Since the mechanism of platelet involvement in the formation of thrombi has been considered to be similar to that in platelet aggregation, the mechanism of platelet aggregation was extensively studied at the molecular level by conventional aggregometry, in order to develop effective antiplatelet agents [6]. Investigators clarified that the conformation of one of the platelet surface glycoproteins (GP), namely GP IIb/IIIa (integrin $\alpha_{IIb}\beta_3$), changed after platelet activation by chemical agonists increasing its affinity to fibrinogen [3, 4]. Since fibrinogen has at least two binding sites for GP IIb/IIIa, activated platelets could bind together sharing the same fibrinogen molecule (Fig. 1). The investigators also demonstrated that another kind of platelet aggregation, mediated by binding of von Willebrand factor (VWF) to the platelet receptor protein GP Ibα, could also be detected by the turbidimetric platelet aggregation assay system using specific modulators such as ristocetin or botrocetin instead of the conventional platelet-activating agents [7, 8]. This turbidimetric platelet aggregometry method was widely used as a platelet function test, because it was relatively easy to perform, and reliably assessed the bleeding tendency in congenital disorders, including Glanzman's thromboasthenia, afibrinogenemia, von Willebrand disease and Bernard-Soulier syndrome [3, 4, 8].

Since turbidimetric platelet aggregometry was so widely utilized and was established as a common platelet function test, it is not surprising that the assay system was also used for the development of antiplatelet agents intended for the prevention of thrombotic diseases. This is based on the concept that the mechanism involved in platelet aggregation also represented that underlying platelet thrombus formation *in vivo*. However, there are no data demonstrating the relationship between the extent of platelet aggregation and the onset of thrombotic diseases [6, 9]. Indeed, until very recently, it was accepted without question that the traditional antiplatelet agent aspirin exerted its antiplatelet effects by inhibiting arachidonic-acidic and collagen-induced platelet aggregation [10, 11]. The same was also true for the thienopyridine anti-platelet agents ticlopidine and clopidogrel, in that they were considered to exert their antiplatelet effects by blocking ADP-induced platelet aggregation [12, 13]. Until now, strong anti-platelet-aggregation agents were expected to be potent antithrombotic agents. Based on this concept, anti-GP IIb/IIIa agents, which can inhibit platelet aggregation completely by blocking fibrinogen binding to the activated GP IIb/IIIa, were developed as antithrombotic agents [6, 9]. Clinical experiences with these specific anti-GP IIb/IIIa agents that could potently inhibit platelet aggregation in cases with acute coronary syndromes [14, 15] along with recent advances in the understanding of the mechanism of platelet thrombus formation *in vivo* [2, 16, 17] have led to questioning of the initial assumption that the mechanism of platelet aggregation represents that underlying platelet thrombus formation *in vivo*. Indeed, strong inhibition of platelet aggregation by anti-GP IIb/IIIa agents, which was expected to produce potent preventive effects on arterial thrombosis, was shown to be effective only in limited clinical situations [14, 15, 18, 19]. Plasma ligand binding to the activated GP IIb/IIIa is the final common pathway for platelet aggregation. However, antiplatelet agents that blocked this interaction were shown not to be sufficiently potent in the prevention of acute coronary syndromes [14, 15], suggesting that assays of platelet aggregation alone can not predict the onset of atherothrombotic diseases, such as acute coronary syndromes.

WHAT IS THE DIFFERENCE BETWEEN PLATELET AGGREGATION AND PLATELET THROMBUS FORMATION *IN VIVO*?

As shown in (Fig. 1), platelet aggregation in conventional aggregometry after platelet activation by agonists, such as ADP and thrombin, was exclusively mediated by the binding of fibrinogen to the activated GP IIb/IIIa. However, the conditions necessary for platelet aggregation are substantially different from those necessary for initiation of platelet thrombus formation *in vivo* (Table 1). One of the most important differences is that platelet thrombus formation *in vivo* is initiated by endothelial injury, such as that occurring after atheroma rupture [1, 20, 21], and not by platelet activation by agonists which is the case with aggregometry (Fig. 2). The roles played by soluble agonists such as ADP and thrombin, although still important [22-24], seem to be relatively smaller *in vivo* than in aggregometry, perhaps because a platelet thrombus *in vivo* is initiated in the presence of arterial blood flow which would immediately dilute the soluble agonists. In platelet thrombus formation occurring on the exposed subendothelial matrix under blood flow conditions, platelet activation is initiated by an interaction of platelets with the exposed subendothelial matrix constituents, such as collagen [25, 26], and immobilized plasma proteins, such as VWF [27, 28]. Soluble activating agents, although they may play important roles in stabilizing platelet thrombi, play a relatively limited role in platelet activation *in vivo*. It is important to note that the necessary interactions for the initiation of platelet thrombus formation *in vivo*, which cannot be assessed by conventional aggregometry, such as platelet interactions with

Table 1. Difference between Platelet Aggregation and Platelet Thrombosis *In Vivo*

	Platelet Aggregation	Platelet Thrombosis *In Vivo*
Blood flow	No significant blood flow	Presence of arterial blood flow
Vessel wall injury	Not involved	Thrombus formation was initiated by vessel wall injury
Soluble agonists	ADP, thrombin, epinephrine, etc.	Not directly involved
Other blood cells	Only platelets	Erythrocytes and leukocytes are involved

subendothelial matrix exposed at sites of vascular damage, could be very promising potential targets for the development of antiplatelet agents [26].

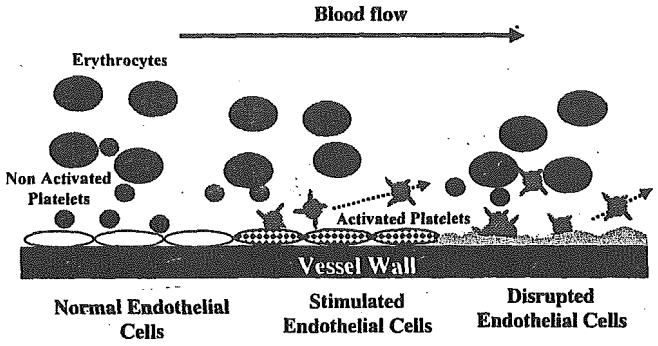


Fig. (2). Role of Platelets in Arterial Thrombus Formation.

Under arterial blood flow conditions, larger sized particles, such as erythrocytes, tend to accumulate close to the center of blood flow. Platelets, on the other hand, tend to flow peripherally. Although no interactions occur between platelets and normal endothelial cells, stimulated endothelial cells expressing adhesion molecules, such as von Willebrand factor on their surface transiently trap platelets and trigger their activation. More marked platelet accumulation occurs when the endothelial cells are disrupted and the thrombogenic subendothelial matrix made up of collagen is exposed to the bloodstream.

There is another important difference between platelet thrombus formation *in vivo* and platelet aggregation in aggregometry, namely the effects of blood flow. As shown in (Fig. 2), platelets play important roles in hemostasis and thrombus formation in the presence of blood flow, because the coagulation cascade does not function effectively under conditions of blood flow, because of the dilution of coagulation factors under these conditions. Investigators, including us, consider that these differences may be important [2, 17, 29, 30]. Indeed, experiments on animal models of arterial thrombosis clearly demonstrated that factors that are not known to be involved in platelet aggregation in conventional aggregometry, such as VWF, play crucial roles in arterial thrombosis *in vivo* [31-33]. Further investigation revealed that immobilized VWF on the exposed subendothelial matrix and its interaction with platelet GP Ibα and GP IIb/IIIa play essential roles in the initiation of platelet thrombus formation at sites exposed to high shear stress conditions [27, 28, 34]. This concept provides us with important clues for developing newer, safer and more effective anti-platelet agents, because with this new understanding, it may now be possible to develop selective antiplatelet agents to prevent arterial thrombosis at sites exposed to high shear stress conditions, without influencing hemostasis at low-shear sites [29].

Taking into consideration the above two important factors involved in the onset of arterial thrombosis *in vivo*, namely endothelial damage and blood flow, we and several other investigators attempted to clarify the mechanism of platelet thrombus formation *in vivo* by using a parallel-plate flow chamber, in which subendothelial matrix at sites of endothelial damage was exposed to shear stress conditions (Fig. 3) [2, 16, 26-28, 30, 34, 35]. The mechanism of

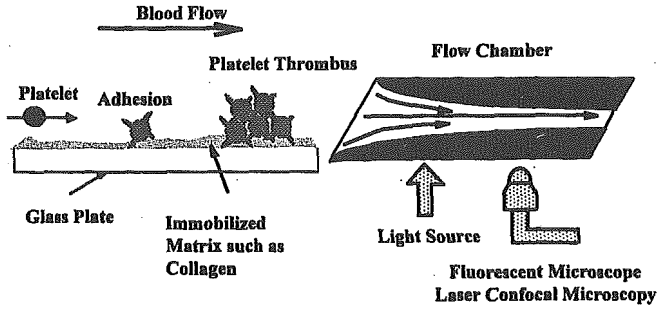


Fig. (3). Parallel- Plate Flow Chamber to Analyze the Mechanism of Platelet Thrombus Formation.

Subendothelial matrix, namely, type I collagen, was immobilized on a glass plate, to create a model of the damaged vascular surface. Whole blood containing platelets rendered fluorescent by the addition of mepacrine was perfused on the collagen surface at a wall shear rate controlled by the parallel-plate flow chamber. Platelet adhesion and thrombus formation on the immobilized collagen surface was then visualized by fluorescence microscopy or laser confocal microscopy.

platelet thrombus formation, as suggested by the use of this assay system, was shown to be far more complex than that of platelet aggregation, which is exclusively mediated by the binding of fibrinogen to the activated GP IIb/IIIa. Many platelet surface proteins such as GP Ibα, GP IIb/IIIa [27, 28, 34], GP VI [26], epinephrine receptors [36] and ADP receptors [22, 23] and their stimulation by various ligands, including VWF, fibrinogen, collagen, catecholamines, and thrombin are involved. Of the many receptor-ligand interactions, that between VWF and GP Ibα rather than those between collagen and GP VI are perhaps the most important. This is because the former is the initial and necessary interaction that stops flowing platelets on the matrix surface against a strong shearing force that works towards detaching the platelets from the surface [37] and the latter cause triggers the strongest signaling to the activated platelets resulting from their firm adhesion on the surface [25, 26]. The mechanism of platelet thrombus formation on to the exposed subendothelial matrix under blood flow conditions proposed by us is shown in (Fig. 4) [2].

HOW DO THROMBI GROW?

Once a constituent of the subendothelial matrix, such as collagen, is exposed to the bloodstream, platelets begin to tether to the exposed surface, through interaction of the GP Ibα receptor with the VWF bound with the collagen surface. Then, platelets are activated by synergistic signaling generated by the VWF-GP Ibα, VWF-GP IIb/IIIa, collagen-GP IV interactions etc, which result in shape changes, firm adhesion on to the surface and cluster formation of the platelets, to form platelet thrombi (Fig. 5). This activation process may occur in stepwise manner. Shear-induced platelet activation, for example, is initiated by phosphatidyl

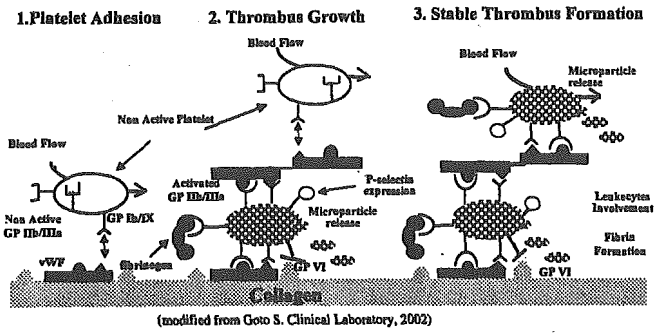


Fig. (4). Mechanism of Platelet Thrombus Formation on the Exposed Subendothelial Matrix under Blood Flow Conditions.

Platelets initially get tethered to the immobilized von Willebrand factor (VWF), which in turn is bound to exposed collagen at the site of vascular damage via the specific receptor GP Ibα. Then, the platelets become firmly adherent to the collagen surface through interactions between VWF-activated GP IIb/IIIa and collagen-GP VI and collagen-$\alpha_2\beta_1$. Then, some as yet unclarified signals cause platelet activation that induces the formation of firm platelet thrombi on the exposed subendothelial matrix.

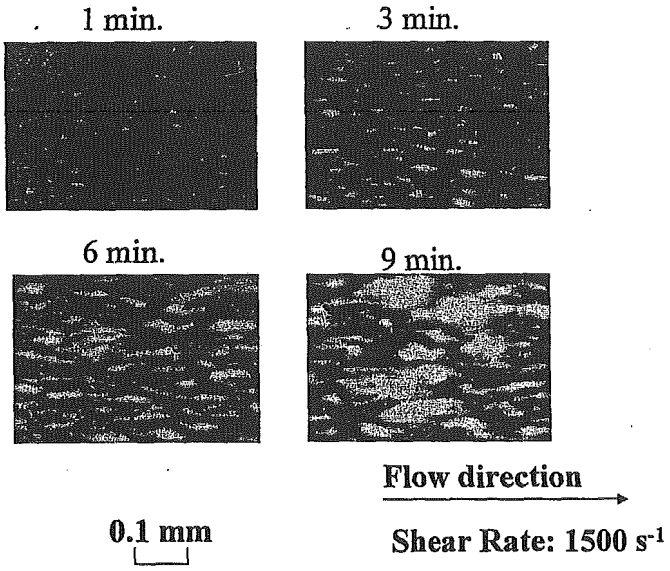


Fig. (5). Two-Dimensional Platelet Thrombus Growth on the Surface of Immobilized Collagen.

Following perfusion of blood containing platelets rendered fluorescent by the addition of mepacrine and anticoagulatated by the specific antithrombin agent Argatroban, platelets adhered to the collagen surface and aggregated to form platelet thrombi, as shown in the figure. On the surface of immobilized VWF (data not shown), however, no clustering could be seen, even though firm platelet adhesion was noted.

serine platelet surface expression detected by annexin V binding followed by a more significant process such as microparticle release and platelet shape change [38]. Activated platelets firmly adhered to the exposed subendothelial matrix surface through various receptors including GP IIb/IIIa [25]. As shown in (Fig. 6), platelets not only adhered directly to the matrix surface and grew in a two-dimensional direction, but also bind to other platelets already adhering to the surface, to exhibit three-dimensional growth. A three-dimensional imaging technique involving ultra-fast laser confocal microscopy with a piezo-motor control unit developed by us was used to observe the three-dimensional growth of platelet thrombi [39]. It has also been shown that platelet cohesion, previously supposed to be similar to platelet aggregation, is also initially mediated by a VWF-GP Ibα interaction [16]. Fibrinogen plays an important role in stabilizing platelet thrombi, but works rather later in the process of thrombosis [27]. Nevertheless, it is important to note that even the mechanism of platelet cohesion was found not to be similar to that of platelet aggregation under conditions of blood flow.

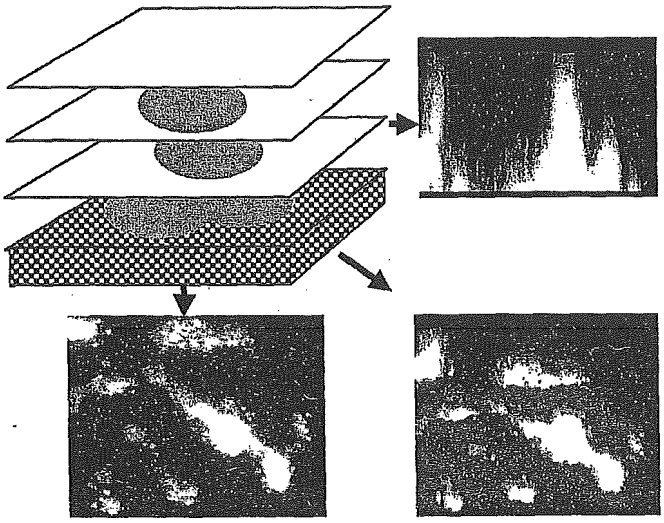


Fig. (6). Three-Dimensional Imaging of Platelet Thrombi Formed on the Surface of Collagen under Blood Flow Conditions.

Ultrafast multi-pinhole laser confocal microscopy with a piezo-electric motor control unit developed by us (and previously described) enabled us to obtain three-dimensional projection images of platelet thrombi formed on the surface of collagen, as shown in the figure.

To understand the mechanism of thrombus growth, the role of the coagulation system, especially that of thrombin formation on the surface of platelet thrombi should also be considered from two distinct aspects; its role in platelet activation and platelet thrombus growth, and its role in fibrin formation around platelet thrombi. Recently, Falati S, *et al.* clearly demonstrated that fibrin formation around platelet thrombi is an essential process in the formation of arterial occlusive thrombi [40]. Indeed, arterial occlusive thrombi *in vivo*, such as coronary thrombi, are always mixed thrombi containing activated platelets and fibrin [41-43]. Thrombin and fibrin formation around activated platelets and platelet thrombi [44-46] play essential roles in thrombus formation *in vivo*, although the mechanism is still to be elucidated. The role played by tissue factor is of particular

interest, because several investigators have demonstrated the accumulation of tissue factor, which directly activates the coagulation system, around activated platelets not only *in vitro* [47-48], but also *in vivo* [40]. The functional blocking of tissue factor might be a good target for developing the next-generation antithrombotic agents [49, 50].

HOW DO THE CURRENTLY AVAILABLE ANTI-PLATELET AGENTS PREVENT THROMBOTIC DISEASE?

(1) Aspirin

After aspirin was clearly demonstrated to have preventive effects on death and recurrence of coronary ischemia in acute myocardial infarction patients in ISIS-2 [51], a large number of clinical trials of aspirin have been conducted under many different conditions. The beneficial effects of aspirin in preventing cardiovascular thrombotic events during the acute phase of myocardial infarction, in old cases of myocardial infarction, in stroke patients, and in other high-risk patients, such as patients with diabetes mellitus, have been clearly established by meta-analyses, published by both the Antiplatelet Trialists' Collaboration (APT) in 1994 [52] and the Antithrombotic Trialists' Collaboration (ATT) in 2002 [53]. Accordingly, it has been established beyond doubt that aspirin is effective in preventing arterial thrombotic disorders, including myocardial infarction and stroke. In spite of its established clinical benefits, the exact mechanism by which aspirin prevents arterial thrombosis has yet to be clarified. Traditional research has demonstrated that aspirin irreversibly blocks the action of the enzyme cyclooxygenase-1, a crucial enzyme involved in the generation of the potent platelet agonist thromboxane A_2 (Fig. 7) [54]. The cyclooxygenase-1 mediated activation pathway plays an important role in platelet activation, but at a relatively later phase, because a previous report suggested that aspirin did not inhibit platelet shape change occurring as a result of platelet activation [55]. Many investigators still suppose that aspirin exhibits its antiplatelet actions through inhibition of thromboxane A_2 biosynthesis. This hypothesis is supported by a recent publication, which reported that the recurrence rate of thrombotic disease tended to be higher in patients resistant to inhibition of thromboxane A_2 production by aspirin [56]. While it is true that aspirin inhibits platelet aggregation associated with platelet activation induced by arachidonic acid or collagen [57], there still remains the question of whether the inhibition of platelet aggregation is indeed the mechanism underlying the antithrombotic actions of aspirin, because the orally available anti-GP IIb/IIIa agents which can inhibit platelet aggregation more strongly than aspirin, do not prevent thrombotic events as effectively as aspirin [58]. Since aspirin is an anti-inflammatory agent, some investigators proposed that the stabilization of

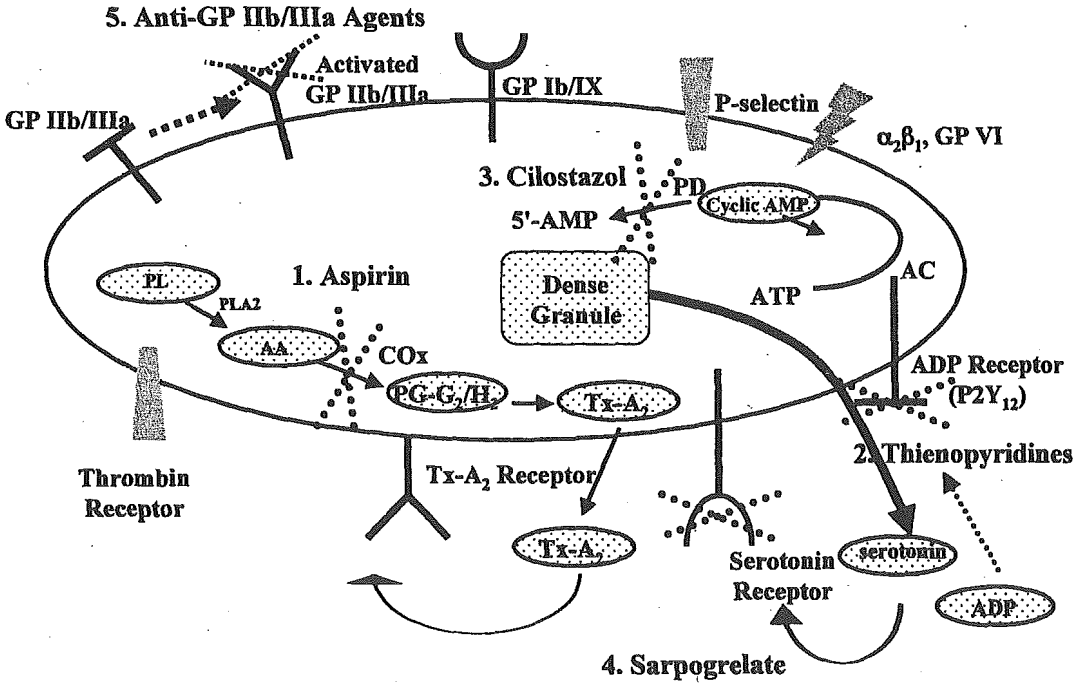


Fig. (7). Target of Anti-Platelet Agents.

This figure summarizes the platelet receptors and signaling pathways necessary for thrombus formation and the major potential targets of antiplatelet agents. Aspirin is an irreversible inhibitor of cyclooxygenase, which is a rate-limiting enzyme in the production of the potent platelet stimulator thromboxane A_2. Thienopyridine antiplatelet agents, namely ticlopidine and clopidogrel, inhibit the platelet surface ADP receptor $P2Y_{12}$ through their active metabolite(s) generated in the liver. Cilostazol is a specific inhibitor of phosphodiesterase-3 and inhibits platelet function by increasing the intracellular c-AMP levels. Sarpogrelate is a specific $5\text{-}HT_{2A}$ receptor inhibitor, which blocks serotonin-mediated platelet activation. Anti-GP IIb/IIIa agents inhibit platelet aggregation by blocking the binding of plasma ligands (mostly fibrinogen) to activated GP IIb/IIIa.

atheroma plaques by the anti-inflammatory effects of aspirin might play some role in the prevention of thrombotic events by this drug [59]. This hypothesis, however, stands on weak ground as it is considered that other non-steroidal anti-inflammatory agents, including ibuprofen, do not prevent cardiovascular thrombotic events [60] and even sometimes increase the incidence of such events [61]. Further evaluation is awaited for a complete understanding of the mechanism of actions of one of the oldest and most commonly used and well-established antiplatelet agents.

(2) Thienopyridine Antiplatelet Agents (ticlopidine and clopidogrel)

The beneficial effects of ticlopidine and clopidogrel in the prevention of arterial thrombotic disease has also been demonstrated by the APT 1994 [52] and ATT 2002 [53] although it still remains unclear as to whether or not the effects of these agents are superior to those of aspirin. Pharmacological investigations have revealed that both agents are pro-drugs [12, 62] and that their antiplatelet effects are exerted via the blockade of the $P2Y_{12}$ ADP receptor by their active metabolites [62, 63]. So far, at least three distinct ADP receptors have been identified in platelets [64] and the $P2Y_{12}$ receptor has been demonstrated to be involved in the augmentation of platelet aggregation with a modest influence on platelet shape change or release reactions as assessed by aggregometry [13, 64, 65]. Since the effects of stimulation of this receptor on platelet aggregation may not reliably represent its effects on arterial thrombosis *in vivo*, we tested the role of $P2Y_{12}$ stimulation in the process of platelet thrombus formation on the surface of exposed subendothelial matrix under blood flow conditions, and revealed that stimulation of this receptor may also play crucial roles in platelet activation initiated by the interaction of platelets with immobilized matrix, even in the absence of the exogenous addition of ADP [66]. We have also shown that $P2Y_{12}$ stimulation by ADP released from activated platelets plays crucial roles in not only platelet adhesion and thrombus formation, but also in platelet activation resulting in P-selectin surface translocation and procoagulant microparticle release [23]. In conventional aggregometry, anti-GP IIb/IIIa agents appear to be highly potent antiplatelet agents, because they inhibit platelet aggregation induced by any kind of activating agents. But, in the prevention of platelet thrombus formation *in vivo*, agents inducing $P2Y_{12}$ inhibition might have stronger effects, because they inhibit not only platelet aggregation, but also events upstream of platelet activation, such as phosphatidylinositol 3-kinase activation [67], α-granule degranulation and microparticle release [68] that result in platelet-derived procoagulant activity [23].

Thienopyridine antiplatelet agents are widely used in combination with aspirin in patients undergoing coronary stenting [69]. The stronger effects in the prevention of subacute stent thrombosis (SAT) achieved with a combination of antiplatelet agents, initially demonstrated with aspirin plus ticlopidine [70], and now also confirmed with aspirin plus clopidogrel [71], as compared to that achieved with warfarin underlines the more important role of platelets than the coagulation system in stent thrombosis. However, the exact mechanisms underlying the strong antithrombotic effects of combinations of antiplatelet agents could not be clarified by conventional aggregometry. The superior effects of a combination of antiplatelet agents as compared to that of aspirin or ticlopidine alone were also recently demonstrated in a study using an *ex vivo* assay system [72], although the exact mechanism involved still remains to be elucidated. Furthermore, the superior antithrombotic effects achieved by inhibition of events upstream of platelet activation by a combination of aspirin and a thienopyridine antiplatelet agent were also demonstrated clinically by the strong preventive effects of aspirin and clopidogrel on cardiovascular thrombotic events in acute coronary syndrome patients in the ACS-CURE study [73].

(3) Anti-GP IIb/IIIa Agents

Anti-GP IIb/IIIa agents were developed as strong antiplatelet drugs because they blocked the final common pathway of fibrinogen binding to the activated GP IIb/IIIa necessary for platelet aggregation [9, 74, 75]. Accordingly, they had the ability to completely inhibit platelet aggregation, regardless of the activating agents used. Prof. Coller and his colleagues were the first to develop a clinically available anti-GP IIb/IIIa agent by combining the mouse-derived monovalent Fab of anti-human GP IIb/IIIa (7E3) and human IgG [75]. This chimeric human mouse Fab c-7E3 was supposed to have reduced immunogenicity, even though it is not completely humanized [76]. After initial clinical trials had demonstrated the safety of this agent, large-scale randomized clinical trials performed in patients who underwent coronary interventions [77, 78]. Surprisingly, the incidence of acute thrombotic complications after coronary intervention decreased by more than 50% with the use of this chimeric Fab as an anti-GP IIb/IIIa agent (patent name: abciximab) [78]. Subsequently, two small-molecule anti-GP IIb/IIIa agents, named tirofiban and eptifibatide, were approved by the FDA, because they were also found to significantly reduce the incidence of acute thrombotic complications after coronary intervention [75]. It is now common understanding that anti-GP IIb/IIIa agents, which block platelet aggregation, are effective in the prevention of acute thrombotic complications after coronary intervention in patients with acute coronary syndromes and stable effort angina.

The initially successful results in patients, who underwent coronary intervention, led one to believe that the anti-GP IIb/IIIa agents would be the ultimate anti-platelet agents. However, two negative findings broke the myth. The GUSTO-IV ACS trials planned to show the superior effects of the anti-GP IIb/IIIa agent abciximab over conventional antithrombotic treatment (aspirin plus heparin) in patients with acute coronary syndromes not undergoing coronary intervention; contrary to expectation, abciximab did not show any additional benefit over those of the conventional antithrombotic regimen [14]. In addition, the inferior thrombosis-preventive effects of orally available anti-GP IIb/IIIa agents as compared to those of aspirin have been shown in several clinical trials [15]. These reports, in addition to the results of the GUSTO-IV ACS trial, led us to wonder whether the mechanism of acute thrombotic complications in patients undergoing and not undergoing

coronary intervention might not be the same. Nevertheless, the limited antithrombotic effects of anti-GP IIb/IIIa agents, even though they can completely inhibit platelet aggregation, might be understood as explained in (Fig. 8). There is no doubt that platelets are the most important players in the onset of thrombosis, even though it is also important to understand that they are not the only players. Indeed, combination therapy with an antiplatelet agent and an anticoagulant has been shown to be more effective than either an antiplatelet agent or an anticoagulant agent alone [79]. We assume that upstream inhibition after platelet activation, not only blocking aggregation, but also platelet procoagulant activity resulting from platelet activation, might be superior to blocking the final common pathway for platelet aggregation.

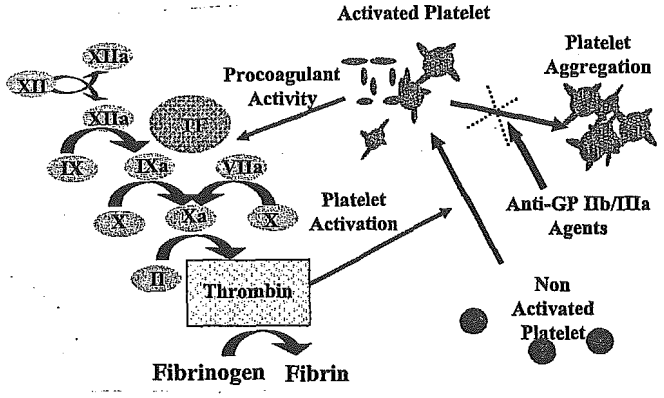
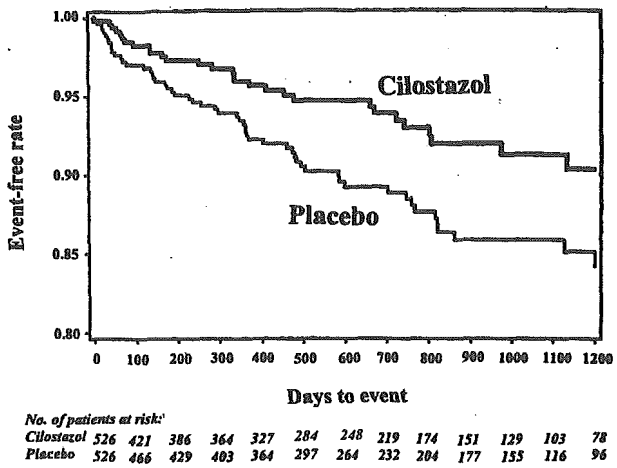


Fig. (8). Interrelation between Platelet Thrombus Formation and Activation of the Coagulant Cascade

There is a positive feedback system between platelet thrombosis and the coagulation cascade. Activated platelets not only tend to aggregate, but also provide procoagulant activity to activate the coagulation cascade. Once the coagulation cascade is activated, platelet activation is induced by thrombin generated on its surface. Anti-GP IIb/IIIa agents are strong inhibitors of platelet aggregation, but cannot inhibit platelet activation or platelet expression of procoagulant activity.

(4) Other Anti-platelet Agents

Of the many other antiplatelet agents available, cilostazol and sarpogrelate merit attention. Cilostazol is a specific phoshphodiesterate-3 inhibitor that exerts its antiplatelet effects by increasing the intra-platelet cAMP level [80]. This agent not only inhibits platelet aggregation, but also platelet activation. Moreover, cilostazol has a potential to prevent atherothrombotic disease by improving endothelial cell function [81]. Large-scale randomized trials recently conducted in Japan demonstrated that cilostazol reduces the recurrence rate of stroke by almost 50% (Fig. 9) [82]. Several animal experiments and small-scale clinical trials have also demonstrated that cilostazol might even prevent the later events of restenosis [83-85]. It is also worthwhile to note that this agent is ideal for the treatment of peripheral arterial disease, because it can prevent both vascular spasm and platelet plugging.



(from Goto F, et al. J. Stroke and Cerebrovasc, 2000)

Fig. (9). Cilostazol Stroke Prevention Trial.

This figure is reproduced from "Cilostazol stroke prevention study: A placebo-controlled double-blind trial for secondary prevention of cerebral infarction, " by Goto F, et al., published in the Journal of Stroke and Cerebrovascular Disease (2000). One thousand and fifty patients with a history of stroke in the previous 1 to 6 months were recruited for the trial. The preventive effect of oral cilostazol therapy was compared with placebo. As shown in the figure, the recurrence rate was significantly reduced in the cilostazol group.

Another anti-platelet agent currently available in some countries is sarpogrelate. This agent is a specific inhibitor of 5-HT_{2A} receptors, and thereby blocks serotonin-induced platelet aggregation [86]. Since serotonin is stored in the dense granules and released upon stimulation, like ADP, the mechanisms underlying the antiplatelet effects of specific anti-serotonin agents might be similar to those of the thienopyridine agents. A precise understanding of the role played by serotonin in platelet thrombus formation *in vivo* is awaited to improve our understanding of the mechanism of actions of this sarpogrelate.

FUTURE DIRECTIONS IN THE DEVELOPMENT OF NEWER ANTI-PLATELET AGENTS

Until now, most of the antiplatelet agents developed are those that primarily inhibit platelet aggregation. As described in this review, the mechanism of platelet aggregation *in vitro* is not similar to that of platelet thrombus formation *in vivo*. We now know that multiple receptor-ligand interactions are involved in the process of platelet thrombus formation *in vivo*, occurring on the exposed subendothelial matrix under blood flow conditions. All the relevant molecules involved in the multiple-synergistic platelet activation pathway, shown in (Fig. 7), may be exploited as novel targets for antiplatelet agents. The following are of particular interest:

(1) Von Willebrand Factor-GP Ibα

Since platelet tethering mediated by the VWF-GPIbα interaction is the initial step of the platelet activation occurring at sites exposed to high shear stress conditions, direct inhibitors of this interaction, or inhibitors of the specific signaling (yet to be clarified) triggered by this interaction may prove to be effective anti-platelet agents. These molecular targets have an advantage over those of the conventional anti-GP IIb/IIIa agents, because the VWF-GP Ibα interaction only occurs at sites exposed to a high shear stress, such as in atherosclerotic stenotic coronary arteries that could be inhibited without concomitant inhibition of normal hemostatic plug formation. Both humanized anti-VWF and anti-GP Ibα antibody are currently under development [87-89].

(2) ADP-P2Y Receptor

Thienopyridine antiplatelet agents are prodrugs of anti-$P2Y_{12}$ agents. Strong antithrombotic effects are achieved with only partial inhibition of $P2Y_{12}$ [90]. There still is the possibility that specific $P2Y_{12}$ inhibitors may be developed as stronger antithrombotic agents [91]. The use of direct $P2Y_{12}$ inhibitors under close monitoring, or the development of other P2Y receptor inhibitors ($P2Y_1$, $P2X_1$) may prove to be newer strategies for antiplatelet therapy.

(3) Collagen Receptors

Unlike platelet aggregation, platelet thrombus formation is initiated by exposure of the subendothelial matrix to the bloodstream. Platelet interaction with the constituents of this matrix, especially collagen, might be a new target that could be exploited in the development of antithrombotic agents. Of the collagen receptors known, GP VI is of particular interest in this connection, because it plays a crucial role in platelet activation and thrombus formation on the surface of collagen [25, 26]. We expect that anti-GP VI drugs may be developed as strong potent antiplatelet agents with the least risk of bleeding, because GP VI-deficient humans and animals only show a modest bleeding tendency.

(4) Others

Thrombin plays a role not only in fibrin formation, but also in platelet activation. Drugs that inhibit thrombin formation, thrombin-thrombin receptor interaction, and thrombin receptor stimulation-triggered signaling, can all be developed as antiplatelet agents. Serotonin receptor inhibitors, catecholamine receptor-inhibitors, as well as inhibitors of many intra-platelet activation signaling molecules might be considered as next generation antiplatelet agents.

CONCLUSIONS

1) The mechanism of platelet thrombus formation *in vivo* is different from that underlying platelet aggregation in conventional aggregometry. Therefore, the platelet aggregation assay is not a suitable screening test for the assessment of antiplatelet agents, even though the assay has long been used as a conventional method for assessing platelet function and is still useful for understanding the mechanisms involved in the onset of bleeding disorders.

2) Multiple synergistic stimulation of platelet receptors by the corresponding ligands is involved in the process of platelet activation. The von Willebrand factor-GP Ibα interaction and the collagen-GP VI interaction are the two most important receptor-ligand interactions in the process of platelet thrombus formation on the surface of collagen under conditions of high shear stress.

3) The aforementioned receptor-ligand interactions, as well as the intraplatelet signaling triggered by these interactions may be exploited as targets in the development of newer antiplatelet agents. Furthermore, the antiplatelet actions of already established antiplatelet agents, such as aspirin and thienopyridine agents, should be reassessed with regard to their effects on platelet thrombus formation on the surface of exposed subendothelial matrix under blood flow conditions.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research in Japan (09770506, 11770367, 13670744, 15590771), the Tokai University School of Medicine Research Aid, 1997, 1998 and 1999; a grant JSPS-RFTF97I00201 from the Japanese Society for the Promotion of Science, a grant from the Takeda Science Foundation, 1998; a grant from the Yamanouchi Foundation for Research on Metabolic Disorders, 1998; a grant from the Japanese Medical Association, 1998; a research fund of the Mitsukoshi Health and Welfare Foundation, 1999; the Suzuken Memorial Fund, 1999; a research fund from the Japanese Council on Molecular Cardiology, 2000, a research fund from the Mitsui Life Insurance Foundation; a research fund from the Japan Foundation of Cardiovascular Research 2000; a research fund from the Mochida Memorial Medical and Pharmaceutical Foundation, 2001; Grant for Advanced Medicine Supported by the Ministry of Health, Labor and Welfare 2001 and 2002; support from the Department of Oriental Medicine (Tsumura) 2003; and a Grant from NOVARTIS Foundation (Japan) for the Promotion of Science 2003.

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Understanding the Mechanism and Prevention of Arterial Occlusive Thrombus Formation by Anti-Platelet Agents

S.Goto*

Division of Cardiology, Department of Medicine, Tokai University School of Medicine, Kanagawa, Japan

Abstract: For many years, platelet aggregation, which is mediated exclusively by the binding of fibrinogen to activated glycoprotein (GP) IIb/IIIa, has been used for the screening of antiplatelet agents. However, clinical experience with anti-GP IIb/IIIa agents, which can completely inhibit platelet aggregation, has shown that these drugs are not the most ideal agents for the prevention of atherothrombosis. Recently, many investigators have reported that platelets play a major role in thrombus formation at sites exposed to blood flow, and also that there is a crucial difference between the mechanism of platelet thrombus formation under blood flow conditions *in vivo* and that of platelet aggregation occurring in conventional aggregometry. Indeed, multiple receptor-ligand interactions, including von Willebrand factor (VWF) binding with platelet GP Ibα and GP IIb/IIIa, collagen binding with collagen receptors, as well as stimulation of platelet receptors, such as adenosine 5'-diphosphate (ADP) receptors, appear to be involved in the process of *in vivo* arterial thrombus formation. Moreover, not only platelets, but also the coagulation cascade activated by the procoagulant activity expressed on the surface of activated platelets, are believed to play a crucial role in the formation of occlusive thrombi. These findings suggest that drugs which block events upstream of the final common pathway for platelet aggregation might be better antiplatelet agents than those that merely inhibit platelet aggregation. We may then expect new antiplatelet agents on the horizon that exert their actions against both thrombus formation under blood flow conditions and against the procoagulant activity appearing on the surface of activated platelets.

Key Words: von Willebrand factor, platelet, arterial thrombosis, shear stress, endothelial cell

INTRODUCTION

Due to the increased incidence of various risk factors including diabetes mellitus, hypertension, decreased physical activities, the risk of atherothrombotic disease such as myocardial infarction and ischemic stroke is increasing world-wide, especially in industrialized countries. Thrombosis due to the presence of vulnerable athermanous plaque plays important in the onset of these disorders. It is rather difficult to inhibit the progression of atherosclerosis so far because it is a kind of aging process. On the hand, recent progress in understanding the mechanism of thrombus formation give us certain hope to develop agents, which can prevent arterial thrombosis efficiently. We show herein the mechanism as well as the possible target of new generation of anti-atherothrombotic agents.

MECHANISM OF ARTERIAL PLATELET THROMBUS FORMATION.

(1). Important Role of von Willebrand Factor and Its Receptors on the Platelet Surface.

Arterial thrombosis, including thrombosis in the coronary arteries and carotid arteries, is now believed to be initiated by disruption of the vascular endothelium associated with plaque rupture or plaque erosion [1,2]. Platelets located adjacent to the endothelium start to accumulate at sites where the subendothelial matrix, an important component of which is collagen, becomes exposed to the bloodstream as a result of endothelial damage (Fig. (1)). This is the initial event in atherothrombosis [3]. The mechanism of arterial platelet thrombus formation on the exposed subendothelial matrix, which forms the basis of atherothrombosis , has been investigated extensively in both animal thrombosis models and in *ex vivo* and *in vitro* studies using human platelets.

In a canine model of coronary thrombosis, besides the commonly used antiplatelet agent aspirin and agents inhibiting platelet aggregation such as anti-glycoprotein (GP) IIb/IIIa agents, specific monoclonal antibodies and drugs inhibiting von Willebrand factor (VWF) binding with platelet GP Ibα which do not inhibit agonist-induced platelet aggregation at all, were also demonstrated to be effective in preventing atherothrombosis [4-6]. These results, along with the report that a specific monoclonal antibody against the human A1 domain of VWF inhibited occlusive thrombus formation in the dog coronary artery and also prevented thrombus formation and neointimal proliferation in a guinea pig model, [7-9] strongly support the contention that the A1 domain of VWF and its interaction with its corresponding platelet receptor GP Ibα plays a crucial role in the formation of platelet thrombi. In addition to these findings in animal experiments, *ex vivo* flow chamber experiments conducted using blood samples obtained from

*Address correspondence to this author Division of Cardiology, Department of Medicine, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan; Tel: +81-463-93-1121 Ext.2200; Fax: +81-463-93-6679; E-mail: shinichi@is.icc.u-tokai.ac.jp

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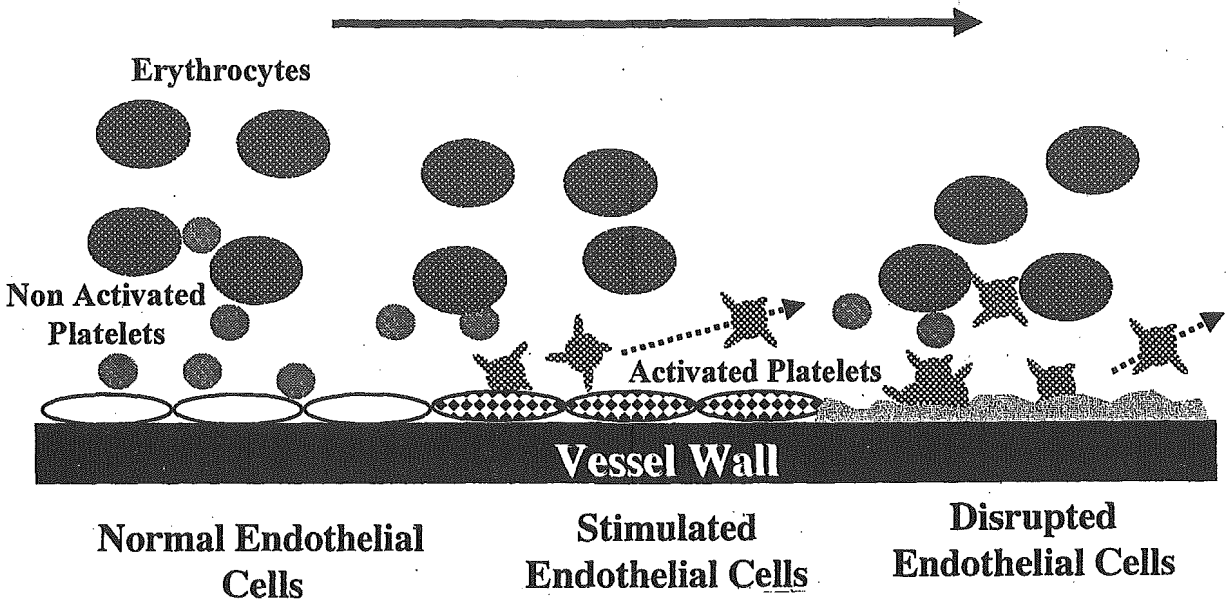

Fig. (1). Role of Platelets in Arterial Thrombus Formation. Under arterial blood flow conditions, platelets tend to flow peripherally. No interaction occurs between platelets and endothelial cells when the endothelial cell functions are normal. On the other hand, when the endothelial cells are disrupted, platelets begin to interact transiently with VWF expressed on stimulated endothelial cells, or to accumulate on the matrix surface. Note that platelet thrombus formation occurred in the presence of damaged endothelium.

human donors have also shown that besides GP IIb/IIIa, GP Ibα, which transiently binds with VWF, [10,11] also plays an important role in the activation of platelets [12-18] and the formation of platelet thrombi (Fig. (2)) [2,3,10,19-23]. Based on these experimental results, we propose the following mechanism for platelet thrombus formation; [2,20] when components of the subendothelial matrix, *e.g.*, collagen, become exposed to the bloodstream as a result of atheroma rupture, platelets flowing adjacent to the vessel wall start to accumulate on the exposed matrix surface under conditions of high shear stress and become activated. These activated platelets then become firmly adherent to the matrix surface through more stable interactions, such as collagen binding with its receptors, [24] and VWF binding with GP IIb/IIIa [21]. Then, other platelets coming in contact with the adherent platelets interact with them via binding of the GP Ibα on their surface with the VWF bound on the surface of the adherent platelets. It is important to note that even platelet cohesion, which was previously believed to be mediated exclusively by fibrinogen binding with GP IIb/IIIa, [25-27] requires the VWF-GP Ibα interaction under conditions of high shear stress [28]. In other words, the fibrinogen-GP IIb/IIIa interaction is evidently necessary for the stabilization of platelet thrombi [21]. Thus, VWF appears to play a crucial role in not only the initial tethering of the platelets to the matrix surface, but also in the accumulation of platelets on the surface of other platelets already adherent on the matrix surface (Fig. (3)).

In addition to its role in arresting platelets on the exposed matrix surface or on the surface of already adherent platelets, the VWF-GP Ibα binding is also believed to trigger activation signals. As a matter of fact, GP Ibα has a functional link with the cytoskelton through actin-binding proteins [29]. Furthermore, GP Ibα can also bind directly to the 14-3-3-adapter protein ζ (14-3-3 ζ), which is supposed to play an important role in the initiation of activation signaling [30]. In addition to the role of the signals originating from the cytoskelton, recent studies also suggest the possible role of membrane protein association mediated by cholesterol-rich microdomains named lipid rafts. One study has demonstrated the presence of the GP Ib/IX complex in the lipid rafts, [31] and others have suggested the possible role of other raft-associated proteins, such as GP VI/FcRγ, in GP Ib/IX complex-mediated intracellular signaling [32]. Once these mechanisms are clarified in detail, we might be able to find antiplatelet targets in VWF-GP Ibα–mediated signals [34]. Regarding drug development, it is important to note that crystallographers have demonstrated the three-dimensional structure of the GP Ibα-VWF complex, [33] and it may be possible to develop small-molecule inhibitors of the VWF-GP Ibα complex as potential antiplatelet agents 35,36].

(2). Role of Multiple Synergistic Stimulation of Various Platelet Receptors in Stabilizing Platelet Thrombi.

The VWF-GP Ibα interaction plays an important role in the formation of the initial platelet thrombi; however, it is important to understand that the thrombi formed via the mediation of only this interaction are unstable and easily dissociated [21]. Synergistic stimulation of other receptors, some examples of which are shown in Fig. (4), is necessary for the formation of stable platelet thrombi. Of these, the following are of particular interest, because they are either targets of antiplatelet agents already available, or may serve as potential targets of newer generation antiplatelet drugs.

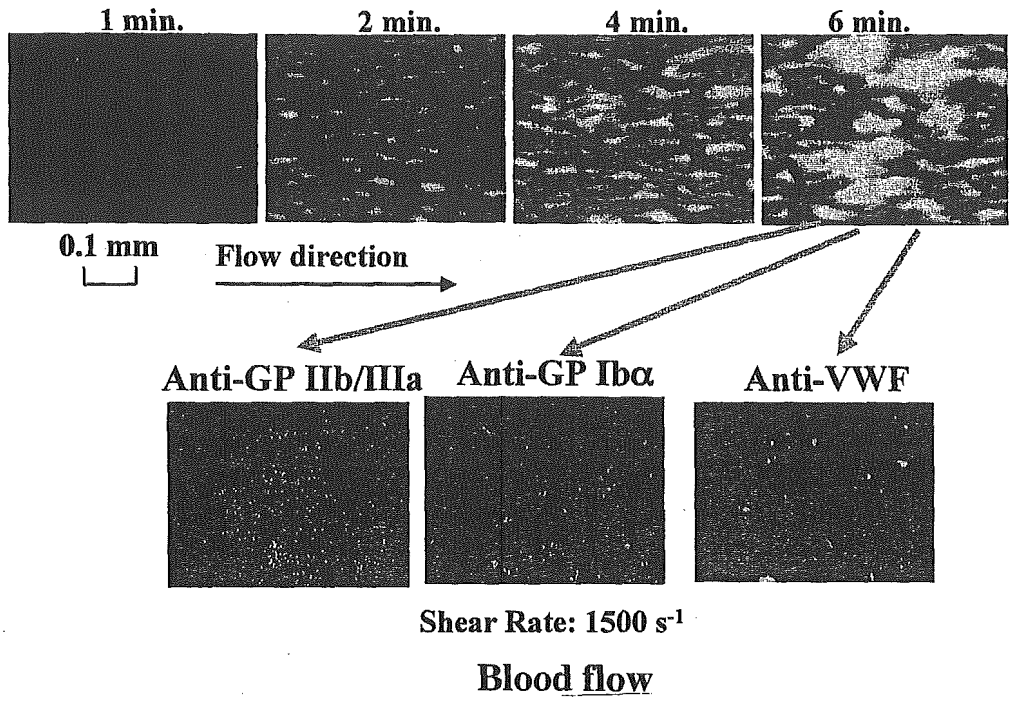

Fig. (2). Platelet Thrombus Formation on the Collagen Matrix under Blood Flow. Platelet thrombus formation on the surface of collagen was observed following perfusion of whole blood containing fluorescinated platelets (upper panel). The thrombus formation was completely inhibited by blockade of the VWF-GP Ibα interaction, as well as by that of GP IIb/IIIa.

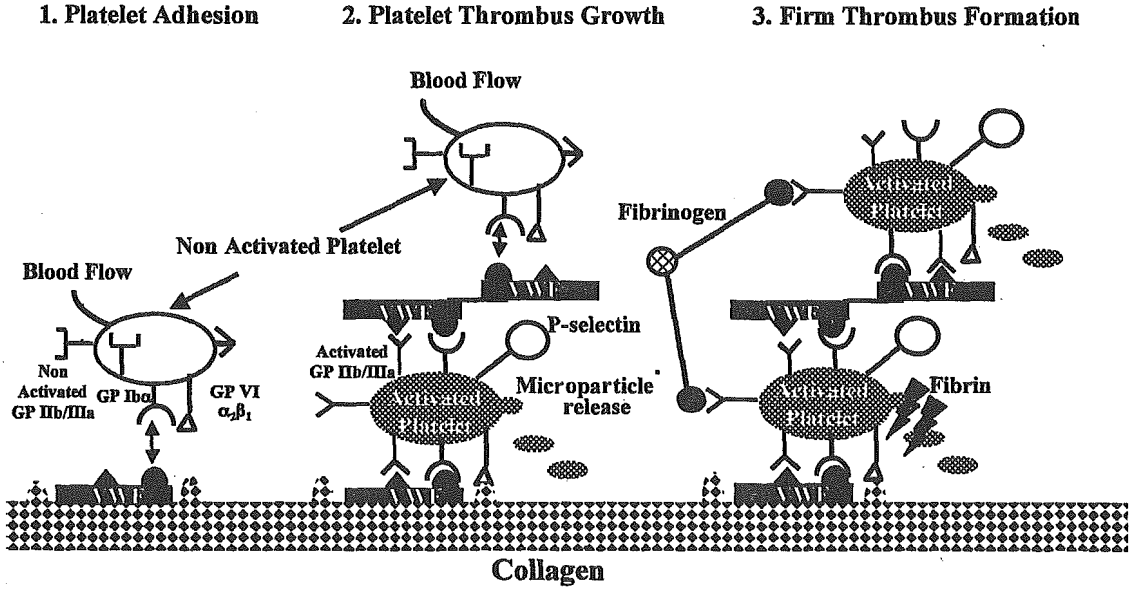

Fig. (3). Mechanism of Platelet Thrombus Formation on the Exposed Subendothelial Matrix under Blood Flow Conditions. Platelets initially become tethered to immobilized von Willebrand factor through GP Ibα, then become firmly adherent to the surface of the matrix through VWF-activated GP IIb/IIIa and collagen-GP VI interactions. Activated platelets express P-selectin, so that inflammatory cells, such as leukocytes, also become involved in the thrombus formation. Moreover, activated platelets contribute to fibrin formation by expressing procoagulant activity mediated by the phospholipids on their surface, and microparticle release.

A. ADP Receptors

Three different ADP receptors have been cloned so far, namely $P2X_1$, $P2Y_1$ and $P2Y_{12}$ [37-39]. The antiplatelet effects of the widely used thienopyridine antiplatelet agents ticlopidine and clopidogrel are mediated by blockade of $P2Y_{12}$ by their active metabolite(s) [40,41]. Biochemical experimental reports have revealed a link between the $P2Y_{12}$ receptor and the Gi-coupled adenylate cyclase [37,38]. It may be reasonable to suppose that the antiplatelet effects of thienopyridine agents are mostly mediated by increase in the intracellular levels of c-AMP, although several reports have

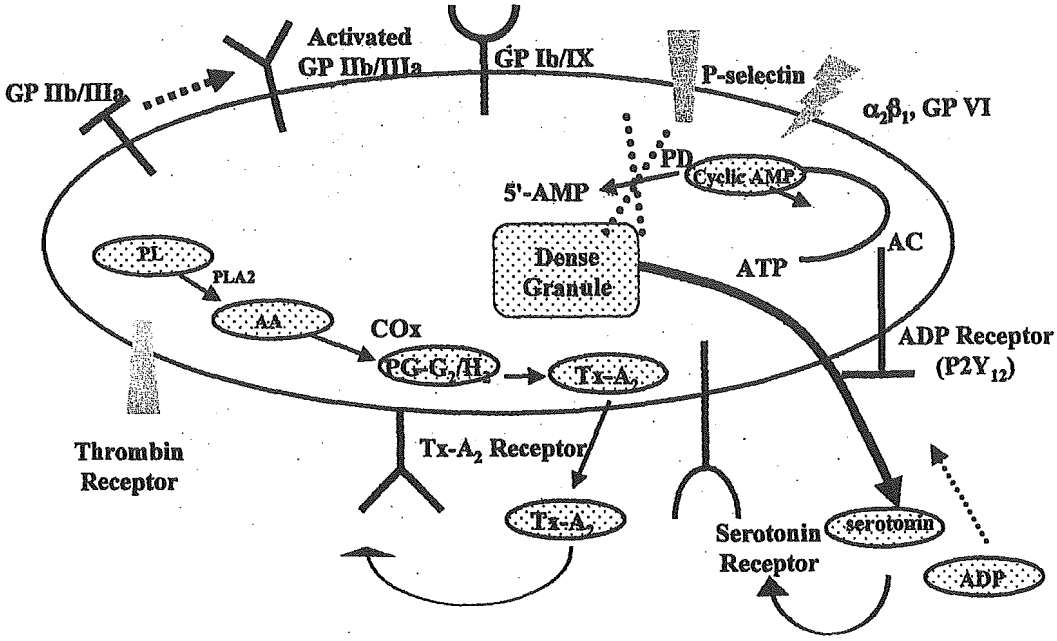

Fig. (4). Synergistic Stimulation of Various Receptors is Involved in the Process of Platelet Thrombus Formation. Stimulation of various receptors, examples of which are shown in this figure, is involved in the process of platelet activation.

suggested the involvement of other mechanisms as well [42,43]. Nevertheless, the inhibitory effects of the thienopyride antiplatelet agents are not limited to inhibition of platelet aggregation induced by exogenously added ADP; indeed, we have clearly demonstrated that platelet activation initiated by the VWF-GP Ibα interaction under high shear stress conditions in the absence of exogenous addition of ADP is also inhibited by more than 50% when $P2Y_{12}$ is blocked by a specific inhibitor [44]. Moreover, platelet thrombus formation occurring on the surface of type I collagen under blood flow conditions, which is obviously not directly related to the exogenous addition of ADP, was also inhibited by $P2Y_{12}$ blockade [45,46]. These experimental findings lend theoretical support to the clinical experience with clopidogrel in the prevention of atherothrombotic diseases, such as the acute coronary syndrome [47]. These important role of ADP receptors in platelet thrombus formation was recently reviewed elsewhere in detail by Nurden AT and Nurden P [48].

B. Thromboxane A_2 Receptor

The role played by thromboxane A_2 receptor in the process of platelet thrombus formation is less completely clarified than that of the ADP receptors. It is well known that aspirin, which is a commonly used antiplatelet agent worldwide, inhibits the production of thromboxane A_2 by blocking cyclooxygenease [49]. This suggests the possible role of $Tx-A_2$ and the $Tx-A_2$ receptor in the process of arterial thrombus formation. However, in spite of clear demonstration of the role of aspirin in the prevention of atherothrombotic disease, [50,51] the precise mechanisms underlying the inhibition of platelet thrombus formation by this drug are still not clearly understood. Unlike $P2Y_{12}$ inhibitors, aspirin does not inhibit shear-induced VWF-mediated platelet activation [13] or aggregation [52]. Although aspirin is known to inhibit arachidonic- acid-induced and collagen-induced platelet aggregation, [49] the relevance of such inhibitory effects in the prevention of arterial thrombus formation *in vivo* remains unclear. Moreover, specific $Tx-A_2$ receptor inhibitors, which are supposed to be stronger antiplatelet agents than aspirin, have not been shown to have superior preventive effects against atherothrombotic diseases to those of aspirin [53,54]. Further investigations are necessary for a complete under-standing of the role played by $Tx-A_2$ receptor stimulation in the process of arterial thrombus formation *in vivo*.

C. Collagen Receptors

Another important receptor that is probably involved in the process of arterial thrombus formation is the collagen receptor, because collagen is one of the most important constituents of the subendothelial matrix exposed at sites of endothelial damage. Both integrin $\alpha_2\beta_1$ and GP VI are involved in platelet adhesion on the surface of collagen [24]. Numerous investigators have demonstrated the importance of GP VI in platelet thrombus formation, especially under blood flow conditions [32,55-57]. GP VI, which is linked with FcRγ, [58] plays an important role in not only platelet activation induced by platelet interaction with collagen, but also in VWF-mediated platelet activation, [35] as explained above. This important role of GP VI in platelet thrombus formation was recently reviewed by Nieswandt and Watson. [59] Antiplatelet agents targeted against GP VI might be safe and effective next-generation agents, because they would be expected to prevent platelet thrombus formation only at sites where the subendothelial matrix becomes exposed as a result of endothelial damage, to more strongly inhibit thrombus formation under high shear stress conditions, such as in

atherosclerotic stenotic coronary arteries, and to be associated with only a modest bleeding tendency, as seen in GP VI-deficient animals and humans [60].

D. Fibrinogen Receptor (GP IIb/IIIa)

Since platelet aggregation is exclusively mediated by fibrinogen binding with activated GP IIb/IIIa regardless of the initial activation signal, this receptor ligation is considered to be the final common pathway for platelet aggregation [25-27]. Blockers of GP IIb/IIIa were therefore expected to be among the strongest antiplatelet agents developed yet. After some clinical experience, however, we have begun to understand the efficacy and limitations of the anti-GP IIb/IIIa agents in the clinical setting, and also the role of GP IIb/IIIa in the process of arterial thrombus formation *in vivo*. We describe in detail the current status of this understanding in the following section.

ROLE OF PLATELET GP IIB/IIIA RECEPTOR IN ARTERIAL THROMBUS FORMATION: NEW CONCEPTS AFTER CLINICAL EXPERIENCE WITH ANTI-GP IIB/IIIA AGENTS.

(1). Initial Expectation

Although we now understand that the conditions necessary for platelet aggregation are far different from those necessary for platelet thrombus formation *in vivo*, many investigators previously believed that platelet aggregation, as observed in conventional aggregometry, in which platelet activation is induced by chemical agonists such ADP or thrombin, alone was involved in platelet thrombus formation. In this context, aspirin, the most commonly used antiplatelet agent worldwide, was considered to exert its antiplatelet effects by inhibiting arachidonic-acid- and collagen-induced platelet aggregation. Also, the antiplatelet effects of the thienopyridine antiplatelet agents ticlopidine and clopidogrel were considered to be exerted via inhibition of ADP-induced platelet aggregation. Since platelet aggregation is exclusively mediated by fibrinogen binding with GP IIb/IIIa, it was expected that platelet thrombus formation would be prevented when the fibrinogen-GP IIb/IIIa ligation was blocked by specific inhibitors.

Of the three distinct classes of anti-GP IIb/IIIa agents currently approved for clinical use by the FDA, [61-63] abciximab, a human-mouse chimeric Fab of IgG originally developed as the anti-human mouse monoclonal antibody 7E3 by Dr. Barry Coller, [64] was the first one tested in clinical trials [65,66]. This chimeric human mouse Fab (Fab c-7E3) was supposed to have reduced immunogenicity, [67] although this molecule has not yet been completely been humanized. After initial clinical experience demonstrated the safety of this agent, large-scale randomized clinical trials were performed in patients undergoing coronary intervention. [65,66] Surprisingly, it was found that the incidence of acute thrombotic complications after coronary intervention decreased by more than 50% following administration of this chimeric Fab of anti-GP IIb/IIIa (patent name: abciximab). Subsequent to the approval of abciximab, two small-molecule anti-GP IIb/IIIa agents named tirofiban and eptifibatide were also approved by the FDA, because these agents were also found to significantly reduce the incidence of acute thrombotic complications in patients with the acute coronary syndrome [68,69].

(2). Clinical Usefulness and Limitations of Anti-GP IIb/IIIa Agents

Anti-GP IIb/IIIa agents are strong antiplatelet agents, because they block the final common pathway of fibrinogen binding to GP IIb/IIIa that is necessary for platelet aggregation. We now have reproducible data demonstrating the effects of anti-GP IIb/IIIa agents on the prevention of acute thrombotic complications after coronary intervention [61,62]. However, recent clinical experience has revealed the limitations of these anti-GP IIb/IIIa agents in the prevention of occlusive arterial thrombosis, *e.g.*, that involved in the development of acute myocardial infarction in high-risk patients such as those with unstable angina, when no coronary intervention is performed [70]. Furthermore, none of the clinical trials of orally available anti-GP IIb/IIIa agents conducted so far have been demonstrated to be superior to aspirin in the prevention of cardiovascular events [71]. How can these differential effects of anti-GP IIb/IIIa agents in different clinical settings be explained?

As clearly demonstrated recently by Falati *et al.*, formation of arterial occlusive thrombi cannot be completed by platelet activation alone [72]. Fibrin formation, mostly on the platelet thrombi, is extremely important for the formation of thrombi large enough to occlude arteries or arterioles. While anti-GP IIb/IIIa agents inhibit the final common pathway for platelet aggregation, they do not inhibit the procoagulant activity expressed on the surface of activated platelets, except for those agents that also inhibit $\alpha_v\beta_3$ [73]. This implies that the formation of arterial occlusive thrombi, mostly composed of fibrin, cannot be effectively prevented by inhibition of the final common pathway for platelet aggregation. Aspirin and thienopyridine antiplatelet agents might be more effective for this purpose, because they inhibit relatively upstream events in the process of platelet activation. Then, how can the strong efficacy of anti-GP IIb/IIIa agents be explained in the prevention of thrombotic complications after interventional treatment? We speculate that the effects under this condition may depend on the inhibition of distal emboli: 1) Anti-GP IIb/IIIa agents have been shown to prevent thrombotic complications occurring after coronary intervention: The most frequently occurring and most effectively prevented event was non-Q wave myocardial infarction diagnosed based on the elevation of the serum CK, the development of which is associated with not only thrombotic occlusion, but also with distal embolization; [66] 2) using recently developed distal protection devices, platelet thrombi could be detected in almost all the cases after coronary intervention: 3) The myocardial perfusion images obtained by PET have demonstrated the effects of anti-GP IIb/IIIa agents in the prevention of distal emboli in patients undergoing atherectomy [74]. Similar protective effects, although less marked, may be observed in patients undergoing coronary intervention.

FUTURE DIRECTIONS IN THE DEVELOPMENT OF ANTIPLATELET AGENTS FOR THE PREVENTION OF ATHEROTHROMBOSIS.

(1). Drugs Specifically Inhibiting the Development of Arterial Thrombosis

Antiplatelet agents, such as anti-GP IIb/IIIa, have been developed as agents that block platelet functions, in general, to prevent platelet aggregation. These strong antiplatelet agents, however, are associated with a strong bleeding tendency. Presently, we are beginning to understand the specific processes involved in the development of arterial thrombosis as compared to those involved in the formation of hemostatic platelet plugs. In my personal opinion, the following differences should be taken into consideration while developing antiplatelet agents for specifically inhibiting atherothrombosis associated with a minimal bleeding tendency.

(A). Platelet Procoagulant Activity

Since the formation of arterial occlusive thrombi is characterized mainly by excess fibrin formation [72] that is initiated by the procoagulant activity of platelets adhering to the surface of exposed subendothelial matrix, [78,75,76] regulation of the platelet procoagulant activity may be the most important target for the development of antiplatelet or antithrombotic agents to specifically inhibit the formation of arterial occlusive thrombi. Although the mechanisms are not fully understood, exposure of the negatively charged phopholipids on the surface of platelets, microparticle release and the accumulation of tissue factor on the surface of activated platelets [18,75-77] have been suggested to play important roles in the expression of procoagulant activity on the surface of activated platelets. We have shown previously that among the three anti-GP IIb/IIIa agents currently available for clinical use, tirofiban has the weakest activity against expression of platelet procoagulant activity induced by the VWF-GP Ibα interaction under high shear stress conditions [73]. This may contribute to the superior preventive effects of abciximab over tirofiban in the prevention of acute thrombotic events, as demonstrated in large-scale randomized head to head comparisons; [78] the difference in the effectiveness between the two drugs was, however, no longer significant after 6 months [79]. We may therefore expect to develop a new generation of specific antiplatelet agents associated with a minimal bleeding tendency to prevent arterial thrombotic occlusion by developing agents inhibiting platelet procoagulant activity. Further investigations must be conducted to gain a complete understanding of the mechanism of expression of the platelet procoagulant activity.

(B). Receptors Involved in Platelet Thrombus Formation on the Exposed Subendothelial Matrix Under Blood Flow Conditions.

Since arterial thrombosis, such as in the acute coronary syndrome, is initiated by exposure of the subendothelilal matrix under conditions of arterial blood flow, drugs inhibiting the platelet-matrix interaction under blood flow conditions might have specific inhibitory effects on the onset of atherothrombosis. In this regard, there are several potentially important targets, some of which have already been discussed. VWF and its receptor GP Ibα are unique targets, because they play a role only under conditions of high shear stress. It might be useful to develop agents that block the VWF-GP Ibα interaction, which would be expected to be effective only under conditions of high shear stress, such as in the case of coronary thrombosis occurring at sites of atherosclerotic stenosis. Moreover, the bleeding tendency observed in patients completely deficient in VWF or GP Ibα is usually relatively mild as compared to that seen in patients deficient in GP IIb/IIIa. These findings lend support to the hypothesis that the VWF-GP Ibα interaction, or the direct signals generated by this interaction might serve as potentially useful targets for the development of relatively selective antiplatelet agents for the prevention of atherothrombosis.

As discussed previously, collagen receptors, in particular, GP VI, might also be potentially suitable target for the development of antiplatelet agents specifically effective in the prevention of arterial thrombosis, because collagen exposure to the bloodstream after atheroma rupture is the initial event that triggers the development of acute myocardial infarction. The bleeding tendency caused by blockade of GP VI is expected to be relatively mild, because patients deficient in GP VI and knockout mice deficient in GP VI have been shown to exhibit only a modest bleeding tendency [55]. Recently, some very interesting findings were published by Nieswandt *et al.* They showed that injection of anti-GP VI antibody, without directly inhibiting the collagen-GP VI interaction, not only attenuated the platelet surface expression of GP VI, but also the production of GP VI mRNA in megakaryocytes [80].

CONCLUSIONS

(1) The mechanisms of arterial thrombus formation *in vivo* are different from those underlying platelet aggregation in conventional aggregometry. Not only GP IIb/IIIa ligation, but also numerous other receptor-ligand interactions are involved in the former.

(2) Anti-GP IIb/IIIa agents, developed as strong inhibitors of platelet aggregation, have been reported to be effective in the prevention of thrombotic complications after coronary intervention, but their effects on the prevention of atherothrombosis are limited. Drugs inhibiting activation events upstream of the final common pathway might be more effective.

(3) Future antiplatelet agents should selectively prevent atherothrombosis without being associated with bleeding tendency. In this regard, the procoagulant activity expressed on the platelet surface, as well as molecules specifically involved in platelet thrombus formation on the exposed subendothelial matrix under blood flow conditions, might serve as good targets.

ACKNOWLEDGEMENT

This work was supported in part by a Grant-in-Aid for Scientific Research in Japan (09770506, 11770367,

15590771), the Tokai University School of Medicine Research Aid 1997, 1998 and 1999, grant JSPS-RFTF97I00201 from the Japanese Society for the Promotion of Science, grant from the Takeda Science Foundation, 1998, grant from the Yamanouchi Foundation for Research on Metabolic Disorders, 1998, grant from the Japanese Medical Association, 1998; research fund of the Mitsukoshi Health and Warfare Foundation, 1999, the Suzuken Memorial Fund, 1999, research fund from the Japanese Council on Molecular Cardiology, 2000, a research fund from the Mitsui Life Insurance Foundation, a research fund from the Japan Foundation of Cardiovascular Research, a grant from the Mochida Memorial Foundation for Medical and Pharmaceutical Research, a grant for Advanced Medicine Supported by the Ministry of Health, Labor and Welfare, support from the Department of Oriental Medicine (Tsumura) 2003, and a grant from Novartis Foundation (Japan) for the Promotion of Science.

ABBREVIATIONS

GP= Glycoprotein

VWF = Von Willebrand factor

ADP = Adenosine 5'-diphosphate

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Hemostatic effects of fibrinogen γ-chain dodecapeptide-conjugated polymerized albumin particles in vitro and in vivo

Yosuke Okamura, Shinji Takeoka, Yuji Teramura, Hitomi Maruyama, Eishun Tsuchida, Makoto Handa, and Yasuo Ikeda

BACKGROUND: Prototypes of platelet (PLT) substitutes have been studied and the focus was on a dodecapeptide, HHLGGAKQAGDV (H12), which is a fibrinogen γ-chain carboxy-terminal sequence (γ 400-411) and exists only in the fibrinogen domain.

STUDY DESIGN AND METHODS: H12 was conjugated to the surface of polymerized albumin particles (polyAlb) as biocompatible and biodegradable particles with a mean diameter of 260 ± 60 nm, and the hemostatic ability of H12-conjugated polyAlb (H12-polyAlb) under flow conditions and thrombocytopenic rats have been studied.

RESULTS: H12-polyAlb enhanced the in vitro thrombus formation of activated PLTs on a collagen-immobilized plate when exposed to the flowing thrombocytopenic imitation blood. Furthermore, the analysis of the tail bleeding time of rats that were made thrombocytopenic by busulfan injection showed that H12-polyAlb had a hemostatic effect. Based on the bleeding time and the amount injected, the hemostatic capacity of 20 H12-polyAlb was estimated to correspond to that of one PLT.

CONCLUSION: These results were important first steps toward the development of PLT substitutes and indicated that H12-polyAlb may be a suitable candidate for an alternative to human PLT concentrates transfused into thrombocytopenic patients in the future.

Platelet (PLT) transfusion plays an important role in supportive therapy of patients with thrombocytopenia caused by hematologic malignancies, cancer, or during surgical procedures and radiotherapy. The shortage of PLTs, however, has always been a serious problem because of the short storage life of PLT concentrates (72 hr in Japan). In addition, the risk of viral and bacterial infections by transfusion is a serious concern. PLT substitutes such as solubilized PLT membrane protein-conjugated liposomes (plateletsome),[1] infusible PLT membranes,[2] fibrinogen-bonded red blood cells (RBCs),[3] fibrinogen-coated albumin microcapsules (synthocyte),[4] and arginine-glycine-asparaginic acid (RGD) peptide-bound RBCs (thromboerythrocyte)[5] have been developed to solve these problems. Despite their usefulness in enhancing PLT aggregation and reducing bleeding

ABBREVIATIONS: cH12 = control H12; cH12 polyAlb = cH12-conjugated polyAlb; GP = glycoprotein; H12 = HHLGGAKQAGDV dodecapeptide; H12-polyAlb = H12-conjugated polyAlb; polyAlb = polymerized albumin particles; PRP = platelet-rich plasma; SPDP = *N*-succinimidyl 3-(2-pyridyldithio)propionate.

From the Graduate School of Science and Engineering and the Advanced Research Institute for Science and Engineering, Waseda University, Tokyo; and the Department of Internal Medicine and the Department of Transfusion Medicine & Cell Therapy, School of Medicine, Keio University, Tokyo, Japan.

Address reprint requests to: Shinji Takeoka, Graduate School of Science and Engineering, Waseda University, Tokyo, 169-8555, Japan; e-mail: takeoka@waseda.jp.

This work was supported in part by Health and Labor Sciences Research Grants (Research on Pharmaceutical and Medical Safety, S.T. M.H., and Y.I); Ministry of Health, Labor and Welfare, Japan; and grants-in-aid from the JSPS, Japan (No. 15300171, S.T), Ministry of Education, Culture, Sports, Science and Technology (Leading Project for Biosimulation, M.H) and 21COE "Practical Nano-Chemistry" and "Consolidated Research Institute for Advanced Science and Medical Care" from MEXT (S.T), Japan.

Received for publication June 28, 2004; revision received December 16, 2004, and accepted December 16, 2004.

doi: 10.1111/j.1537-2995.2005.00173.x

TRANSFUSION 2005;45:1221-1228.

time in vivo, these PLT substitutes consist of materials derived from blood components.

PLT membrane glycoprotein (GP) Ibα and GPIa/IIa are the receptors for von Willebrand factor (VWF) and collagen, respectively.[6-8] We have focused our studies on recombinant forms of these proteins (rGPIbα[9,10] or rGPIa/IIa complex[11-13]) and have conjugated them to biocompatible carriers such as polymerized albumin particles (polyAlb)[11,14,15] and phospholipid vesicles (liposomes).[12,13,16] In particular, we found that rGPIa/IIa-conjugated polyAlb reduced the bleeding time of thrombocytopenic mice in vivo.[11]

PLTs that adhere to the collagen-immobilized surface are activated, and the conformation of GPIIb/IIIa that exists on PLT membrane changes from a silent state to an activated state.[17] This leads to the binding of fibrinogen and VWF[18,19] followed by PLT aggregation.[20,21] Fibrinogen contains three putative PLT interaction sites, namely, a tetrapeptide containing RGD sequences such as RGDF and RGDS at α95-98 and α572-575, respectively,[22] and a dodecapeptide (HHLGGAKQAGDV, H12) corresponding to a γ-chain carboxy-terminal segment (γ400-411).

We have also developed fibrinogen-conjugated polyAlb, which was shown to facilitate the accumulation of flowing PLTs into polyAlb aggregates after their attachment to an activated PLT-immobilized surface in vitro.[14] These findings confirmed that such conjugates could emulate the function of PLTs in primary hemostasis. Fibrinogen from human blood, however, is not stable, and its activity in solution is extremely low.[14]

Recently, we focused on H12, which exists only in fibrinogen domain.[23-30] Based on our result obtained from flow cytometric analyses of agglutination, the H12-conjugated latex beads showed minimal interaction with nonactivated PLTs in comparison with RGD-conjugated latex beads.[31] Furthermore, H12-conjugated latex beads enhanced the in vitro PLT thrombus formation on collagen-immobilized plates when exposed to flowing thrombocytopenic imitation blood.[31]

In this study, we conjugated H12 to the surface of polyAlb to produce biocompatible and biodegradable particles and evaluated their effect on enhancement of thrombus formation on a collagen-coated surface in the presence of the H12-polyAlb when exposed to flowing thrombocytopenic imitation blood in vitro. We prepared thrombocytopenic rats by busulfan administration, intravenously administered the H12-polyAlb into the rats, and measured the tail bleeding time for evaluation of the hemostatic properties of the particles.

MATERIALS AND METHODS

Reagents

A fibrinogen γ-chain dodecapeptide (C-HHLG GAKQAGDV, H12) or a reverse sequence of H12 (C-VDGAQKAGGLHH, control H12 [cH12]) was synthesized with a solid-phase synthesizer by BEX (Tokyo, Japan). *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and an anticoagulant D-Phe-Pro-Arg-chloromethylketone were purchased from Pierce Chemical Co. (Rockford, IL) or Calbiochem (San Diego, CA). 3,3′-Dihexyloxacarbocyanine iodide, which is a PLT fluorescent dye, was purchased from Molecular Probes (Eugene, OR). Both busulfan and polyethylene glycol (PEG; mean molecular weight, 400) were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human serum albumin (rHSA) was donated by Mitsubishi Pharma (Osaka, Japan).

Preparation of H12-polyAlb or cH12-conjugated polyAlb

A solution of rHSA (250 mg/mL) was dialyzed against distilled water for 6 hours at 4°C to remove stabilizers such as *N*-acetyl-D,L-tryptophan and sodium caproate. The rHSA solution (25 mL) was diluted with saline to 10 mg per mL, and the pH was adjusted to 10.7 (at room temperature) by titration with 0.1 N NaOH (800 μL). The solution was heated to 80°C for 10 minutes, rapidly cooled in an ice bath, and then brought to room temperature. The pH was adjusted to 6.1 at room temperature by dropwise addition of 0.1 N HCl (900 μL) and then the solution was stirred at 40°C for 120 minutes until the turbidity reached 0.4 ± 0.1. Excess iodoacetamide (25 mg) was added to terminate polymerization, and the solution was dialyzed against PBS at 4°C for 24 hours. A 25-mL dispersion of polyAlb ([rHSA] = 9.0 mg/mL, pH 7.4) was thus prepared. Mean diameter was determined by a dynamic scattering method (Coulter N4 Plus submicron particle sizer, Beckman-Coulter, Miami, FL). H12 was conjugated to the surface of polyAlb as previously described.[15] A solution of SPDP in ethanol (20 mmol/L, 15 μL) was added to the polyAlb suspension (18 mg/mL, 10 mL), and the suspension was stirred for 30 minutes at room temperature. The unreacted SPDP and the by-products were separated by repeated centrifugation and washing with saline (30 000 × *g*, 10 min, 4°C, three times), and the pyridyl disulfide-bonded polyAlb was collected. A suspension of pyridyl disulfide–bonded polyAlb (15 mg/mL, 10 mL) was mixed with a solution of H12 (100 mmol/L, 20 μL) and allowed to react at 20°C for 12 hours. The unreacted reagents were removed by repeated centrifugation and washing with saline (30 000 × *g*, 10 min, 4°C) to obtain the purified H12-conjugated polyAlb (H12-polyAlb, 10 mg/mL, 10 mL). The concentration of the H12 conjugated on the polyAlb was determined by the quantification of the 2-thiopyridone that was liberated by the thiol-disulfide exchange reaction, with high-pressure liquid chromatography on a TSK-GEL G3000SW$_{XL}$ column (7.8 mm o.d. × 300 mmh in PBS at 1 mL/min), by measuring the absorbance of the column effluent at 343 nm. The cH12-conjugated polyAlb (cH12-

polyAlb) were prepared by the same method as mentioned above.

PLT aggregation study

Blood withdrawn from healthy volunteers was mixed with 10 percent volume of 3.8 percent (wt/vol) sodium citrate. PLT-rich plasma (PRP) was prepared by centrifugation ($100 \times g$, 15 min, 22°C), and the PLT concentration of PRP was adjusted to 200×10^3 per μL by PLT-poor plasma prepared by centrifugation ($2200 \times g$, 10 min, 22°C). The PLT concentration was determined with an automated hematology analyzer (K-4500, Sysmex, Kobe, Japan). A 20 μmol per L ADP solution was added to the PRP containing H12 or cH12 solutions adjusted to final concentration of 1 mmol per L, and the light transmittance was measured with an aggregometer (Hema Tracer T-638, Nico Bioscience, Tokyo).

Preparation of a collagen-immobilized surface

Collagen I-A (3.0 mg/mL, Cellmatrix, Nitta Gelatin, Osaka, Japan) was suspended in PBS at 4°C to give a final concentration of 30 μg per mL. A glass plate (diameter, 24 mm; thickness, 0.5 mm) was immersed into the collagen suspension at 4°C for 8 hr, carefully rinsed with PBS, and then immersed in a bovine serum albumin solution (20 mg/mL) at room temperature for 2 hr.

Measurement of the interaction of PLTs with the collagen surface in the presence of H12-polyAlb with thrombocytopenic imitation blood

Blood withdrawn from healthy volunteers was treated with the thrombin inhibitor D-Phe-Pro-Arg-chloromethylketone (final concentration, 40 μmol/L) and was filtered through a white blood cell (WBC) removal filter (NEO1J, Nihon Poll, Tokyo, Japan), which could remove PLTs as well as WBCs. The residual PLT concentration of the filtered blood was determined to be $5.0 \times 10^3 \pm 3.0 \times 10^3$ per μL, and the final PLT concentration was adjusted to 20×10^3 per μL by addition of PRP, which was prepared by centrifugation ($100 \times g$, 15 min, 22°C) of sodium citrate-treated blood. The PLT concentration was determined with an automated hematology analyzer (K-4500). This blood preparation was termed as thrombocytopenic imitation blood.

The thrombocytopenic imitation blood and H12-polyAlb (10 mg/mL, 70 μL) mixtures were placed in a recirculating chamber mounted on an epifluorescent microscope (ECLIPS TE300, Nikon, Tokyo, Japan) equipped with a CCD camera, and the interaction of PLTs with the collagen immobilized on the surface was observed. Single-frame images of adhesion and aggregation of PLTs in the presence of H12- or cH12-polyAlb were obtained with an image processor (Argus-50, Hamamatsu Photonics, Hamamatsu, Japan), and the surface coverage of the adhered PLTs on the plate was calculated with an image processor (Argus-20, Hamamatsu Photonics). All perfusion studies were performed at 37°C.

Measurement of the tail bleeding time of the thrombocytopenic rats

All animal studies were approved by the Animal Subject Committee of Keio University, School of Medicine, and performed according to NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication 85-23, Rev. 1985). Experiments were carried out with male Wistar rats (230-250 g, CLEA Japan, Tokyo, Japan). A busulfan solution was prepared at a final concentration of 5 mg per mL in PEG (mean molecular weight, 400).[32,33] Rats were anesthetized with diethyl ether and injected on Day 0 and Day 3 with 10, 15, or 20 mg per kg on each dosing day, to produce a total dosage of 20, 30, or 40 mg per kg busulfan, respectively. Blood samples for cell counting were obtained from ether-anesthetized rats by inserting a 25-gauge needle into a tail vein, and the cell concentration was determined with an automated hematology analyzer (K-4500).

On Day 10, thrombocytopenic rats were anesthetized with a general anesthetic sedative (Sevofrane), and the sample suspension was infused into the tail vein. The samples were H12-polyAlb, cH12-polyAlb, or polyAlb at a dose of 4 mL per kg; saline was used to obtain the control value. Five minutes after administration, a 2.5 mm length × 1.0 mm depth template-guided incision (Quikheel, Becton-Dickinson, San Jose, CA) was made 1 cm from the tip of the tail. A tail was immersed in a 50 mL cylinder of saline, and the time taken for bleeding to stop was measured. In addition, cell concentrations were determined with an automated hematology analyzer (K-4500) before (–5 min) and after (30 min) samples injection.

Statistical analysis

Significance of the Day 10 group versus the normal group as shown in Fig. 4, and the H12-polyAlb group versus the saline group, the polyAlb group, and the cH12-polyAlb group as shown in Fig. 5, was tested with Tukey-Kramer tests. A p value of less than 0.05 was considered to be significant. Analytic software was used (StatView, SAS Institute Inc., Cary, NC).

RESULTS

PLT aggregation study

By use of an aggregometer, we confirmed that H12 showed a concentration-dependent suppression of PLT aggrega-