

**Table 1.**  
Age- and Sex-Related Distribution of Protein C and S Levels in General Population

Age group, y	Protein C, %				Protein S, %			
	Men		Women		Men		Women	
	n	Mean $\pm$ SD	n	Mean $\pm$ SD	n	Mean $\pm$ SD	n	Mean $\pm$ SD
30-39	66	118.9 $\pm$ 20.9	88	108.8 $\pm$ 19.2	46	100.7 $\pm$ 20.0	62	79.1 $\pm$ 17.0
40-49	262	126.0 $\pm$ 19.0	388	114.4 $\pm$ 19.3	165	102.6 $\pm$ 21.6	252	79.4 $\pm$ 17.7
50-59	373	123.8 $\pm$ 19.7	593	129.4 $\pm$ 20.2	231	98.5 $\pm$ 21.5	338	82.8 $\pm$ 17.1
60-69	660	117.8 $\pm$ 19.6	745	131.3 $\pm$ 19.8	390	93.6 $\pm$ 20.5	442	85.6 $\pm$ 18.5
70-79	555	113.1 $\pm$ 20.1	491	128.2 $\pm$ 19.7	324	85.1 $\pm$ 18.6	278	83.6 $\pm$ 17.2
80-89	167	107.1 $\pm$ 18.7	117	122.7 $\pm$ 18.9	96	78.3 $\pm$ 17.8	66	82.0 $\pm$ 16.6

Prevalence of deficiency estimated in the Japanese general population was then compared with the prevalence reported for the white general population (Table 2). The prevalence values for antithrombin and protein C deficiencies were quite similar, both conditions affecting 1 of 500 to 700 individuals. Differences were nevertheless noticeable with respect to the other factor measured. Plasminogen deficiency in particular has a high prevalence among Japanese but not among whites. Protein S deficiency may also have a higher prevalence among Japanese, even if caution is exercised about such a conclusion, because the assay used and the criteria used to define deficiency differ between studies.

We measured plasminogen, antithrombin, and protein C activity in 108 patients with deep vein thrombosis to estimate prevalence of deficiency (Table 3). Comparison of prevalence in the general population with that in the deep vein thrombosis group revealed that antithrombin and protein C deficiencies were genetic risk factors associated with deep vein thrombosis in the Japanese population (odds ratios, 38 and 52, respectively) [16]. In contrast, there was no evidence of a link between plasminogen deficiency and risk of deep vein thrombosis [15].

## 5. Genetic Changes in Thrombosis-Related Proteins in the Japanese Population

Factor V Leiden and prothrombin G20210A are genetic risk factors for deep vein thrombosis in white populations, but mutations have not been found in the Japanese population. No other genetic variations have been formally identified

**Table 2.**  
Comparison of Prevalence of Deficiencies of Proteins C and S, Antithrombin, and Plasminogen between Japanese and Westerners\*

Deficiency	Population	General	
		Population, %	DVT Group, %
Protein C	Japanese	0.13	6.5
	Westerners	0.15-0.33	3.2
Protein S	Japanese	1.12	ND
	Westerners	0.03-0.13	1.3-2.2
Antithrombin	Japanese	0.15	5.6
	Westerners	0.17	1.1
Plasminogen	Japanese	4.29	2.8
	Westerners	0.3-0.5	ND

\*Data from the literature [15-17,19,21,23,53]. Prevalence in the Japanese population was estimated from the plasma activity of each factor. DVT indicates deep vein thrombosis; ND, not determined.

as genetic risk factors. Nevertheless, 5 genetic changes in thrombosis-related genes that may have an effect on the occurrence of deep vein thrombosis are known to be present in the Japanese population.

A missense mutation causing an Ala to Thr change at position 620 (A620T) of mature plasminogen has been identified in a Japanese patient with recurrent deep vein thrombosis [25,28]. The mutation was formerly called A601T and was referred to as plasminogen Tochigi, but the numbering standards adopted by the Nomenclature Working Group recommend that the A of the ATG of the initiator Met codon be denoted nucleotide +1 and that the initial Met residue amino acid be denoted +1 [29], causing us to rename several of the mutants we characterized. A patient with the A620T mutation exhibited decreased plasminogen activity, but antigen level was within normal limits [30]. In the mini-plasmin crystal structure, the mutation is located just before the active His residue (Ala55 in the chymotrypsin numbering system) [31]. Small-scale studies have shown that allele frequency for the plasminogen Tochigi mutation is between 0.011 and 0.021 [32,33]. The mutation has been found with an allele frequency of approximately 0.015 in the Chinese Han population and with a frequency of 0.016 in the Korean population [33] but has not been found in white populations [32].

ADAMTS13 is a von Willebrand factor (VWF)-cleaving protease [34]. Defects in the ADAMTS13 gene cause thrombotic thrombocytopenic purpura, a disease characterized by thrombocytopenia and microangiopathic hemolytic anemia with variable degrees of renal failure, neurological dysfunction, and fever. A missense mutation causing replacement of Pro475 by Ser (P475S) in the Cys-rich domain of ADAMTS13 was identified with an allele frequency of approximately 0.05 in the Japanese population [35]. Results of in vitro studies indicated the mutation has low VWF-cleaving activity. Homozygotes for this mutation retain ADAMTS13 activity and thus do not have the thrombotic thrombocytopenic purpura phenotype. Although polymorphism is found in Chinese populations at a lower frequency, it has not been identified in white populations [36-38].

Protein S is an important regulator of coagulation, and a missense mutation causing Lys196 to be replaced by Glu (K196E mutation, formerly known as protein S Tokushima and referred to as K155E mutation) within the second epidermal growth factor-like domain of protein S has been identified in Japanese patients with deep vein thrombosis [39,40]. As in the A620T mutation in plasminogen, protein S activity was decreased in carriers of the K196E mutation, but

**Table 3.**

Comparison of Prevalence of Protein C, Antithrombin, and Plasminogen Deficiencies between Deep Vein Thrombosis Group and General Population\*

	No. of heterozygotes (prevalence)		Odds ratio, 95% CI (vs general population)	P
	Patients with DVT (n = 108)	General population (n = 4517)		
Protein C	7 (6.48%)	6 (0.13%)	52.1 (17.2-157.9)	<.0001
Antithrombin	6 (5.56%)	7 (0.15%)	37.9 (12.5-114.8)	<.0001
Plasminogen	3 (2.78%)	194 (4.29%)	0.6 (0.2-2.0)	.5958

\*Data from Okamoto et al [15] and Sakata et al [16]. DVT indicates deep vein thrombosis; CI, confidence interval.

antigen levels were within normal limits [39,41]. Frequency of the allele has been reported to be 0.008 [39], and in vitro studies have revealed that the K196E mutation has decreased APC cofactor activity and poorly accelerated inactivation of prothrombinase [42]. Prothrombinase is the only prothrombin activator complex of the clotting cascade. It is composed of factor Xa bound to FVa on the phospholipid surface of thrombin-stimulated platelets.

A substitution in the 5'-untranslated region of the factor XII gene has been reported. It consists of a C-to-T change at nucleotide position -4 (-4C→T, formerly referred to as 46C→T) [43]. T-allele frequency is 0.73 in the Japanese population, and polymorphism is also found in white populations but with a lower frequency (0.2). Transcription of the T-allele is lower than normal, presumably because of creation of an additional ATG initiation codon and/or impairment of the consensus sequence that initiates translation according to the scanning model. Plasma level of factor XII ultimately decreases, as does, consequently, its associated activity [43]. However, the implications of this mutation in the development of thrombotic disorders is unclear [44].

A 4G/5G polymorphism within the promoter region of plasminogen activator inhibitor 1 (PAI-1) has also been reported. Individuals carrying the 4G allele exhibit higher plasma PAI-1 levels than 5G allele carriers, and the prevalence of the 4G allele appears significantly higher in patients with myocardial infarction than in population-based controls [45]. An in vitro study showed that a promoter region with the 4G allele is unable to bind a repressor protein. The consequence is that the basal level of PAI-1 transcription increases. The frequencies of 4G allele in the white and the Japanese populations have been reported to be 0.52 to 0.53 and 0.61, respectively [45-47]. As for factor XII polymorphism, the relation between the 4G allele and thrombotic disorders needs to be clarified [46].

### 6. Genetic Risk of Vein Thrombosis in the Japanese Population: Emerging Evidence of Protein S K196E as a Risk Factor

To address whether the 5 genetic variations described earlier are genetic risk factors for deep vein thrombosis in the Japanese population, we performed a case-control study [48]. Between December 2002 and October 2004, 161 patients with deep vein thrombosis (78 men and 83 women working under the auspices of the Ministry of Health Labor and Welfare of Japan) were registered by the Study Group of Research on Measures for Intractable Diseases. We evaluated the genetic contribution of the 5 polymorphisms by comparing their

prevalence among the 161 patients who had deep vein thrombosis with their prevalence among 3655 population-based controls. Only the E allele of the protein S gene was found to be a genetic risk factor for deep vein thrombosis in the Japanese population (Table 4). No other polymorphism was found to be statistically significant between these 2 groups. Another recent study reached the same conclusion [49].

Among the population-based controls, 66 of 3651 individuals were heterozygous for the E-allele polymorphism, and none were homozygous. This finding corresponds to an allele frequency of 0.009 within the Japanese population, consistent with the previously reported frequency of 0.0082 [39]. Extrapolating from these values, we estimate that approximately 1 of every 12,000 Japanese individuals is homozygous for the E allele, representing a total of as many as 10,000 individuals. Thus a substantial number of Japanese carry the E allele for protein S and risk of development of deep vein thrombosis. Given the frequency of this mutation and its strong correlation with deep vein thrombosis, it may be advisable to screen individuals for the E allele and recommend that carriers avoid environmental risk factors associated with deep vein thrombosis.

### 7. Perspectives on Protein S K196E Mutation Research

Protein S K196E mutation is an established genetic risk factor for deep vein thrombosis among Japanese persons and has been confirmed in 2 independent studies [48,49]. Whether the K196E mutation is also a risk factor for arterial occlusive disease and other thrombotic diseases, such as recurrent abortion, is unknown. In the case of factor V Leiden, a possible link between mutation and arterial occlusive disease is a controversial issue. It is clear that the K196E mutation in protein S is not as dramatic as would be complete deficiency of protein S or C: no homozygote carrier was identified in infants with severe purpura fulminans. It is likely that the risk of thrombosis in individuals with K196E protein S is greatly influenced by acquired risk factors. Presence of protein S K196E in addition to other risk factors associated with thrombosis, such as pregnancy, use of oral contraceptives, trauma, and an additional defect in protein C, protein S, or antithrombin, may dramatically exacerbate thrombosis tendency. Predisposing factors may act synergistically to increase the risk of thrombosis due to protein S K196E mutation.

Protein S K196E mutation was originally identified in 1993 in Japanese patients with deep vein thrombosis and belonging to 2 independent families [39,40]. In vitro studies showed that protein S with E196 has diminished capability to act as an APC cofactor. In addition, compared with the wild type, protein S

**Table 4.**  
Number and Genotype Frequency of 5 Polymorphisms in Deep Vein Thrombosis and Control Groups

	Plasminogen (A620T)	ADAMTS13 (P475S)	Protein S (K196E)	Factor XII (-4C→T)	Plasminogen Activator Inhibitor 1 (4G/5G)
Deep vein thrombosis					
Major homozygous	152	139	146	63	61
Heterozygous	9	20	13	75	69
Minor homozygous	0	1	2	23	30
Total	161	160	161	161	160
Minor allele frequency	0.028	0.069	0.053	0.376	0.403
General population					
Major homozygous	3501	3290	3585	1513	1468
Heterozygous	149	332	66	1651	1686
Minor homozygous	0	17	0	486	497
Total	3650	3639	3651	3650	3651
Minor allele frequency	0.020	0.050	0.009	0.359	0.367
$\chi^2$	0.987	2.179	75.464	0.372	3.402
P	.320	.336	<.001	.830	.183

with E196 has reduced ability to inhibit the prothrombinase complex, suggesting that mutant protein S cannot interact efficiently with both APC and factor Xa [42,50]. However, protein S activity in the plasma of carriers with the mutant allele is controversial. In 1 family, carriers had protein S activity within the normal range [39], whereas in the other family protein S activity was lower than normal [41]. Although protein S K196E mutation is directly linked to development of deep vein thrombosis, measurable protein S activity is not always reduced. This finding indicates that protein S activity may not be the proper tool for detecting plasma deficiency. Therefore genetic analysis or other direct discrimination methods must be used for proper identification of protein S K196E carriers.

Overall, the protein S K196E mutation seems to occur mainly in eastern Asian populations, but its exact geographical distribution is an important issue. The A620T mutation in plasminogen occurs with an allele frequency of 0.014 to 0.015 in the Chinese Han population and with an allele frequency of 0.016 [33] in the Korean population. It is likely that the allele frequency is similar in the Japanese population. In contrast, ADAMTS13 polymorphism has an allele frequency of 0.05 in the Japanese population but is found at a lower frequency in the Chinese population [37]. After haplotype analysis, the origin of the factor V Leiden mutation was traced back 100,000 years, to a period after the out-of-Africa migration. A more recent mutation is that of the Z allele of  $\alpha_1$ -antitrypsin, which occurred only 6000 years ago in northern Europe [51,52]. Using a similar approach, it would be interesting to trace the origin of the protein S K196E mutation, when and where it occurred first, and how it spread in the Asian population.

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## Platelet-Directed Gene Therapy

Tsukasa Ohmori Yoichi Sakata

Research Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University School of Medicine, Tochigi, Japan

### Key Words

Lentiviral vector · Hematopoietic stem cells ·  
Stem cell transplantation · Hemophilia

### Summary

Beyond their prominent role in hemostasis and thrombosis, platelets are characterized by expert functions in assisting and modulating vascular integrity, inflammatory reactions and immune responses. These pleiotropic functions are partly achieved by the release of a multitude of secretory proteins at the site of vascular injury. Since platelets can circulate throughout the body and release a number of mediators on demand, targeting platelets as a circulating delivery system would seem a reasonable approach to modify hemostasis and thrombus formation. Gene transfer in platelets requires gene transduction into hematopoietic stem cells (HSCs) using integrating vectors that directly regulate the expression of the targeted substance by a platelet-specific promoter, because platelets are anucleate cells and their precursor megakaryocytes have a limited life span. Recent studies show that gene transduction of HSCs results in sufficient genetic information been given in platelets so that they synthesize sufficient transgene products during megakaryopoiesis. Indeed, phenotype correction of a mouse model of inherited platelet disorder and hemophilia A by platelet-directed gene transduction has been demonstrated. This review highlights the cellular advantages of platelets as delivery vesicles of a specific factor, the recent advances of transgenic mice, and transduction of HSCs to establish the efficient expression of the targeted protein in platelets.

### Schlüsselwörter

Lentiviraler Vektor · Hämatopoetische Stammzellen ·  
Stammzelltransplantation · Hämophilie

### Zusammenfassung

Neben ihrer zentralen Rolle in der Hämostase und Thrombose sind Thrombozyten auch von einzigartiger Bedeutung für die Unterstützung und Modulation der vaskulären Integrität sowie von inflammatorischen und immunologischen Reaktionen. Die pleiotropen Wirkungen basieren teilweise auf der Freisetzung einer Vielzahl von sezernierten Proteinen an Orten vaskulärer Verletzungen. Aufgrund der typischen Zirkulation der Thrombozyten im gesamten Körper und der gezielten Freisetzung von Mediatoren, stellen die Thrombozyten ein ideales zirkulierendes System zur Abgabe von Substanzen mit dem Ziel der Modifikation der Hämostase und der Thrombusbildung dar. Der Gentransfer in Thrombozyten setzt eine Gentransduktion in hämatopoetischen Stammzellen (HSZs) mit Hilfe von integrierenden Vektoren voraus, die eine direkte Regulation der Expression von Zielsubstanzen über thrombozytenspezifische Promotoren steuern, da Thrombozyten als anukleäre Zellen und auch die Megakaryozyten als Vorläuferzellen nur eine sehr begrenzte Lebensdauer aufweisen. Aktuelle Untersuchungen zeigten, dass bei der Gentransduktion von HSZs eine ausreichende Menge genetischer Information an Thrombozyten übertragen wird und eine genügende Menge des Transgens während der Megakaryopoese synthetisiert wird. In der Tat konnte eine phänotypische Korrektur nach dem auf die Thrombozyten gerichteten Gentransfer in einem Mausmodell mit hereditärem Thrombozytendefekt und Hämophilie A nachgewiesen werden. Dieser Übersichtsartikel beleuchtet neben den zellulären Vorteilen von Thrombozyten als zirkulierende Vesikel für die Übermittlung hämostasespezifischer Faktoren auch die aktuellen Entwicklungen im Bereich der transgenen Mausmodelle sowie den Wissensstand der Transduktion hämatopoetischer Stammzellen zur Etablierung einer effizienten Expression von Zielproteinen in Thrombozyten.

## Introduction

Platelets are an essential element of the body's hemostasis system, but through their involvement in thrombosis are also a major cause of morbidity and mortality [1, 2]. Since platelets are anucleate cells and have a limited lifespan of 7–10 days, they must be continually manufactured from their precursor megakaryocytes and released into circulating blood from bone marrow [3]. When stimulated, circulating platelets adhere and aggregate with each other to generate primary hemostasis and release a variety of substances, thus initiating the coagulation cascade and protecting the integrity of the vasculature [1, 2]. Platelets have an attractive future as a delivery system of various substances because they circulate throughout the body and specifically and locally release appropriate substances at the site of thrombus formation. These platelet-specific characterizations indicate that platelet-directed gene therapy is a very attractive therapeutic application for both inherited platelet disorders and coagulation factor deficiencies. In this review, the first section covers the mechanisms by which platelets are activated, and the involvement of platelets in the coagulation cascade for a better understanding of why platelets are utilized as targeted cells. We then discuss results from the use of transgenic mice, recent advances in the transduction of hematopoietic stem cells (HSCs) by viral vectors, and the application of platelet-directed gene therapy.

## Platelet Activation and Release Reaction

Circulating platelets do not normally encounter the connective tissue matrix that lies beneath vascular endothelial cells [4]. Once a break within the integrity of this vascular lining occurs, platelets are exposed to, and interact with, collagen via interactions of the glycoprotein (GP) Ib $\alpha$  / GPV / GPIX complex on the platelet surface with von Willebrand factor (VWF) (fig. 1) [5]. Platelet interactions with collagen not only provide a surface for platelet adhesion through the GPIb $\alpha$ /GPV/GPIX complex (CD42b+CD42c/CD42d/CD42a), but also serve as a strong stimulus for platelet activation through its collagen receptors GPVI and GPIIb/IIIa (also known as integrin  $\alpha$ 2 $\beta$ 1, CD29/CD49b) [6, 7]. This results in signaling pathways that induce platelets to change their shape, spread along collagen fibrils and secrete thromboxane A<sub>2</sub> (TxA<sub>2</sub>) and adenosine diphosphate (ADP) into circulation [4, 6, 7]. The released TxA<sub>2</sub> and ADP stimulate neighboring platelets, causing them to become activated and in turn secrete additional TxA<sub>2</sub> and ADP. Activated platelets directly bind to the abundant plasma protein fibrinogen, via the platelet receptor GPIIb/IIIa (also known as integrin  $\alpha$ IIb $\beta$ 3; CD41/CD61) [8]. This platelet-fibrinogen-platelet interaction initiates the process of platelet aggregation (fig. 1).

Another important function of platelets is the release of a variety of substances that modulate the coagulation cascade

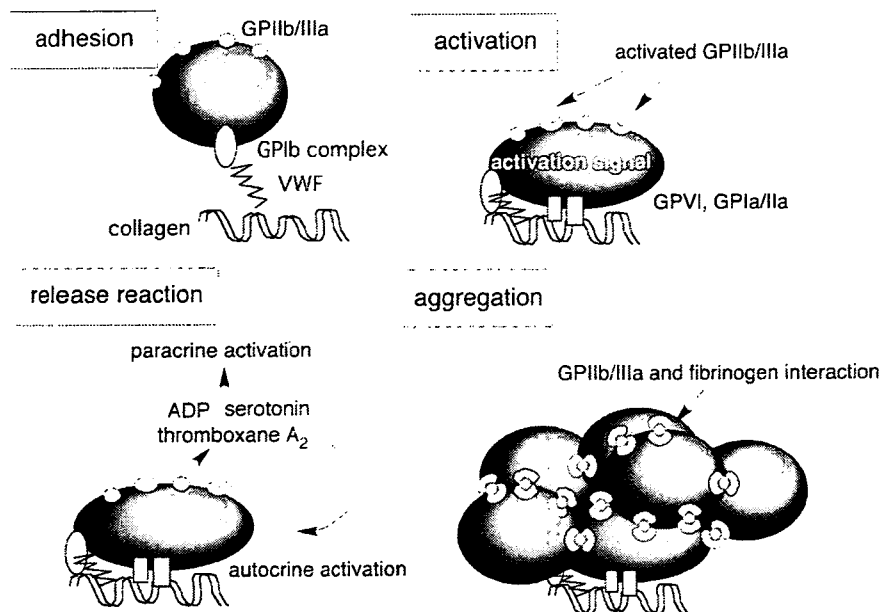
**Table 1.** Major bioactive substances and glycoproteins within platelet granules

	Bioactive substances	Glycoproteins
$\alpha$ -Granules	<i>Cytokine, growth factors</i> platelet factor-4 (PF-4) $\beta$ -thromboglobulin ( $\beta$ -TG) thrombospondin (TSP) platelet-derived growth factor (PDGF) vascular endothelial cell growth factor (VEGF) insulin-like growth factor (IFG) fibroblast growth factor (FGF) hepatocyte growth factor (HGF) RANTES <i>Coagulation factor, fibrinolytic factor</i> PAI-1, coagulation factor V VWF, fibrinogen	GPIIb/IIIa GPIb/VI/IX GPIV GPVI GLUT-3  P-selectin PECAM-1 CD40L
Dense granule	ADP, ATP serotonin calcium	LIMP-1(CD63) Ral, Rab
Lysosome	$\beta$ -hexyaminidase $\beta$ -glycerophosphatase collagenase	LIMP-1, -2, -3

and/or functions of platelets and other cells [9, 10]. These can regulate thrombus formation and affect its mechanical properties as well as contribute to cell-adhesive events, immunity, and the growth of vascular cells. During circulation, platelets are reactive to various stimuli and release materials stored in specific granules. Platelets thus transport specific compounds throughout the body and release a variety of substances at sites of vascular injury.

Platelets contain 3 types of granules within cytoplasm; lysosomes, dense granules, and  $\alpha$ -granules (table 1). These three specific granule populations store different types of constituents, some at high concentrations. Dense granules contain the small non-protein molecules responsible for autocrine and/or paracrine platelet activation, including serotonin and ADP [9, 10]. Recently, Slc35d3, an orphan member of a nucleotide sugar transporter family, was shown to specifically regulate the contents of platelet-dense granules [11].  $\alpha$ -Granules, the most abundant granules in platelets, contain proteins (e.g., fibrinogen, fibronectin, vitronectin, VWF) that enhance the adhesive process, along with a large number of growth factors and cytokines that interact with other cells (table 1) [9].  $\alpha$ -Granules also have coagulation and fibrinolytic factors that modulate thrombus formation. Platelets contribute approximately 20% of the factor V (FV) present in whole blood, with nearly all of it in  $\alpha$ -granules. Platelet FV is stored within platelets as partially proteolyzed molecules, ranging in molecular mass from 115 to 330 kDa [12]. Platelet FV exhibit significant cofactor activity upon release from platelets demonstrating a 2- to 3-fold increase in cofactor activity upon further ac-

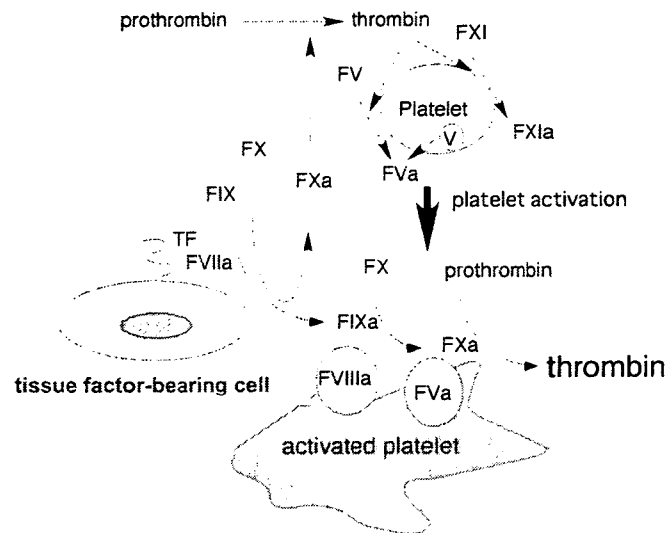
**Fig. 1.** Platelet activation at sites of vascular injury. The initial interaction of platelets with subendothelial collagen under high shear conditions is mediated by the plasma protein VWF, which binds collagen and platelet GPIb (adhesion, top left). This unstable interaction facilitates transient tethering and rolling. GPIb-mediated adhesion is superseded by more stable binding to collagen by GPVI and GPIIb/IIIa (activation, top right). Collagen receptors mediate platelet activation signalling, which results in spreading and in the secretion and release of thromboxane A<sub>2</sub> and ADP (release reaction, bottom left). Finally, GPIIb/IIIa affinity becomes up-regulated, resulting in fibrinogen-mediated platelet aggregation through binding to GPIIb/IIIa (aggregation, bottom right)



Building upon lessons learned about the role of the SNARE complex (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) in neuronal cell exocytosis [15], there has been a substantial increase in our understanding of platelet secretion. Platelets have the three basic components of the SNARE machinery: t-SNAREs (target receptors), v-SNAREs (vesicle-associated membrane receptors), and soluble components (including NSF and NSF-attachment proteins) [16]. The SNARE machinery regulates the association and subsequent fusion of vesicles with membranes. The molecular mechanisms of the platelet release reaction have been reviewed in detail elsewhere [10, 16].

### Involvement of Platelets in Coagulation Cascade

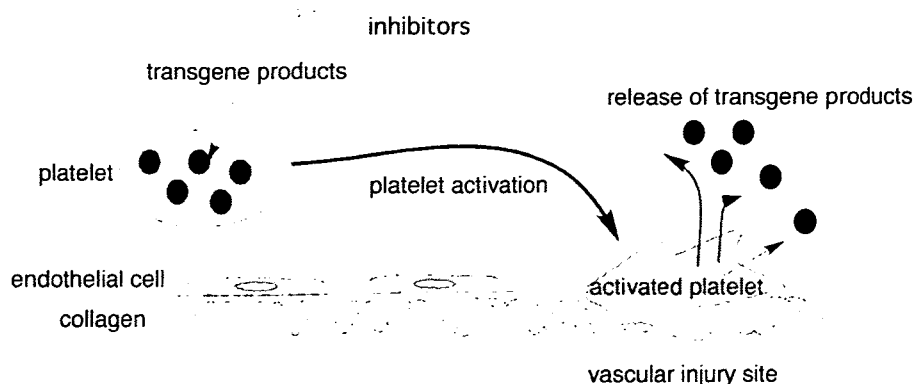
Platelets also play an important role in localizing clotting reactions to the sites of vascular injury (fig. 2). Platelets adhere and aggregate at the same sites where tissue factor (TF) is exposed [17, 18]. Once platelets are activated, cofactor-activated FV (FVa) (released from activated platelets) and activated factor VIII (FVIIIa) are rapidly colocalized to the platelet membrane surface [19, 20]. Cofactor binding is mediated in part by the exposure of phosphatidyl serine on the platelet membrane, a process resulting from a flip-flop mechanism whereby phosphatidyl serine on the inner leaflet of the membrane bilayer flips to the outside [20]. Activated factor IX (FIXa) formed by the factor VIIa (FVIIa) / TF complex binds to the surface of activated platelets. Specific receptors on the activated platelets bind FIXa and promote the formation of FIXa/FVIIIa complexes. Once the platelet tenase complex is assembled, factor X (FX) is recruited from the plasma and activated to FXa on the platelet surface. FXa then associates



**Fig. 2.** Involvement of platelets in the coagulation cascade. Cofactors FVa (released from activated platelets) and FVIIIa are rapidly colocalized to the platelet membrane surface. FIXa formed by the FVIIa/TF complex binds to the surface of activated platelets. Activated platelets bind FIXa and promote the formation of FIXa/FVIIIa complexes. Once the platelet-tenase complex is assembled, FX is activated to FXa on the platelet surface. FXa then associates with FVa on the surface to generate a burst of thrombin sufficient to clot fibrinogen.

with activated factor Xa (FXa) or thrombin [13]. Platelet-derived FV appears to support hemostasis even in patients with an acquired FV inhibitor [14], suggesting that platelets can deliver coagulation factor and protect degradation by any circulating inhibitors. Lysosomal granules contain glycosidases and proteases that have an unclear function in platelet biology [9].

**Fig. 3.** Advantages of platelet-directed gene therapy. Platelets provide a way to enhance the local concentrations of target substances at sites of vascular injury while minimizing the influence of plasma proteins that may inhibit their activities. Platelet-mediated protein delivery may also inhibit the emergence of neutralizing antibodies because platelets store target substances protected within their cytoplasm.



with FVa on the surface to generate a burst of thrombin sufficient to clot fibrinogen and form a hemostatic plug [20]. As well, factor XI (FXI) can bind to GPIb on platelet surfaces and be activated by thrombin, bypassing the need for factor XIIa [21]. This suggests that FXI acts an enhancer of thrombin generation on the platelet surface. In hemophilia patients, an individual has a markedly decreased ability to generate FXa on the platelet surface, resulting in decreased prothrombinase activity [20].

### Advantage of Platelets as a Delivery System – Lessons from Transgenic Mice

As described above, activated platelets aggregate and release a number of mediators that modify vascular integrity and hemostasis at sites of vascular injury [9, 10]. There are several advantages of the platelet-release reaction as a delivery system for a specific factor (fig. 3). One is that it provides a way to enhance the local concentration of target substances at sites of vascular injury. Given the evidence that platelets play a central role and provide the scaffold for the coagulation cascade, this would be a reasonable approach for delivering therapy to individuals deficient in the coagulation factor. Platelet-mediated protein delivery may also abolish the emergence of neutralizing antibody that often limits applications of hemophilia gene therapy. In patients with neutralizing antibodies, platelets are a very attractive delivery system because they specifically store protein in the bloodstream and then specifically release it at sites of thrombus formation, thereby minimizing the influence of any circulating inhibitors.

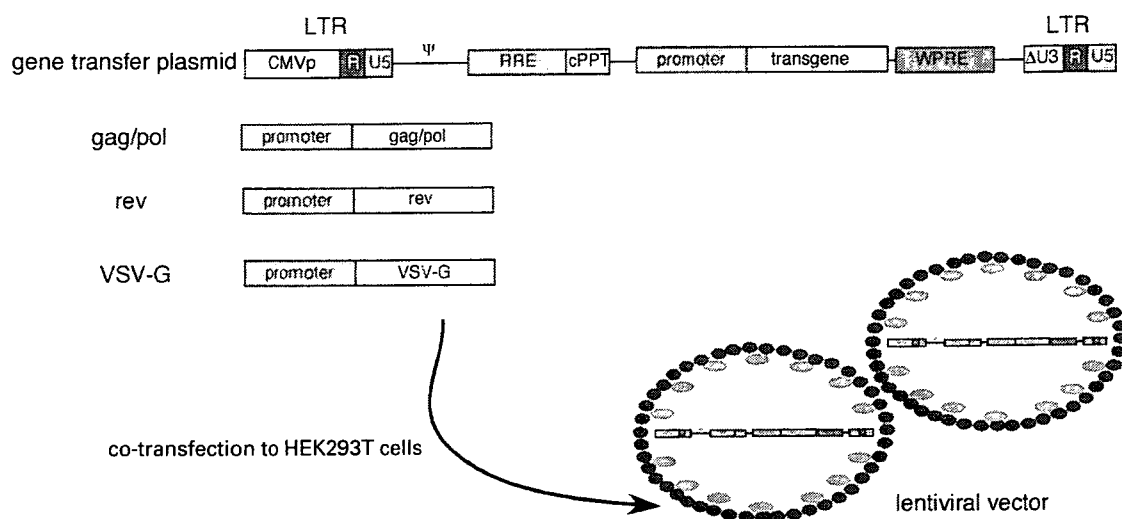
In 2003, Poncz and coworkers [22] proposed and demonstrated the feasibility of gene transfer into platelets and their precursor megakaryocytes using transgenic mice. They showed that platelet-directed gene transfer enable the storage of targeted substances within platelets. Platelet expression of urokinase-type plasminogen activator (u-PA) using a megakaryocyte-specific platelet factor 4 (pf4) promoter enabled u-PA to be stored in platelets and then released within developing thrombi when the platelets became activated [22]. The platelet

u-PA not only resulted in a mild bleeding diathesis in adult transgenic mice, but transfusion of the platelets into wild-type animals blocked untoward thrombosis, suggesting that platelets can store a thrombolytic protein and release the protein of interest at sites of developing thrombi [22]. Further, platelet-specific expression of FVIII could be achieved in a transgenic setting with the resultant FVIII predominantly or exclusively stored in platelet granules rather than being released into the plasma [23]. When transgenic mice were crossed onto a FVIII null background, whole blood clotting time was partially corrected [23]. These data suggest that the platelet-specific expression of FVIII can be achieved by platelet-specific promoter and predominantly or exclusively stored in the platelet granules rather than being released into plasma. Recently, Shi et al. [24] clearly demonstrated that ectopically expressed FVIII in platelets could treat hemophilia in the absence as well as in the presence of circulating inhibitors using a transgenic model. The expression of human B-domain-deleted FVIII driven by GPIIb promoter can correct the bleeding phenotype of FVIII-deficient mice in spite of the lack of detectable FVIII in plasma, as described above. Correction of the hemorrhagic phenotype in hemophilia A can be achieved by bone marrow cell transplantation or platelet transfusion from transgenic mice [24]. Of note, targeting FVIII expression to platelets still supports hemostasis under conditions of a high titer of FVIII-neutralizing antibodies [24]. These findings have facilitated the development of methods for gene therapy that employ platelets to deliver therapeutic agents such as specific coagulation factor to sites of vascular injury.

The conditional expression of targeted protein in megakaryocytes and platelets has recently been demonstrated in transgenic mice. Nguyen et al. [25] reported that the tetracycline/doxycycline system in conjunction with the pf4 promoter yields conditional overexpression of genes *in vivo*. Alternatively, the bacterial artificial chromosome-derived pf4-Cre transgene allowed efficient and lineage-restricted excision of a loxP target gene [26]. These transgenic mice promise to be a very useful tool to study megakaryopoiesis, platelet formation, and platelet function.



**Fig. 4.** Structure and production of lentiviral vector. A minimal lentiviral vector plasmid consisting of the CMV/LTR chimera promoter followed by the packaging signal ( $\Psi$ ), rev-binding element RRE for cytoplasmic export of the RNA, the transgene expression consisting of internal promoter and transgene, and the 3' self-inactivating LTR. All genes coding for enzymatic or structural HIV or SIV proteins have been removed. Together with the vector plasmid, a packaging plasmid encoding gal-pol, rev, and an envelope-expressing plasmid are co-transfected to HEK293T cells. Lentiviral vector is produced in the supernatant from the HEK293T cells.



## Methods for Platelet-Directed Gene Therapy

### Gene Transfer Vectors

Since platelets and their precursor megakaryocytes have a finite lifespan, HSCs are a preferable target for genetic transfer to establish long-term expression of the targeted protein in platelets *in vivo* [27]. HSC gene transfer, using viral vectors, has been actively investigated for more than 20 years, and oncoretroviral vectors have been most vigorously pursued [28–30]. However, recent clinical studies suggest that the standard transduction protocols used in conjunction with oncoretroviral vectors generally do not lead to levels of gene transfer that are clinically relevant [29, 31, 32]. Oncoretroviruses require cell division for integration. As repopulating HSCs are largely quiescent, oncoretroviral vectors are largely inefficient for such targets [33]. On the other hand, lentiviruses are capable of infecting certain types of quiescent cells [34–36]. Thus, there has been significant interest in the application of lentivirus-derived vectors to the transduction of HSCs. Indeed, the use of lentiviral vectors has been shown to achieve high-level expression of transgenes in HSCs [37–39]. As well, lentiviral genomes contain fewer CpG dinucleotides than oncoretroviral vectors, leading to the experimental observation that lentiviral vectors are more resistant to silencing [40]. Although adeno-associated virus (AAV) vectors are an alternative for the transduction of HSCs, there is controversy regarding the ability of AAV to transduce HSCs [41, 42]. The structure of a lentivirus vector plasmid and the packaging and helper plasmid is shown in figure 4. To prevent the generation of replication-competent lentiviruses, the native sequence in lentiviral vector constructs is considerably deleted and/or altered [43–45]. Cis-elements within the transfer vector are minimized and necessary trans-elements are supplied from

packaging and envelope helper constructs. Lentivirus vector was produced in supernatants from HEK293T cells transfected with these vector plasmids [43, 44, 46]. The minimal transgene expression cassette of gene transfer vector contains the long-terminal repeat (LTR), the packaging signal ( $\Psi$ ), a heterogenous promoter, and the transgene of interest. The central polypurine tract (cPPT) sequence enhances lentiviral vector efficiency by facilitating nuclear translocation of preintegration complexes [47, 48]. The lentivirus native envelope is typically replaced with a helper plasmid expressing heterologous envelope GP. This process, termed pseudotyping, can greatly modify both the cell and the host range tropism of the vector [49]. The vesicular stomatitis virus GP (VSV-G) has been extensively used to transduce HSCs [50, 51]. Other viral envelopes have successfully been applied to pseudotype lentiviral particles, including those from lyssaviruses, arenaviruses, flaviviridae, baculovirus, and alphaviruses [52–54]. Of the lentiviral vectors, human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), and feline immunodeficiency virus (FIV) have been used for various basic research investigations [44, 52, 54]. There are reasons, other than commercial, to select the lentiviral vector with different types of lentiviral vector. We used the SIV lentiviral system for efficient platelet-targeting gene transduction because of its probable safety [44]. The SIV lentiviral system was derived from SIVagmTYO1, and is non-pathogenic to both its natural host and to experimentally infected Asian macaques [44]. Replication-competent virus particles were not detected in vector-infected cells, and the risk of development of replication-competent lentivirus particles in HIV carrier patients may be theoretically lower than that for the HIV-based vectors [55]. It is also worth noting that HIV-1-based lentiviral vector was found to transduce cytokine-mobilized rhesus

macaque CD34<sup>+</sup> cells very poorly [56]. On the other hand, SIV vectors efficiently transduce macaque CD34<sup>+</sup> cells as assayed *in vitro* [56], suggesting that SIV vectors appear promising for evaluating gene therapy approaches in non-human primate models.

#### *Risk of Insertional Mutagenesis and Recent Vector Modifications to Improve Safety*

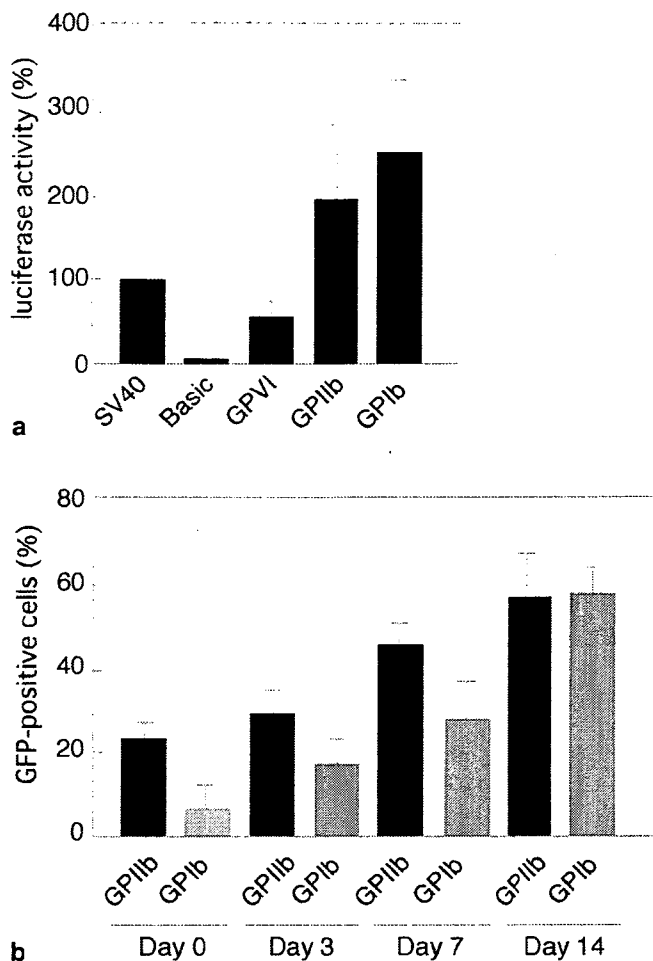
While lentiviral vectors and retroviral vectors offer a means to permanently correct genetic diseases by stably expressing a transgene by integration to chromosomal DNA, all current integrating gene transfer vectors carry a finite risk of insertional mutagenesis [57]. Insertional mutagenesis resulting in retroviral enhancer-mediated activation of the T-cell proto-oncogene LMO2 has occurred in children with X-linked severe combined immunodeficiency (X-SCID) after retroviral gene therapy, indicating the potential genotoxicity of retroviral integration in HSCs [57, 58]. Molecular analysis of affected patients cells from X-SCID gene therapy suggest that the problems are likely disease-specific [58] because there have been no reports of adverse effects from insertional mutagenesis in patients with adenosine deaminase deficiency treated with oncoretroviral vector [59]. Clonal dominance in humans with chronic granulomatous disease and the ability of retroviral integration to immortalize normal bone marrow cells with an integrating virus has the potential to alter subsequent biologic behavior [60, 61]. On the other hand, there would be insufficient numbers of gene-corrected cells to achieve a sustained therapeutic effect in the absence of such clonal expansions. Only long-term follow up of patients will determine the true safety of gene transduction to HSCs.

Studies on the integration preferences of oncoretroviruses and lentiviruses suggest that the patterns of integration of both vectors are quite different. The integration of oncoretrovirus into chromosomes was favored near transcription units, based on the association of the integration site with a DNAase I-hypersensitive site and CpG islands, a situation often associated with transcribed genes [62, 63]. On the other hand, the lentiviral vector integration sites were more evenly distributed throughout the coding sequences of targeted genes [57, 63]. Thus, compared to oncoretroviral vectors, lentiviral ones may be less prone to insertional mutagenesis. Several strategies can be implemented to decrease the risks of insertional mutagenesis by improving the vector structure. Self-inactivating (SIN) vector systems, in which the U3 region of the viral 3'-LTR is deleted, have been developed and are currently used in a variety of lentiviral vectors [43, 44]. SIN vectors are expected to improve safety profiles by eliminating viral LTR promoter activity. Conventional oncoretroviral vectors, such as those used in the X-SCID trial, used 5'-LTR as a functional promoter for the transgene [64]. Although intact LTR can promote transcription of downstream sequences, and/or an enhance element of LTR can interact with nearby promoters, SIN vectors usually rely on a single internal promoter to drive transcrip-

tion of the transgene [43, 44, 54]. In addition, the use of a cell lineage-specific promoter as an internal promoter in a SIN vector may be safer than using a viral or ubiquitous promoter. A further solution for preventing insertional mutagenesis is the incorporation of chromosomal insulators into the viral vector [54, 57, 65, 66]. Insulators are genetic elements near chromatin domain boundaries that function as barriers against the repressive effects of neighboring inactive chromatin, or they prevent inappropriate activation of a promoter by heterologous enhancers [67, 68]. There are two benefits in utilizing a chromosomal insulator. First, insulators can abolish the potential of a proviral genome to activate a gene encoding a transactivator when integrated near that gene, which can lead to insertional mutagenesis. Second, chromatin insulators protect gene expression by the targeted gene from neighboring silencers, preventing transcriptional silencing of oncoretroviral vectors [69]. To date, the best-characterized chromatin insulator is the 5' DNase I-hypersensitive site 4 (cHS4) core from the chicken  $\beta$ -globin locus control region [67, 68]. Insertion of cHS4 in SIN lentiviral vectors results in higher and less variable expressions of human  $\beta$ -globin, similar to observations with cHS4-containing retroviral vectors carrying the human  $\gamma$ -globin gene [65, 66]. The levels of  $\beta$ -globin expression achieved from insulated SIN lentiviral vectors were sufficient to phenotypically correct the thalassemia phenotype of 4 patients with human thalassemia major *in vitro*; this correction persisted long term for up to 4 months in xenotransplanted mice *in vivo* [38]. Recently, it has been shown that reduction of the risk of insertional mutagenesis in integration-deficient lentiviral vectors can mediate sustained transgene expression *in vivo* in rodent ocular and brain tissues [70]. Mutations in the integrase coding sequence results in integration-defective lentiviral vector, and class I mutations achieved normal DNA synthesis, integration failure, and accumulation of DNA in the cell nucleus as double-stranded circles [71]. The use of this vector system is particularly attractive for postmitotic tissues. However, application to HSC transduction is regarded as an exception because these vectors lack replication signals and are progressively diluted as a result of cell division [72].

#### *Platelet-Specific Promoter*

To establish an efficient transduction of megakaryocytes and platelets, at least a platelet-specific promoter must be utilized. The use of a cell lineage-specific promoter may provide some assurance of vector safety, as describe above. As well, transplantation of HSCs transduced with a lentiviral vector expressing the eGFP gene driven by the cytomegalovirus (CMV) promoter resulted in efficient eGFP expression in CD45<sup>+</sup> cells, but not in platelets [73] (see fig. 6). On the other hand, transduction of HSCs with a lentiviral vector harboring a platelet-specific promoter resulted in efficient gene marking to platelets. It is not entirely understood why the eGFP expression driven by the CMV promoter was significantly decreased in platelets, as opposed to the high transduction effi-



**Fig. 5.** Advantage of GPIIb promoter as a platelet specific promoter. **a** Comparison of platelet-specific promoter activities. Each construct along with a promoter-less vector (basic) or a positive control vector (SV40) was transfected into the UT-7/TPO megakaryocytoblastic cell line. Luciferase activities were measured 48 h after transfection and are shown relative to the activity driven by the SV40 promoter (SV40/Enhancer) ( $n = 5$ ). **b** CD34<sup>+</sup> cell-derived megakaryocytes at day 0, 3, 7, and 14 after the start of megakaryocytic differentiation were transduced with a SIV lentiviral vector equipped with eGFP driven by a GPIIb promoter or GPIb promoter at a MOI of 30. Columns and error bars represent the percentage of GFP-positive cells (mean  $\pm$  SD;  $n = 3$ ).

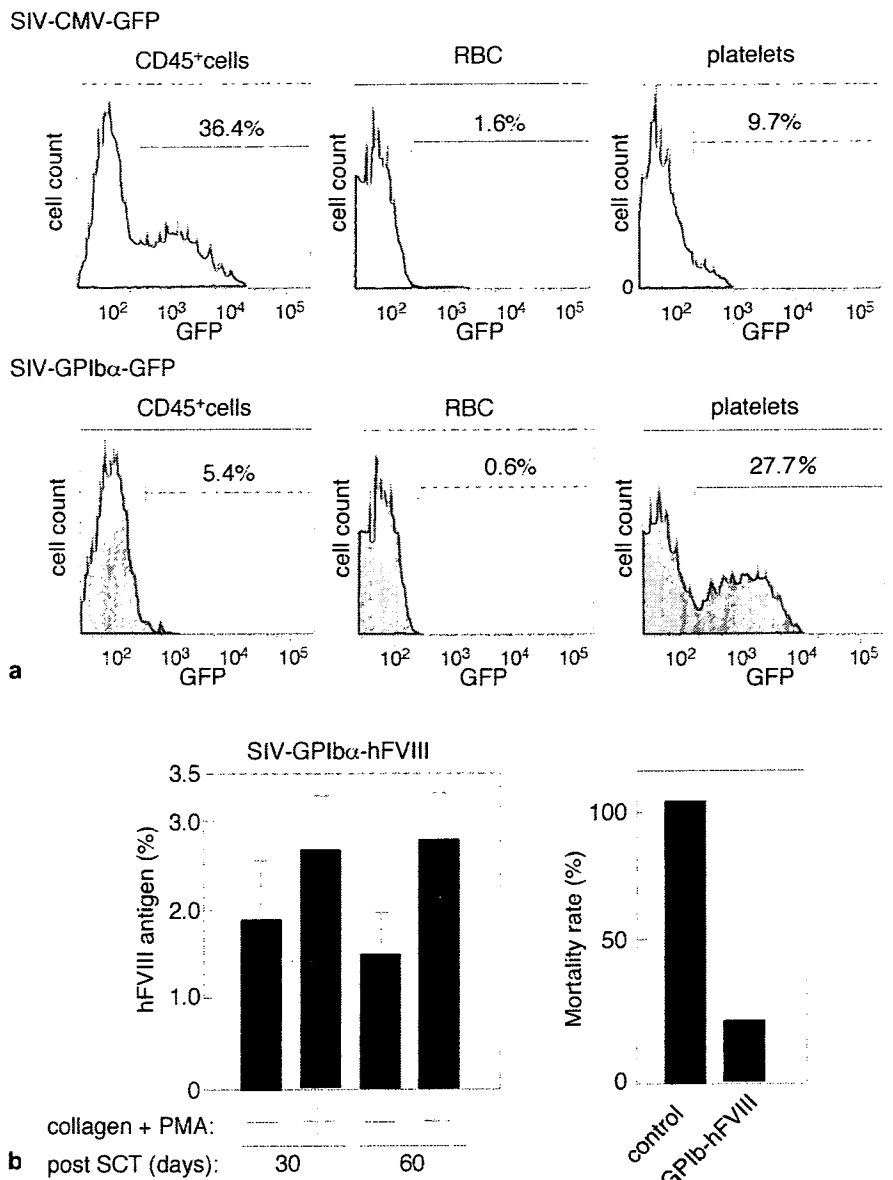
iciencies of CD45<sup>+</sup> cells. Generally, the reduction of transgene expression caused by a shortened protein half-life is even more pronounced in terminally differentiated blood cells [74]. The decreased expression might have been mediated by the down-regulation of the transgene during differentiation; the stability of the encoded protein is at least as relevant for the expression of a transgene as the choice of the promoter or cis-elements influencing RNA processing in differentiated cells [74]. Candidates for a promoter sequence for megakaryocyte- and platelet-specific expression include the promoter sequences of the GPIb/GPIX/GPV complex, GPIIb, p4, and GPVI [25, 26, 73, 75]. These promoter sequences have a potential site to bind the transcription factors including

GATA-1, Ets, FOG-1 and NF-E2, which are essential regulators of distinct stages in megakaryocyte differentiation [3,76]. We are now utilizing the GPIIb promoter as a platelet-specific promoter because the promoter activity of GPIIb is more potent than that of GPIb in UT-7/TPO and CD34<sup>+</sup> cell-derived megakaryocytes [73] (fig. 5a). Another reason is that the GPIIb promoter works at a late stage of megakaryopoiesis [77] (fig. 5b). Although the GPIIb gene is expressed in platelets and megakaryocytes, it is an early gene for megakaryopoiesis [77]. In conditional knockout mice in which the thymidine kinase gene was driven by the GPIIb promoter, the administration of gancyclovir led to a dramatic reduction in the platelet count [78]. In bone marrow, erythroid and myeloid progenitors were also affected, which indicated the presence of GPIIb in progenitor cells [78]. Indeed, 18% of human CD34<sup>+</sup> HSCs already express GPIIb, and so the appearance of GPIb was markedly delayed as compared to that of GPIIb, indicating that GPIb is a later marker of megakaryocytic maturation [73, 77]. Therefore, we believe that platelet-directed gene therapy using the GPIIb promoter will allow more specific and restricted expression of gene products in megakaryocytes and platelets.

### Phenotype Correction of Mouse Models of Hemorrhagic Diseases by Platelet-Directed Gene Therapy

#### *Inherited Platelet Disorder*

Abnormalities of platelet function manifest themselves primarily as excessive hemorrhage at mucocutaneous sites, with petechiae, epistaxis, gingival hemorrhage, and menorrhagia most common [79]. Glanzmann's thrombasthenia (GT) is a rare autosomal-recessive hereditary disorder characterized by the severe reduction of platelet aggregation due to qualitative or quantitative abnormalities of GPIIb/IIIa [80]. More than 100 distinct genetic defects have been characterized for GT, with an even distribution in both genes [80]. For gene therapy for GT, megakaryocyte-specific expression of GPIIb/IIIa is potentially important because the expression of the platelet-specific integrin in neutrophils, erythrocytes, or monocytes might alter the adhesive properties of these cells, resulting in the unexpected progression of thrombosis. Wilcox and colleagues [27, 75, 81–83] have consistently reported the feasibility of gene therapy for inherited platelet disorder. They initially used a SIN oncoretroviral vector encoding the GPIIIa gene or the  $\beta$ -galactosidase gene driven by GPIIb promoter and found that the transduced CD34<sup>+</sup> HSCs with the vector specifically express the targeted gene transcript after differentiation into megakaryocytes [75]. When a retroviral vector containing the GPIIIa gene driven by the GPIIb promoter was transduced into CD34<sup>+</sup> cells from a GT patient with defects in the GPIIIa gene, GPIIb/IIIa were actually detected after in vitro megakaryocyte differentiation [81]. Recently, the same authors showed that transplantation of transduced cells with a



**Fig. 6.** Efficient expression of transgene in platelets using lentiviral vector harboring GPIb $\alpha$  promoter. **a** KSL cells were transduced with a SIV lentiviral vector equipped with eGFP driven by a CMV (SIV-CMV-eGFP) or GPIb $\alpha$  (SIV-GPIb $\alpha$ -eGFP) promoter at a MOI of 30. Representative flow cytometric analyses of eGFP-positive cells in CD45<sup>+</sup> lymphocytes and granulocytes, red blood cells (RBC), and platelets in peripheral blood after transplantation are shown. **b** Phenotypic correction of hemophilia A in mice by platelet-directed gene therapy. Blood from FVIII-deficient mice transplanted with KSL cells transduced without or with SIV-GPIb $\alpha$ -hFVIII was stimulated in the presence or absence of 50  $\mu$ g/ml of collagen and 1  $\mu$ mol/l PMA for 15 min. hFVIII antigen levels in platelet-poor plasma were measured (n = 4 per group) (left panel). Mortality rate within 24 h after tail clipping in mice transplanted with control or SIV-GPIb $\alpha$ -hFVIII-transduced KSL cells (n = 10 for control, n = 8 for GPIb $\alpha$ ) (right panel).

lentivirus vector equipped with the human integrin  $\beta$ 3 gene under control of the GPIIb promoter resulted in phenotypic correction of a mouse model of GT, integrin  $\beta$ 3-deficient mice [82]. These data indicate the possibility of gene therapy for better management of patients with inherited platelet disorder, including GT and Bernard-Soulier syndrome.

#### Coagulation Factor Deficiencies

Hemophilia A is an X chromosome-linked bleeding disorder caused by defects in the FVIII gene and affects approximately 1:5,000 males [84]. Hemophilia A is considered suitable for gene therapy because it is caused by a single gene abnormality and therapeutic coagulation factor levels may well vary across a broad range (5–to 100%) [85, 86]. Although sustained therapeutic expression of FVIII has been achieved in preclinical studies using a wide range of gene transfer technologies targeted at different tissues, the emergence of neutralizing anti-

body often limits clinical applications [86–88]. The targeting of HSCs is not an exception; lentiviral FVIII gene transduction of HSCs is able to produce therapeutic levels of FVIII by ubiquitous promoters [89], but the emergence of neutralizing antibodies to FVIII has resulted in decreased levels of FVIII activity [90].

Retrovirus transduction of FVIII driven by the virus promoter into human CD34<sup>+</sup> HSCs enables FVIII-transduced megakaryocytes to store human FVIII with VWF within  $\alpha$ -granules [91]. This is a very important finding because VWF is the natural carrier protein of FVIII, and FVIII interaction with VWF within platelet  $\alpha$ -granules may enable the stable storage of FVIII. As well, transplantation of bone marrow cells transduced with lentiviral vector having FVIII driven by a platelet-specific promoter improve the hemostatic function of FVIII-deficient mice, despite there being undetectable or scant levels of FVIII in plasma (fig. 6b) [73, 92]. FVIII levels in plasma

were significantly increased after platelet activation (fig. 6b) [73]. These data suggest that platelet-derived FVIII is locally released and specifically involved in thrombus formation at the site of thrombus formation. Since this may limit the development of inhibitors by preventing the expression of FVIII in antigen-presenting cells, development of neutralizing antibody against FVIII has not been detected in transplanted mice [73, 92]. 10–30% of populations affected by hemophilia A develop inhibitors to infusion products, which leads to the disruption of coagulation and severe bleeding [84]. Under these conditions, platelet-directed gene therapy for hemophilia A is a very attractive option because platelets could specifically store the protein in the bloodstream and then specifically release it at sites of thrombus formation, thereby minimizing the influence of any circulating inhibitors, as described above (see the section on ‘Advantage of Platelets as a Delivery System – Lessons from Transgenic Mice’). Recently, erythroid-specific FIX expression driven by  $\beta$ -globin promoter resulted in the phenotypic correction of hemophilia B mice [93]. However, given the context of the specific release of targeted protein at the site of thrombus formation, platelet-directed gene therapy has a major advantage as a therapy for inherited coagulation factor deficiencies.

## Concluding Remarks

Platelet-directed gene therapy appears an important strategy to treat various hemorrhagic disorders. The use of lentiviral vectors and platelet-specific promoters has resulted in efficient expressions of targeted proteins in platelets, resulting in the correction of mouse models of GT and hemophilia A. Lentiviral vectors have already entered phase I trials as an

anti-HIV gene delivery into patients with HIV infection and have proven safe, at least in short-term surveys [94]. However, target diseases for platelet-directed gene therapy, including hemophilia and GT, are not generally lethal disorders. At this time, the lack of patient safety data means that the risks are likely to weigh more heavily than the benefits. To begin clinical trials for the correction of human diseases, further studies are needed to resolve a variety of questions. Which amount of corrected stem cells is needed to correct the particular disease phenotype? Can vector modification reduce the risk of insertional mutagenesis? Will an *in vivo* selection of transduced cells be necessary to ensure stable expressions of targeted proteins? We must also consider the risk of stem cell transplantation. The disorders eligible for platelet-directed gene therapy have normal immune reactions, indicating that complete myeloablation to eliminate the recipient marrow inhibits a graft-versus-host disease and ensures the engraftment of transduced HSCs. It seems more practical to use the autologous HSC transplantation protocol of non-myeloablative transplantation to reduce the risk of transplantation side effects. Further evaluations utilizing larger animals such as the cynomolgus monkey or dog will be necessary to determine efficient and safe transplantation protocols.

## Acknowledgements

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REGULAR ARTICLE

## Annexin 2 and hemorrhagic disorder in vascular intimal carcinomatosis

Seiji Madoiwa<sup>a,b</sup>, Tsutomu Someya<sup>e</sup>, Mitsugu Hironaka<sup>e</sup>,  
Hiroshi Kobayashi<sup>f</sup>, Tsukasa Ohmori<sup>a,b</sup>, Jun Mimuro<sup>a,b</sup>,  
Yukihiko Sugiyama<sup>c</sup>, Tatsuo Morita<sup>d</sup>, Yoshioki Nishimura<sup>b</sup>,  
Takahisa Tarumoto<sup>b</sup>, Keiyo Ozawa<sup>b</sup>, Ken Saito<sup>e</sup>, Yoichi Sakata<sup>a,b,\*</sup>

<sup>a</sup> Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical School, Shimotsuke, Tochigi, Japan

<sup>b</sup> Department of Hematology, Jichi Medical School, Shimotsuke, Tochigi, Japan

<sup>c</sup> Department of Urology, Jichi Medical School, Shimotsuke, Tochigi, Japan

<sup>d</sup> Department of Pulmonary Medicine, Jichi Medical School, Shimotsuke, Tochigi, Japan

<sup>e</sup> Department of Pathology, Jichi Medical School, Shimotsuke, Tochigi, Japan

<sup>f</sup> Seirei Hamamatsu General Hospital, Hamamatsu, Shizuoka, Japan

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### KEYWORDS

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Transitional cell carcinoma;  
siRNA

**Abstract** Vascular intimal carcinomatosis refers to a characteristic tumor proliferation on vascular intima that replaces normal endothelium. This pathological event of unknown cause is quite different from tumor thrombotic microangiopathy due to the absence of thrombi on the tumor cell surfaces. We analyzed renal transitional cell carcinoma cases with metastasis to the main pulmonary arteries and marked hyperfibrino(geno)lysis. The fibrinogen-derived products from patients' plasma were identified as D1A/γ, D1/γ, and D1/β by immunoblotting with the NH<sub>2</sub>-terminus of the fragment D specific antibody JIF-23. In all cases, the neoplastic cells with vascular intimal carcinomatosis were stained positive for anti-human annexin 2, which is a unique cell surface co-receptor for plasminogen and tissue-type plasminogen activator. In contrast, normal renal pelvic mucosa or renal transitional cell carcinoma without vascular intimal carcinomatosis did not express any annexin 2. The isolated transitional cell carcinoma cells contained annexin 2 mRNA and expressed its protein. Anti-annexin 2 antibody and transfection of annexin 2 small interfering RNA into these carcinoma cells significantly inhibited tissue-type

\* Corresponding author. Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical School, 3311-1, Yakushi-ji, Shimotsuke, Tochigi 329-0498, Japan. Tel.: +81 285 58 7398; fax: +81 285 44 7817.  
E-mail address: yoisaka@jichi.ac.jp (Y. Sakata).



plasminogen activator dependent plasmin generation. These findings suggest that annexin 2 mediated fibrinolysis on the transitional cell carcinoma cells may play a role in inducing hemorrhagic disorder in vascular intimal carcinomatosis.

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## Introduction

Transitional cell carcinoma accounts for more than 90% of renal pelvic and ureteric tumors, and it is known to spread by direct invasion into the renal parenchyma or surrounding structures by epithelial extension or vascular invasion [1]. Hematogenous metastases from transitional cell carcinoma into the lung cause embolic carcinomatosis [2,3]. The pathophysiological effects of microembolic carcinoma on pulmonary vessels are mechanical occlusion by the tumor, thrombotic occlusion by fibrin, or a florid intimal reaction termed carcinomatous arteriopathy [4]. Vascular intimal carcinomatosis is defined as replacement of endothelium with carcinoma cells proliferating onto the intimal surface [5]. This pathological feature of vascular intimal carcinomatosis is distinctly different from the tumor thrombotic microangiopathy due to the absence of blood clot formation. Transitional cell carcinoma with vascular intimal carcinomatosis has features characteristic of hemorrhagic diatheses, which is thought to result from increased fibrinolysis [5,6]. However, mechanisms that result in these pathological activities are not well understood.

Plasminogen activators such as tissue type plasminogen activator (t-PA) and urokinase type plasminogen activator (u-PA) catalyze the processing of plasminogen to plasmin, a proteolytic enzyme that cleaves fibrinogen and fibrin. A number of pathologic states can result in systemic hyperfibrinolytic (genolysis) presumably through the release of plasminogen activators and production of plasmin in sufficient quantities to overcome their natural inhibitors, plasminogen activator inhibitor-1 (PAI-1) and alpha2-plasmin inhibitor ( $\alpha$ 2-PI). Indeed, t-PA and u-PA secretion and systemic fibrinolytic activation by ovarian, breast, and prostate adenocarcinoma indicate that a bleeding diathesis may be induced by malignant disease [7–10]. Annexin 2 is a calcium-regulated, phospholipid-binding protein on endothelial cells, macrophages, and some other cells. It is a unique cell surface co-receptor for plasminogen and t-PA, and it enhances cell surface plasmin generation [11]. The overexpression of annexin 2 on the surface of acute promyelocytic leukemia cells may lead to uncontrolled production of plasmin, shifting the hemostatic balance toward abnormal bleeding [12].

In the current study, we demonstrate that fatal hemorrhagic diatheses due to hyperfibrinolytic (genolysis) might be attributable to annexin 2 expression on the renal transitional cell carcinoma cells showing vascular intimal carcinomatosis.

## Materials and methods

### Blood samples and quantification of fibrinolysis markers

All samples were obtained with informed consent from patients according to the Declaration of Helsinki. Citrated plasma and serum samples were stored at  $-80^{\circ}\text{C}$  until analyzed. Serum levels of fibrinogen and fibrin degradation products (FDP) were determined by the latex agglutination assay using LPIA-FDP (Dia-latron, Tokyo, Japan). Plasma D-dimer, thrombin-anti-thrombin-III complex (TAT), and plasmin- $\alpha$ 2 plasmin inhibitor complex (PIC) levels were measured by enzyme-linked immunosorbent assay (Kokusai-Shiyaku, Kobe, Japan). Plasma anti-thrombin III activities were measured by TestzymeS ATIII (Daiichi Kagaku, Tokyo, Japan).

### Case histories

The first patient, a 49-year-old male, was referred to our hospital because of iliopsoas muscle bleeding, macroscopic hematuria, and purpura. Although the peripheral platelet count was normal ( $212 \times 10^3/\mu\text{L}$ ), the prothrombin time (PT) and activated partial thromboplastin time (APTT) were prolonged to  $>150$  s (normal: 10.0–11.7 s) and 81.3 s (normal: 28.5–40.0 s), respectively. The fibrinogen level was severely decreased to less than 40 mg/dL with a markedly increased FDP level (Table 1). The levels of plasma TAT and PIC were 19.4 ng/mL (normal:  $<2.4$  ng/mL) and 9.4 (normal:  $<0.9$   $\mu\text{g/mL}$ ), respectively. The activity of plasma anti-thrombin III was not decreased (91.5%; normal: 88.0–116.0%). Protease inhibitors and aminocaproic acid partially inhibited his hypofibrinogenemia state. The patient was diagnosed as having primary hyperfibrinolysis with grade 2 transitional cell carcinoma of the left

Table 1 Clinical findings of vascular intimal carcinomatosis

Case no.	Sex/age	Primary lesion/size	Vascular intimal carcinomatosis	Bleeding	PT/aPTT (s)	Fibrinogen (mg/dL)	FDP ( $\mu\text{g/mL}$ )	D-dimer ( $\mu\text{g/mL}$ )	White blood cell ( $\times 10^7/\text{mL}$ )	Platelet ( $\times 10^3/\text{mL}$ )	CA19-9 (U/mL)	Therapy	Response to therapy
1	M/49	Transitional cell carcinoma of left kidney (grade 2)/ 3.6 $\times$ 3.2 $\times$ 2.5 cm	Left main pulmonary artery and left renal vein	Ecchymosis, hematuria, gingival and intramuscular hemorrhage	>150/81.3	<40	246.0	45.0	13.4	212	367.9	Protease inhibitors, fresh-frozen plasma, platelets, tumor removal	Partial and transient
2	F/44	Transitional cell carcinoma of left kidney (grades 1 and 2)/ 1.0 $\times$ 1.0 $\times$ 1.0 cm	Large sized pulmonary arteries and inferior vena cava	Ecchymosis, nasal and gingival hemorrhage	20.4/51.6	<40	>100	14.2	5.2	195	146.0	Protease inhibitors, fresh-frozen plasma	Partial
3	M/53	Transitional cell carcinoma of right kidney (grade 2)/ 2.5 $\times$ 2.0 $\times$ 1.0 cm	Left main pulmonary artery and right renal vein	Hemoptum, impaired hemostasis after tooth extraction	15.9/44.2	65	551.5	46.3	10.3	211	356.0	Protease inhibitors, fresh-frozen plasma	Partial

renal pelvis by cytological analysis of the urine and computed tomographic scans. Although a left total nephrectomy partially ameliorated his coagulation disturbance, the patient died from multiple organ failure due to severe bleeding.

The second patient, a 44-year-old woman, complained of nasal bleeding, gingival hemorrhage, and skin purpura as previously described [5]. Her PT and APTT were markedly prolonged to 20.4 s and 51.6 s, respectively. The levels of plasma TAT and PIC were 10.1 ng/mL and 8.2 µg/mL, respectively. Her plasma anti-thrombin III activity was normal (97.9%). Although this patient was treated with serine protease inhibitors and continuous infusions of frozen fresh plasma, severe hypofibrinogenemia and elevated FDP level developed, and she later had died of massive cerebral bleeding. The autopsy revealed a papillary shaped white tumor on her left renal pelvis that was diagnosed as grade 1 and 2 transitional cell carcinoma by microscopic examination.

The third case, a 53-year-old male, was admitted due to a recent onset of hemoptysis with dyspnea and impaired hemostasis after tooth extraction. The platelet count was normal, but PT and APTT were considerably prolonged. The fibrinogen level was decreased to 65 mg/dL with marked increases in FDP and a mildly elevated D-dimer level. His plasma TAT and PIC levels were 16.6 ng/mL and 15.0 µg/mL, respectively. The activity of plasma anti-thrombin III was normal (100.6%). The patient was treated with nafamostat mesilate and frequent infusion of fresh frozen plasma for his severe fibrinolytic state, but he died from respiratory failure on the 14th hospital day. Histopathological examination of autopsy specimens showed a sclerotic mass on the pelvis of the right kidney, which was diagnosed as a grade 2 transitional cell carcinoma.

### Plasma derived from patients with hyperfibrinogenolysis

The study was conducted with Ethical Committee approval and under the guidelines of the Declaration of Helsinki. All patients' samples were obtained after informed consents. Blood samples were collected from the patients and quickly anticoagulated with 1/9 volume of 3.8% sodium citrate. Plasma was separated by centrifugation for 30 min at 3000 rpm and 4 °C and then stored at -80 °C until use.

### Immunopurification of fibrinogen and fibrin degradation products from patient plasma

Anti-fibrinogen polyclonal antibody (Calbiochem-Novabiochem, San Diego, CA, USA) was coupled to

CNBr-activated Sepharose 4B (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's protocol. A 200 µL of aliquot of anti-fibrinogen polyclonal antibody-coupled Sepharose was suspended in 1 mL of 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 0.05% Tween 20 (TBS-Tween). Patient plasma (200 µL) was added into the resin suspension and incubated for 16 h at 4 °C. The mixtures were put into a column and extensively washed with TBS-Tween. Bound protein was eluted with 20 mM Tris-HCl, pH 7.6 containing 2% SDS. The elutes were analyzed by immunoblotting with JIF-23 monoclonal antibody that recognizes the amino-terminal structure of the plasminic fragment D species of fibrinogen [13].

### SDS-PAGE and immunoblotting

SDS-polyacrylamide gel electrophoresis was performed with 10–15% resolving gels and 4% stacking gels according to the Laemmli method [14]. Some samples in the gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) by electroblotting, and then developed using specific antibodies (rabbit or mouse) against targeted proteins and peroxidase-conjugated anti-rabbit or anti-mouse IgGs, respectively [15].

### Radioiodination of fibrinogen

Fibrinogen was radiolabeled with Na<sup>125</sup>I by using IODOBEADS (Pierce, Rockford, IL, USA) according to the manufacturer's procedure with the following modifications [16]: 200 µg of fibrinogen in 0.05 M Tris-HCl, pH 7.4, 0.14 M NaCl and 200 µCi of the radioisotope were added to the reaction tube in the presence of a bead coated with iodogen. After iodination for 15 min at 25 °C, the labeled protein was separated from free Na<sup>125</sup>I by gel filtration on a PD-10 column (Pharmacia Biotech, Uppsala, Sweden). The specific activities obtained were  $0.828 \times 10^6$  cpm/µg for fibrinogen.

### t-PA catalyzed plasminogen activation

The t-PA catalyzed plasminogen activation assay was performed as described previously [12]. In brief, cells were preincubated with 100 nM lysine-plasminogen (The Chemo-Sero Therapeutic Institute, Kumamoto, Japan) for 1 h at 25 °C. Then 10 nM t-PA (Calbiochem-EMD Biosciences, La Jolla, CA, USA) and the plasmin substrate H-D-Val-Leu-Lys-pNA (S-2251, Chromogenix, Milano, Italy) were mixed together and then added to the cells in the presence or absence of anti-annexin 2 IgG (Santa Cruz

Biotechnology, Santa Cruz, CA, USA) or control rabbit IgG. The generation of plasmin was monitored by measuring the absorbance at 405 nm at 25 °C.

### Knockdown of annexin 2 gene expression by siRNA

The isolated cells from the patient were cultured with MEM (Gibco-Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, and the cells were transfected with annexin 2 siRNA or a scrambled siRNA (Santa Cruz Biotechnology) according to the manufacturer's protocol. At 30 h after transfection, the transduced cells were analyzed for immunohistochemistry and used for the assay of t-PA-dependent plasminogen activation. Total RNA was extracted from the cells, and reverse-transcriptase polymerase chain reactions (RT-PCR) for annexin 2 were performed as described below.

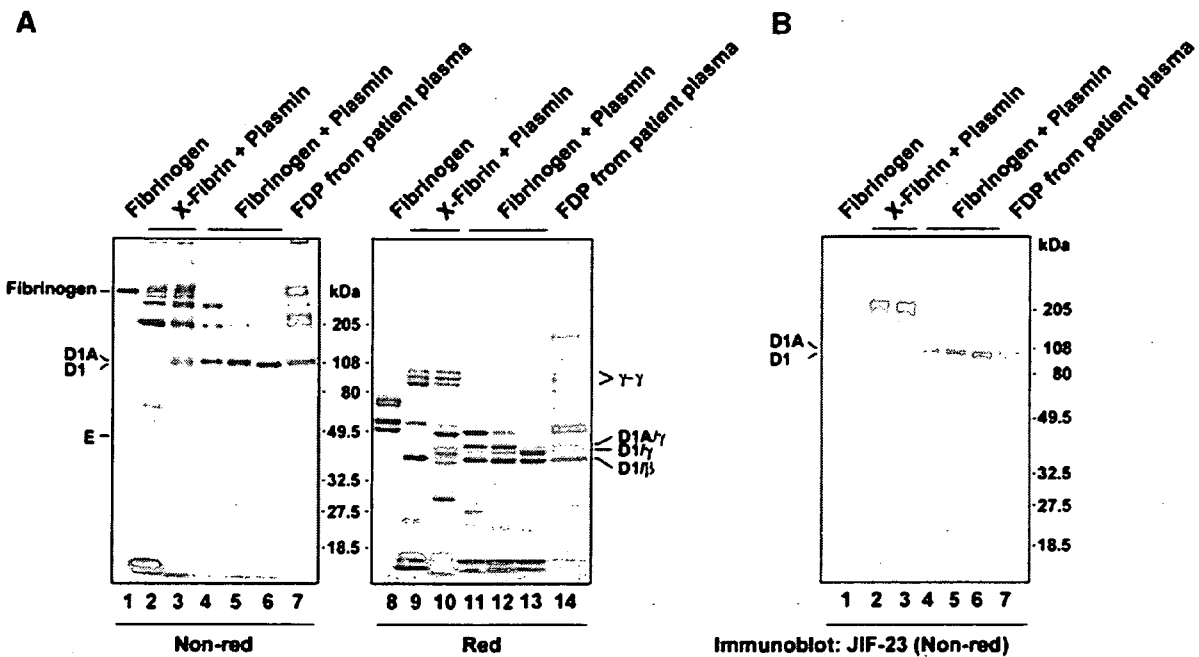
### RT-PCR

Total RNA was isolated from cells according to the methods of Chomczynski and Sacchi [17]. RT-PCR was performed using the following oligonucleotide primers: annexin 2 forward, 5'-ATTGAGATCATCTG-

CTCCAGAAC-3' (nucleotides 434–456) and reverse, 5'-AGTACAGGGACTTGCCGTACTTT-3' (nucleotides 976–998); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-GAAGGTGAAGGTCCGAGTC-3' (nucleotides 39–57) and reverse, 5'-GAAGATGGTGATGGGATTTC-3' (nucleotides 245–264). Each first-strand cDNA was synthesized by RT using a commercial kit (Applied Biosystems, Foster City, CA, USA). Total cellular RNA (1 µg) was reverse transcribed using oligo-dT<sub>16</sub> primers, and amplified by PCR (94 °C for 60 s, primer annealing at 56 °C for 60 s, extension at 72 °C for 60 s) for 30 cycles in a GeneAmp PCR System 9700 (Applied Biosystems) with a final incubation at 72 °C for 10 min. Amplification products were separated on 1.5% agarose gels, stained with ethidium bromide, and photographed.

### Immunohistochemical staining

Formalin-fixed and paraffin-embedded blocks were cut into 4-µm-thick sections, which were then deparaffinized and rehydrated through graded alcohol solutions to water. For histological evaluation, sections were stained with hematoxylin and eosin (H&E), dehydrated, and mounted in Malinol



**Figure 1** Analysis of patient plasma immunopurified with anti-fibrinogen polyclonal antibody-coupled Sepharose. Fibrinogen derived proteins (lanes 7 and 14) were purified from patient's plasma with anti-fibrinogen IgG-coupled Sepharose. Lanes 1 and 8 show untreated fibrinogen. Cross-linked fibrin was treated with 10 nM plasmin in the presence of 1 mM CaCl<sub>2</sub> at 37 °C for 1 h (lanes 2 and 9) or 5 h (lanes 3 and 10). Fibrinogen (1.6 µM) was also treated with 10 nM plasmin in the presence of 1 mM CaCl<sub>2</sub> at 37 °C for 15 min (lanes 4 and 11), 1 h (lanes 5 and 12), or 16 h (lanes 6 and 13). Each sample was stained with Coomassie Brilliant Blue (A) or subjected to immunoblotting with JIF-23 antibody (B). X-Fibrin: cross-linked fibrin, FDP: fibrinogen and fibrin degradation products.