disruption in patients with non-cardiac death, Sato et al. (2009) examined 102 hearts from non-cardiac death autopsy cases and 19 from those who died of acute myocardial infarction (AMI). They found coronary thrombi in 16% of cases with non-cardiac death, and most of them developed on plaque erosion, and the thrombi were too small to affect coronary lumen (Figure 2, Table 1). The disrupted plaques in non-cardiac death case had smaller lipid areas, thicker fibrous caps, and more modest luminal narrowing than those in cases with AMI. A few autopsy studies have examined the incidence of coronary thrombus in non-cardiac death. Davies et al. (1989) and Arbustini et al. (1993) found 3 (4%) mural thrombi in 69, and 10 (7%) thrombi in 132 autopsy cases with non-cardiac death. The all coronary thrombi from non-cardiac death were associated with plaque erosion (Arbustini et al., 1993). Although the precise mechanisms of plaque erosion remain unknown, it is possible that the superficial erosive injury is a common mechanism of coronary thrombus formation. The results suggest that plaque disruption does not always result in complete thrombotic occlusion with subsequent acute symptomatic events, that thrombus growth is critical step for the onset of clinical events, and that at least the regional factors influence the size of coronary thrombus after plaque disruption.

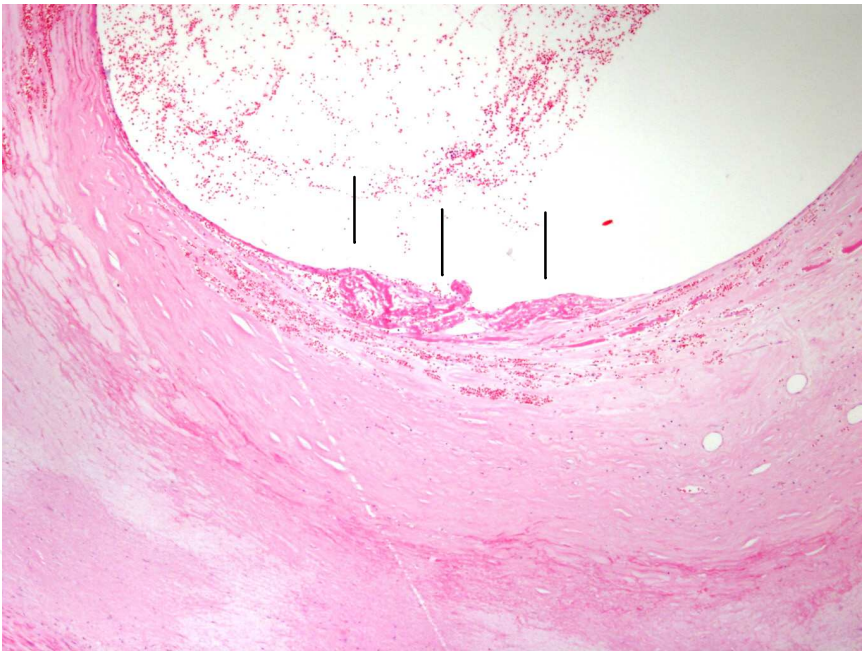


Fig. 2. Human coronary plaque erosion in patient with non-cardiac death.

	Non-cardiac death (n=102)	Acute myocardial infarction (n=19)	P value
Fresh thrombus	10 (10%)	14 (74%)	<0.001
erosion	7 (7%)	4 (21%)	0.07
rupture	3 (3%)	10 (53%)	<0.001
Old thrombus	6 (6%)	5 (26%)	<0.05

(From Sato et al. 2009, with permission)

Table 1. Incidence of thrombosis in non-cardiac death and acute myocardial infarction.

The atherosclerotic lesion shows superficial erosive injury with mural thrombus (arrows). The thrombus is too small to obstruct coronary lumen and induce symptomatic event (hematoxyline eosin stain, from Sato et al. 2009, with permission).

4. Pathophysiology of atherothrombosis

4.1 Triggers on plaque disruption

As described above, atherothrombosis is initiated by plaque rupture or plaque erosion. The plaque disruption is probably affected by vascular wall change and local blood flow. Our recent study revealed that disturbed blood flow could trigger plaque erosion in rabbit femoral artery with SMC-rich plaque. We separately discuss possible factors that affect plaque rupture or plaque erosion in atherosclerotic vessels.

4.1.1 Vascular change in plaque rupture

The thinning and disruption of fibrous cap by metalloproteases together with local rheological forces and emotional status is likely to be involved in plaque rupture. Accumulating evidence supports a key role for inflammation in the pathogenesis of plaque rupture. The inflammatory cells that appear quite numerous in rupture-prone atherosclerotic plaques can produce enzymes degrading the extracellular matrix of the fibrous cap. Macrophages in human atheroma overexpress interstitial collagenases and gelatinases, and elastolytic enzymes. Activated T lymphocytes and macrophages can secrete interferon γ (INF- γ), which inhibits collagen synthesis and induces apoptotic death of SMC (Shah, 2003). Moreover, INF- γ can induce interleukine (IL)-18, an accelerator of inflammation. IL-18 is colocalized with INF- γ in macrophage located at shoulder region, but not at necrotic core, and is associated with coronary thrombus formation in patients with ischemic heart disease (Nishihira et al., 2007). IL-10, an important anti-inflammatory cytokine, also is upregulated in macrophage in atherosclerotic lesion from patients with unstable angina compared with stable angina (Nishihira et al., 2006b). Heterogeneity of macrophages in atherosclerotic plaque could explain the paradoxical findings (Waldo et al., 2008). These evidences indicate that the imbalance of inflammatory pathway appear to participate in the destabilization of the plaque that triggers thrombosis in fibrous cap rupture.

Other possible trigger of plaque rupture is intraplaque hemorrhage. The frequency of previous hemorrhages is greater in coronary atherosclerotic lesions with late necrosis and thin fibrous cap than those lesions with early necrosis or intimal thickening (Kolodgie et al., 2003). Plaque hemorrhage is present in majority (>75%) of acute ruptures, and in 40% of fibrous cap and thin-fibrous cap atheromas. In addition, intraplaque hemorrhage is more frequently seen in patients with AMI compared to patients with healed myocardial infarction or non-cardiac death (Virmani et al., 2003). In coronary culprit lesions obtained by directional coronary atherectomy, intraplaque hemorrhage and iron deposition were more prominent in patients with unstable angina pectoris than with stable angina pectoris. The iron deposition correlated with oxidized low density lipoprotein and thioredoxin, an anti-oxidant protein, and was also associated with thrombus formation (Nishihira et al., 2008b). The pathological findings imply a possible relationship among intraplaque hemorrhage, oxidative stress, and plaque instability. However, the direct evidence that links intraplaque hemorrhage to plaque instability is still lacking.

4.1.2 Blood flow-induced mechanical stress on plaque rupture

Blood flow-induced mechanical stress is an essential factor of development of atherosclerosis and atherothrombosis. The low shear stress and oscillatory shear stress are both important stimuli for induction of atherosclerosis. Using a perivascular shear stress modifier in mice, Cheng et al. (2006) revealed that low shear stress induces larger lesions with vulnerable plaque phenotype (more lipids, more proteolytic enzymes, less SMCs, and less collagen) whereas vortices with oscillatory shear stress induce stable lesions. Chatzizisis et al. (2011) reported development of thin fibrous cap atheroma in coronary artery with low shear stress in pigs. In addition, the shear stress-induced changes in atherosclerotic plaque composition are modulated by chemokines. Inhibition of fractalkine, which is exclusively expressed in the low shear stress-induced atherosclerotic plaque, was reduced lipid and macrophage accumulation in the brachiocephalic arteries in mice (Cheng et al., 2007). Therefore, lower shear stress can induce atherosclerotic lesion prone to plaque rupture. Although it is well recognized that a mechanical stress triggers the disruption of fibrous cap, it remains unclear which factor is mainly responsible for the disruption of the thin fibrous cap. A variety of mechanical factors have been postulated to play a role in plaque rupture, including hemodynamic shear stress, turbulent pressure fluctuation (Loree et al., 1991), sudden increases in intraluminal pressure (Muller et al., 1989), and tensile stress concentration within the wall of the lesion. To investigate the relationship between shear stress distribution and coronary plaque rupture, Fukumoto et al. (2008) analyzed 3-dimensional intravascular ultrasound images in patients with acute coronary thrombosis by a program for calculating the fluid dynamics. The ruptured sites were located in the proximal or top portion of the plaques, and the localized high shear stress is frequently correlated with the rupture sites. This finding is inconsistent with role of low shear stress on atherogenesis. It is possible that the process of initiating plaque rupture is quite different from that of atherogenesis. On the other hand, an excessive concentration of tensile stress within the plaque may be one of the triggers of plaque rupture. When the tensile stress becomes greater than the fragility of the fibrous cap, a plaque disruption may be initiated. The tensile stress is increased by development of a lipid core, thinning of the fibrous cap (Loree et al., 1992). Cheng et al. (1993) analyzed the distribution of circumferential stress in human coronary arteries. The maximum circumferential stress in ruptured plaques was significantly higher than that in stable plaques, although plaque rupture does not always occur at the region of highest stress. These results suggest that a mechanical factor that triggers plaque rupture differ in each case and lesion.

4.1.3 Disturbed blood flow on plaque erosion

Although it has been postulated that erosions result from coronary vasospasm of SMC-rich plaque, the mechanisms of plaque erosion are poorly understood. Approximately 80% thrombi of plaque erosion are nonocclusive in spite of sudden coronary death (Virmani et al., 2000). Platelet rich emboli are found in 74% of patients dying suddenly with plaque erosion compared with plaque rupture (40%). Because activated platelets release vasoconstrictive agents, such as 5-hydroxytryptamine (5-HT, serotonin) and thromboxane A₂, these emboli might increase peripheral resistance leading to alteration of coronary blood flow. 5-HT can induce vasoconstriction and reduce coronary blood flow in human atherosclerotic vessels but not in normal arteries (Golino et al., 1991).

Experimental aortic stenosis can induce acute endothelial change or damage of the normal aorta (Fry, 1968). Therefore, hemodynamic force, particularly disturbed blood flow induced by stenosis or vasoconstriction, could be a crucial factor in generating surface vascular damage and thrombosis. To address the relation between disturbed blood flow and plaque erosion, we investigated the pathological change after acute luminal narrowing in SMC-rich plaque in rabbit. The SMC-rich plaque was induced by a balloon injury of rabbit femoral artery, and expressed TF as human atherosclerotic plaques. Actually, the disturbed blood by acute vascular narrowing induced superficial erosive injury to the SMC-rich plaque at post stenotic regions in rabbit femoral arteries. Figure 3 shows microscopic images of the longitudinal section of the neointima at the post-stenotic region 15 min after vascular narrowing. The endothelial cells and SMCs at this region were broadly detached with time, and associated with platelet adhesion to the sub-endothelium. Apoptosis of endothelial cells

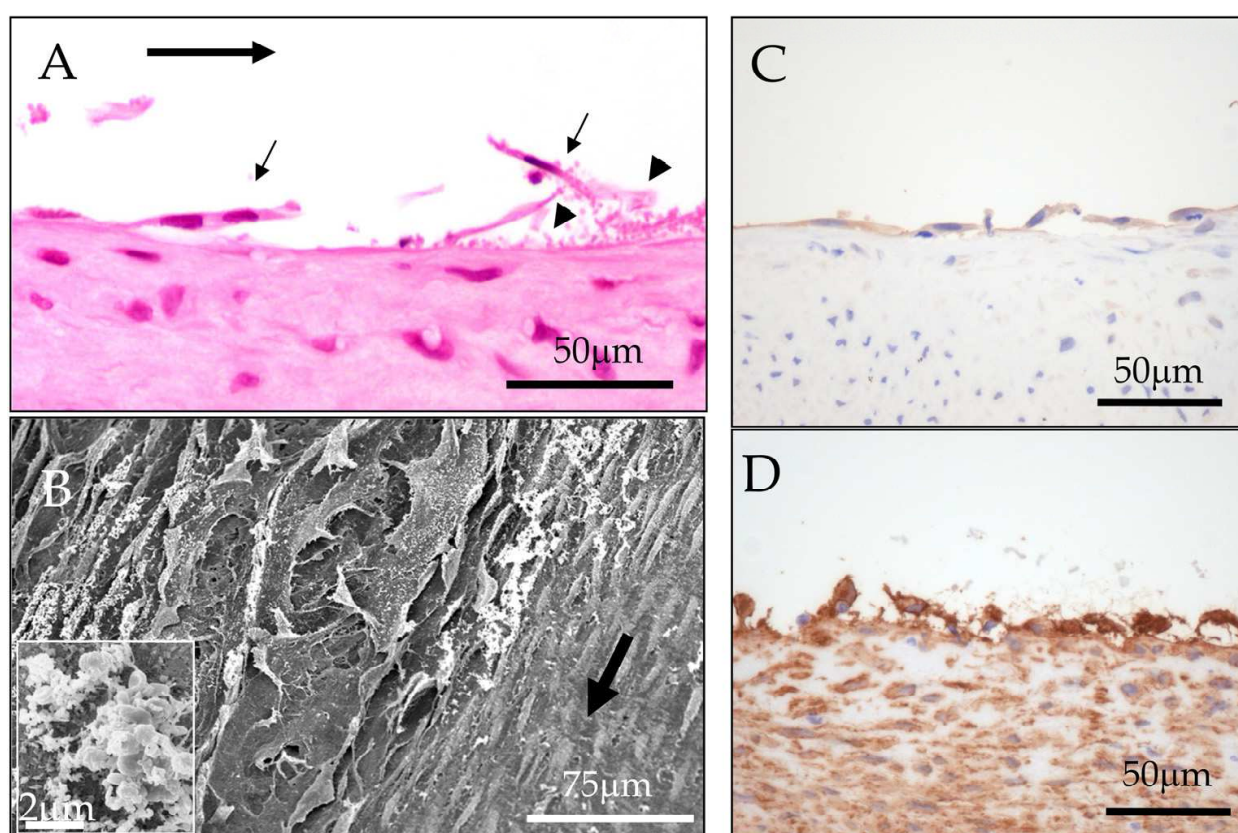


Fig. 3. Representative images of superficial erosive injury of SMC-rich plaque and thrombus formation at the post-stenotic region.

SMC-rich plaque 15 min after vascular narrowing shows endothelial detachment (small arrows) accompanies platelet adhesion (arrow heads) at 1mm from vascular narrowing (A, hematoxyline eosin stain). Detachment of endothelial cells and exposure of subendothelial matrix is accompanied by platelet aggregation on the left side, and residual endothelial cell layer is present on right side (inset, high magnification of aggregated platelets) (B, scanning electron microscopy). Immunohistochemistry for VWF (C, a marker of endothelium) or smooth muscle actin (D, a marker of SMC) confirm detachment of endothelial cells and SMCs at post stenotic region (from Sumi et al. 2010, with permission).

and superficial SMCs was also observed at the post-stenotic region within 15 minutes (Sumi et al., 2010). The vascular narrowing induced large mural thrombi which composed of platelets and fibrin, as human plaque erosion. Thus, disturbed blood flow can induce superficial erosive injury to SMC-rich plaque and thrombus formation at post-stenotic region. Computational fluid simulation analysis indicated that oscillatory shear stress contributes to the development of the erosive damage to the plaque (Sumi et al., 2010). Although direct clinical evidence has not yet supported the notion that coronary artery vasospasm plays a role in plaque erosion, the superficial erosive injury of SMC-rich plaque by disturbed blood flow is similar to those of human plaque erosion (Sato et al., 2005). And, platelet and blood coagulation in coronary circulation are activated after vasospastic angina (Miyamoto et al. 2001, Oshima et al., 1990). Therefore, these evidence suggest that an acute-onset disturbed blood flow due to vasoconstriction could trigger plaque erosion. Hemodynamic factors could play an important role in development of plaque erosion.

4.2 Virchow's triads on thrombus growth

As described above, plaque disruption does not always result in complete thrombotic occlusion. Thrombus growth is considered critical to the onset of clinical events. Although thrombus formation is regulated by the vascular wall thrombogenicity, local blood flow, and blood contents, their contribution to thrombus growth has not been clearly defined. We separately discuss three factors that affect thrombus growth in atherosclerotic vessels.

4.2.1 Vascular factors on thrombus growth

Most fundamental difference between normal artery and atherosclerotic artery is presence of abundant active TF in atherosclerotic lesions (Hatakeyama et al., 1997, Wilcox et al., 1989). It seems that vascular wall TF contribute to thrombus size/propagation on atherosclerotic lesions. However, recent studies indicate that a small amount of TF is detectable in the blood and is capable of supporting clot formation *in vitro*. Plasma TF levels are elevated in patients with unstable angina and AMI and correlate with adverse outcomes (Mackman, 2004). Therefore, it is still controversial whether vascular wall and/or blood-derived TF support thrombus propagation. Hematopoietic cell-derived, TF-positive microparticles contributed to laser injury-induced thrombosis in the microvasculature of mouse cremaster muscle (Chou et al. 2004). In contrast, vascular smooth muscle-derived TF contributed to FeCl₃ induced thrombosis in mouse carotid artery (Wang et al., 2009). We investigated whether plaque and/or blood TF contribute to thrombus formation in rabbit femoral artery with or without atherosclerotic lesions. The atherosclerotic lesions in rabbit femoral arteries were induced by a 0.5% cholesterol diet and balloon injury, and showed TF expression and increased procoagulant activity compared with normal femoral arteries (Figure 4). Balloon injury of the atherosclerotic plaque induced thrombin-dependent large platelet-fibrin thrombi. In contrast, balloon injury of normal femoral artery induced thrombin-independent small platelet thrombi (Figure 5). Moreover, whole blood coagulation in the rabbits was not affected by blood TF inhibition with a TF antibody even in hyperlipidemic condition (Yamashita et al., 2009). Therefore, at least, atherosclerotic plaque-derived TF might contribute to activation of intravascular coagulation cascade and thrombus size/propagation on atherosclerotic lesions.

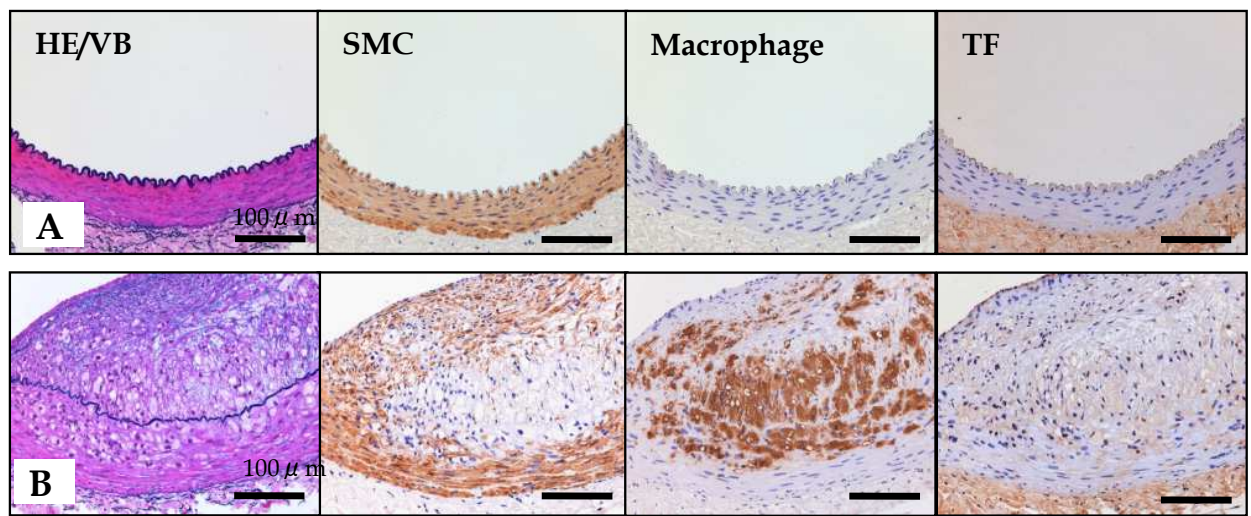


Fig. 4. Histological images of rabbit femoral arteries. Representative immunohistochemical microphotographs of normal (A) and balloon-injured femoral artery at 3 weeks after injury under 0.5% cholesterol diet (B). Atherosclerotic lesion composed of SMCs and macrophages develops in injured artery. TF expression is present in the lesion and adventitia of both arteries. HE/VB, hematoxyline eosin/Victoria blue stain (From Yamashita et al. 2009, with permission).

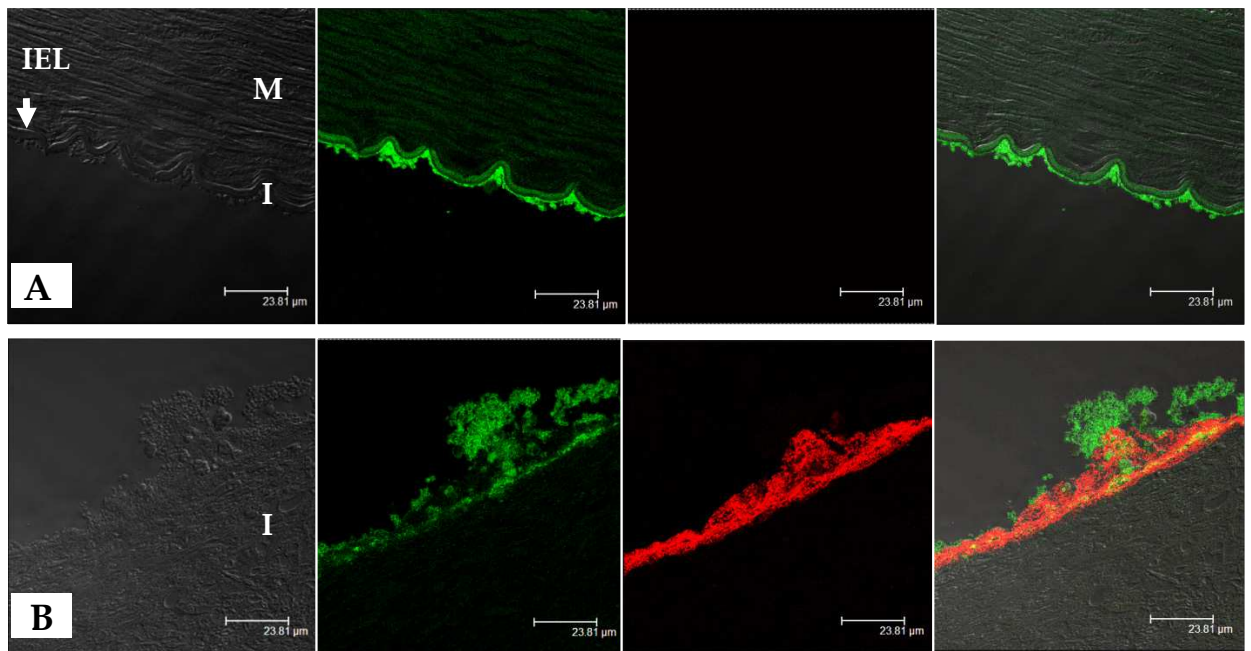


Fig. 5. Immunofluorescence images of thrombus on rabbit femoral artery. Representative immunofluorescent microphotographs of thrombi 15 minutes after balloon injury of normal femoral artery and of atherosclerotic plaque under 0.5% cholesterol diet. Rows show differential interference contrast images, images stained with fluorescein isothiocyanate-labeled GPIIb/IIIa (green), Cy3-labeled fibrin (red), and merged immunofluorescent images. Areas with colocalized factors are stained yellow. The thrombi on normal intima is composed of small aggregated platelet (A), while the thrombi on atherosclerotic plaque is large, and composed of platelet and fibrin (B). I, intima; M, media; IEL, internal elastic lamina. (From Yamashita et al. 2009, with permission).

Several factors can influence TF expression in plaques and atherothrombus formation after plaque disruption. CRP is an inflammatory acute-phase reactant that has emerged as a powerful predictor of cardiovascular disease (Ridker, 2007). CRP is localized in atherosclerotic plaques and is more in thrombotic plaques than non-thrombotic ones (Ishikawa et al., 2003, Sun et al., 2005). The findings imply that CRP is implicated in atherothrombogenesis. To address this issue, CRP-transgenic rabbits were generated, because as human CRP, CRP in rabbits but not in mice works as an acute-phase reactant during inflammation (Koike et al., 2009). In the rabbits, CRP was overexpressed in livers and circulated in blood and deposited in the both SMC-rich and macrophage-rich atherosclerotic lesions. The thrombus size on SMC-rich plaque or macrophage-rich plaque after balloon injury was significantly increased in CRP-transgenic rabbits as compared with wild non-transgenic rabbits (Figure 6). TF expression and its activity in the plaques were significantly increased in CRP-transgenic rabbits. The degree of CRP deposition correlated with TF expression in plaques and thrombus size on injured plaques (Matsuda et al., 2011). On the

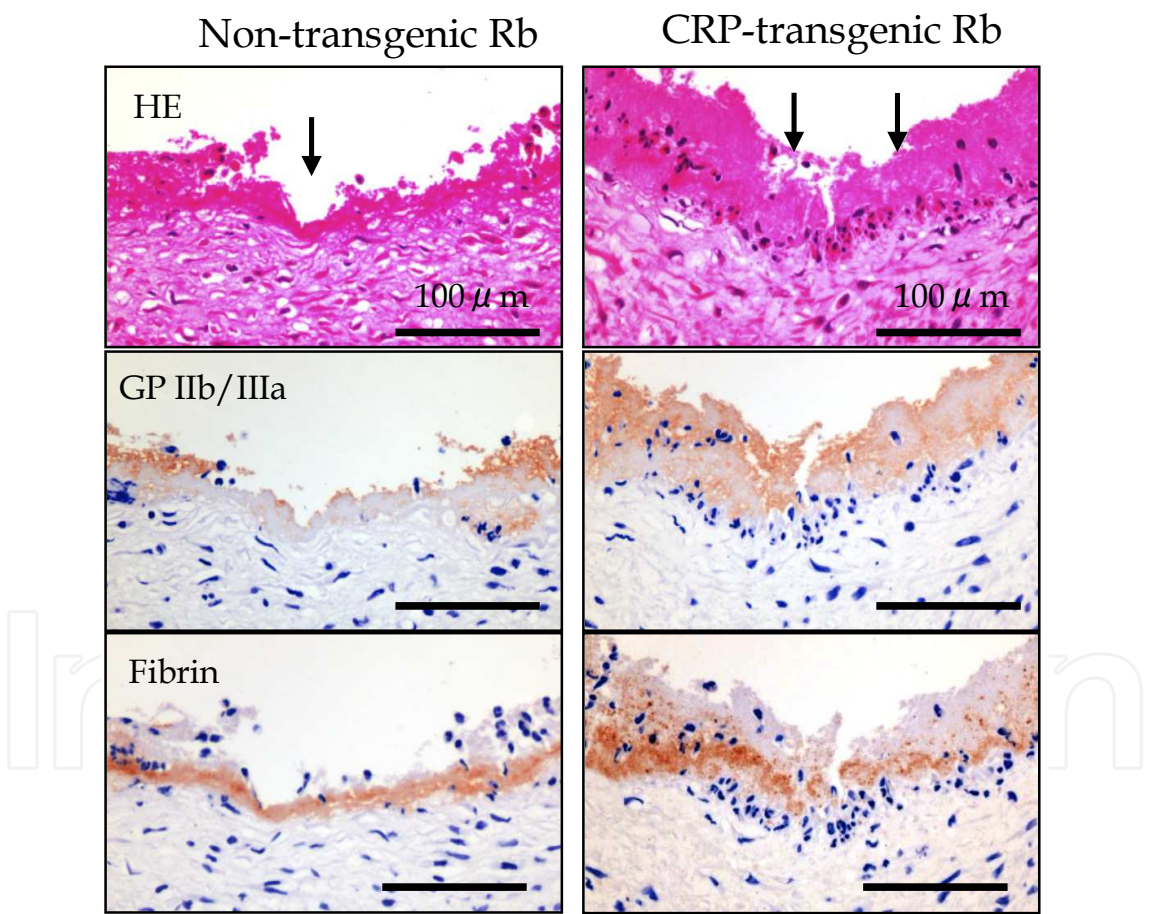


Fig. 6. Thrombus formation on SMC-rich plaque in CRP-transgenic or non-transgenic rabbit femoral artery. The images show thrombus formation on SMC-rich plaque (arrows) 15 min after balloon injury of rabbit femoral arteries. The thrombus size is increased in CRP-transgenic rabbits as compared with non-transgenic rabbit. Immunopositive areas for GPIIb/IIIa and fibrin also increase in CRP-transgenic rabbit (from Matsuda et al. 2011, with permission).

other hand, the CRP overexpression did not enhance atherosclerosis induced by hypercholesterol diets (Koike et al., 2009). CRP localized in atherosclerotic plaques might enhance vascular wall thrombogenicity and thrombus formation after plaque disruption rather than atherogenesis.

4.2.2 Altered blood flow on thrombus growth

Blood flow is a key modulator of thrombus growth. Clinical studies revealed an alteration of coronary blood flow in patients with ischemic heart diseases. Marzilli et al. (2000) reported an approximate 80% reduction in coronary blood flow during ischemia in patients with unstable angina. An autopsy study reported that intramyocardial microemboli were frequently present in sudden coronary death patients (Schwartz et al. 2009). Distal microvascular embolism and/or vasoconstriction could affect blood flow alteration and thrombus formation and growth at the culprit lesions (Erbel & Heusch, 2000). To assess the issue, we examined the effects of the blood flow reduction to thrombus formation in our animal model. Blood flow reduction (>75%) promoted the growth of thrombus, a mixture of platelets and fibrin, on atherosclerotic lesion, which grew to occlusive one. The flow reduction also induced thrombus formation on normal arteries, but the thrombi were very small and composed only of platelets (Yamashita et al. 2004). Therefore, blood flow reduction associated with increased vascular wall thrombogenicity is considered to contribute thrombus growth. We also demonstrated an important role of 5-HT_{2A} receptor on platelets and SMCs in this process via platelet aggregation and thrombogenic vasoconstriction (Nishihira et al., 2006a, 2008a).

In addition to distal vascular resistance, disturbed blood flow by acute vascular narrowing promotes thrombus growth at post stenotic regions. As described above, vascular narrowing of rabbit femoral artery induced superficial erosive injury to SMC-rich plaque at post stenotic regions. The thrombi consisted of a mixture of aggregated platelets and a considerable amount of fibrin. The whole blood hemostatic parameters in the rabbits was not changed after vascular narrowing or anti-rabbit TF antibody treatment, which evidence indicates that TF derived from eroded plaque rather than circulating TF plays an important role in fibrin generation and thrombus growth (Sumi et al. 2010).

The rheological effect on thrombus growth may be partly explained by a shear gradient-dependent platelet aggregation mechanism. Using in vitro and in vivo stenotic microvessels and imaging systems, Nesbitt et al. (2009) revealed a shear gradient-dependent platelet aggregation process which is preceded by soluble agonist-dependent aggregation. Shear microgradient at post stenosis region or down stream face of thrombi induced stable platelets aggregates, and the shear microgradients directly influenced the platelet aggregation size. This process required ligand binding to integrin $\alpha\text{IIb}\beta 3$, transient Ca^{2+} flux, but did not required global platelet shape change or soluble agonists. The findings suggest that platelets principally use a biomechanical platelet aggregation mechanism in early phase of platelet adhesion and aggregation. Vessel and/or thrombus geometry itself may promote thrombus formation.

4.2.3 Blood factors on thrombus growth

As described above, platelet is a major cellular component in coronary thrombus, and platelets play an important role in growing phase of thrombus formation, as well as initial

phase of thrombus formation. Adhesion molecules and its receptors on platelets are essential for thrombus formation, because these molecules support platelet tethering, firm adhesion, aggregation and platelet recruitment to thrombus surface. VWF is a large, multimeric, plasma protein that undergoes a conformational change when bound to matrix under permit its binding to GPIIb/IIIa. Recent studies in vitro and in vivo showed that platelet recruitment on thrombus surface was primary mediated by VWF and GPIIb/IIIa on flowing platelets (Bergmeier et al. 2006, Kulkarni et al. 2000). We demonstrated that a large amount of VWF was localized in coronary thrombi in patients with AMI (Nishihira et al., 2010, Yamashita et al., 2006a), and that monoclonal antibody against VWF A1 domain, which interacts platelet GPIIb/IIIa, significantly suppressed formation of platelet-fibrin thrombi and completely inhibited occlusive thrombus formation in rabbit atherosclerotic lesions (Yamashita et al., 2003, 2004). These findings indicated a crucial role of VWF in thrombus growth via platelet recruitment. The multimer size of VWF can affect thrombus size and is regulated by a plasma protease, a disintegrin and metalloprotease with a thrombospondin type 1 motif 13 (ADAMTS-13). A deficiency of ADAMTS-13 activity causes an increased level of circulating ultralarge VWF multimers, and correlates with the onset of the general thrombotic disease, thrombotic thrombocytopenic purpura (TTP). A clinical evidence suggested dysregulation of VWF multimer size in AMI patient. The ratio of VWF/ADAMTS-13 antigen was higher in patients with AMI than in those with stable angina pectoris, and there was an inverse correlation between plasma VWF antigen and ADAMTS-13 activity in AMI patients (Kaikita et al. 2006). The ADAMTS-13 closely localized with VWF in fresh coronary thrombi from AMI patients (Moriguchi-Goto et al., 2009). A reducing ADAMTS-13 activity by monoclonal antibody against disintegrin-like domain enhanced platelet thrombus growth on immobilized type I collagen at a high shear rate (1500S^{-1}) and platelet-fibrin thrombus formation on injured atherosclerotic lesion of rabbit femoral arteries (Moriguchi-Goto et al., 2009). The study also showed cleavage of large sized VWF multimer during platelet thrombus formation under a high shear rate. The VWF-cleaving site by ADAMTS-13 localized on the surface of platelet thrombus, and the ADAMTS-13 activity was shear dependent manner (Shida et al. 2008). Thus, ADAMTS-13 may work at the site of ongoing thrombus generation and limit thrombus growth.

The recent studies in vitro showed various blood cells, not only monocytes but also neutrophils, eosinophils, and even if platelets, can synthesize TF. Although there is much on debate on the TF expression in blood cells, it is likely that monocytes are the only blood cells that synthesize and express TF (Østerud, 2010). A related topic is contribution of microparticles (MPs) to thrombus formation. MPs are small fragments of membrane-bound cytoplasm that are shed from the surface of an activated or apoptotic cells (Blann et al. 2009). The procoagulant activity of MPs is increased with the exposure of phosphatidylserine and the presence of TF. In fact, MPs have significantly elevated in acute coronary syndrome and ischemic strokes (Geiser et al. 1998, Singh et al. 1995). However, it is still unclear whether the elevated levels of MPs are a cause or consequence of atherothrombosis. Moreover, our animal studies did not support the role of blood-derived TF in atherothrombus formation as described above. Future studies are required to clarify contribution of blood derived TF and/or MPs to thrombus propagation on atherosclerotic lesions.

Among the white blood cells, neutrophils are mostly found in coronary thrombus in patients with AMI, and CD34 positive leukocytes are also found in the thrombus (Nishihira et al., 2010). Recent evidences revealed neutrophils and endothelial progenitor cells influence thrombus growth. Neutrophils can positively or negatively affect thrombus

formation by degradation of coagulation or fibrinolysis factors and promoting platelet function (Kornecki et al., 1988, Moir et al., 2002). Inhibition of interaction between p-selectin and p-selectin glycoprotein ligand 1 reduced fibrin formation in vivo (Palabrica et al., 1992). These adhesion molecules have been implicated in recruitment of leukocytes and leukocyte MPs to thrombi (Vandendries et al., 2004). To reveal the neutrophil-mediated procoagulant mechanisms, Massberg et al. (2010) investigated thrombus formation using neutrophil elastase and cathepsin G deficient mice. Proteolysis of TFPI by these proteases enhanced fibrin and thrombus formation after FeCl₃-induced vessel injury. In addition, activated platelets by collagen accelerated nucleosome externalization by neutrophils. The neutrophil-derived externalized nucleosomes can form neutrophil extracellular traps that provide a scaffold for platelets and red blood cells and histone 3/4 can induce platelet aggregation (Fuchs et al., 2010). On the other hands, neutrophil elastase has fibrinolytic potential, and there is significant correlation between neutrophil elastase-digested fibrin and leukocyte content in human atherothrombi (Rábai et al., 2010). Zeng et al. (2002) investigated contribution of polymorphonuclear leukocytes (PMNs) to fibrinolysis in vivo using plasminogen deficient mice. The PMNs accumulated within the thrombi by 6 hours after FeCl₃-induced vessel injury and peaked at 24 hours. There were no significant differences between the PMNs from plasminogen deficient mice and wild type mice within the 6 hour after thrombus formation, whereas there was significant greater retention of PMNs within the thrombus over 24 hours after thrombus formation. PMNs from both mice showed fibrinolytic activity, but the degradation products were a distinct pattern. Therefore, it is possible that neutrophils works as positive or negative regulator of early or late phase of thrombus formation, respectively.

Endothelial progenitor cells (EPC) contributes to angiogenesis and wound healing (Asahara et al., 1997), and the number of EPCs in blood is associated with cardiovascular risk (Hill et al., 2003). The mechanisms that regulate mobilization, migration, and differentiation of EPCs and their homing to sites of vascular injury are complex and involve several mediators and receptors, such as P-selectin glycoprotein ligand-1, CXC chemokine, and integrins (Chavakis et al., 2005, Massberg et al., 2006). Interaction of thrombus contents and EPCs influences their mobilization and differentiation to mature endothelial cells during vascular injury (de Boer HC et al., 2006). Abou-Saleh et al. (2009) reported that human peripheral blood mononuclear cell derived EPCs bound platelets via p-selectin and inhibit platelet activation, aggregation, and adhesion to collagen in vitro, and that injection of these EPCs reduced thrombus formation after FeCl₃-induced vessel injury of mouse carotid arteries.

Other possible mechanism contributing thrombus propagation in vivo is intrinsic coagulation pathway. The intrinsic coagulation pathway is initiated when coagulation factor XII (FXII) comes into contact with negatively charged surfaces in a reaction involving the plasma proteins, high molecular mass kininogen and plasma kallikrein. Factor XI (FXI) is activated by activated FXII, thrombin, and activated XI. Feedback activation of FXI by thrombin promotes further thrombin generation in vitro (Gailani & Broze, 1991). FXI was present in platelet-fibrin thrombus induced balloon injury of atherosclerotic lesion in rabbits, and anti-FXI antibody reduced thrombus growth without prolonging bleeding (Yamashita et al., 2006b). FXI plays an important role in thrombus growth via further thrombin generation. On the other hand, there are conflicts of evidence that FXII supports arterial thrombus growth. FXII deficient mice were resistant to thrombotic occlusion after FeCl₃ induced vessel injury of carotid arteries (Cheng Q et al., 2010). However, a clinical study demonstrated an inverse relationship between FXII level and risk of myocardial

infarction (Doggen et al., 2006). Moreover, inhibition of FXII did not change platelet aggregation and fibrin formation on atherosclerotic plaque surface under flow in vitro. The effect of FXII on coagulation became obvious only in the absence of TF (Reininger et al., 2010).

5. Conclusion

More than 150 years ago, Virchow described the mechanisms of thrombus formation. It has still remained as a fundamental theory of thrombus formation. To date, pathological and experimental studies have clarified the mechanisms of atherothrombus formation. The thrombus formation is initiated by plaque rupture and plaque erosion. Among the Virchow's triad, vascular and rheological factors are responsible for plaque rupture. Disruption of thin fibrous cap atheroma triggers plaque rupture. On the other hand, disturbed blood by acute luminal change can trigger plaque erosion to SMC-rich plaque. Pathological findings of human atherothrombosis suggest that thrombus growth rather than plaque disruption is a critical step for the onset of cardiovascular events, and that simultaneous activation of coagulation cascade and platelets play an important role in thrombus formation after plaque disruption. All three factors contribute to atherothrombus growth. Our rabbit model of atherothrombosis revealed that excess thrombin generation mediated by plaque TF contribute to large plate-fibrin thrombus formation on atherosclerotic lesion, and that disturbed flow condition after plaque disruption promotes thrombus growth. Recent evidence suggests that leukocytes influence arterial thrombus formation as well as platelet and coagulation/fibrinolysis factors. Differences between hemostasis and thrombus growth may shed light on a novel anti-atherothrombotic drug with a wide safety margin.

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7. References

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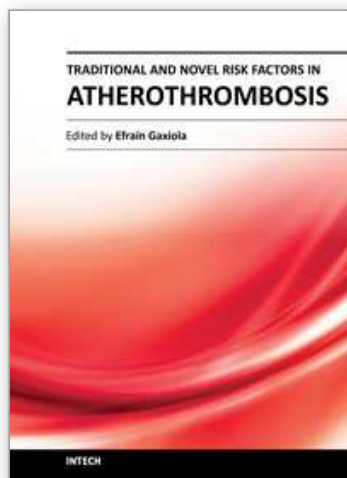
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Traditional and Novel Risk Factors in Atherothrombosis

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Atherothrombosis has reached pandemic proportions worldwide. It is the underlying condition that results in events leading to myocardial infarction, ischemic stroke and vascular death. As such, it is the leading cause of death worldwide manifested mainly as cardiovascular/cerebrovascular death. The complex and intimate relationship between atherothrombosis and traditional and novel risk factors is discussed in the following chapters of Traditional and Novel Risk Factors in Atherothrombosis - from basic science to clinical and therapeutic concerns. Beginning with pathology and pathophysiology of atherothrombosis, plaque rupture/disruption, this book continues with molecular, biochemical, inflammatory, cellular aspects and finally analyzes several aspects of clinical pharmacology.

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University Campus STeP Ri
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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