上市された1996年から2003年までに，インヒビターを保有する先天性血友病および後天性血友病患者でrFVIIaが使用された症例の中から，血栓性事象の自発報告をレビューしている[11]．この期間に，動脈性の血栓性事象が10件（急性心筋梗塞7件および脳血管障害3件），静脈血栓塞栓症（肺血栓塞栓症／深部静脈血栓症）が6件，DICが2件の計18件が報告されている．この間のrFVIIaの全投与回数は，標準用量（体重40kgの症例に90 μg/kgの投与）に換算して70万回以上と推定される．これら18件の事象のうち15件では，心血管系危険因子，肥満および高齢など，何らかの血栓性リスクファクターあるいは乾燥人血液凝固因子抗体迂回活性複合体（APCC）製剤の併用が背景にあり，その他の3件も含めて，rFVIIaの投与が明らかに血栓性事象の発現に直接関与したといえる症例は認められなかった．一方，本調査におけるrFVIIa投与の総回数は約5,000回であるが，血栓塞栓性事象として脳梗塞の疑いと中心静脈カテーテル閉塞が各1件報告された．中心静脈カテーテルの閉塞は，長期留置例ではしばしばみられる．本症例では留置期間は不明であるが，本事象の発現がrFVIIaの最終投与3週間後であり，本剤の血中半減期が3.5時間[6]であることを考慮すると，両者の因果関係はきわめて低いと考えられる．38歳のインヒビターを保有する先天性血友病A症例は，rFVIIa投与1ヵ月後に施行した頭部MRI画像で脳梗塞様所見を認めたが，その8週後のMRI画像では同所見が完全に消失しており，主治医は脳梗塞としては非定型的であると考察している．また，7件の有害事象自発報告の中に血栓塞栓性事象として脳梗塞の疑いが1件あった．本症例は73歳の後天性血友病Aで，既往歴として高血圧，脳梗塞，高脂血症，胃癌，合併症として糖尿病，溶血性貧血および慢性腎不全を有していた．また，本症例はrFVIIa投与17.5時間後に，脳血管障害を疑わせる臨床症状を発症したが，画像による確定診断はなされていない．すなわち，上記脳梗塞の疑いの2件はともに，確定診断はされておらず，rFVIIa投与との明らかな因果関係は認められていない．

安全性に関して，血栓塞栓性事象は，rFVIIaの薬効に並存して発症する可能性のあるものとして，本剤の臨床試験中から市販後の現在に至るまで常に注意が払われている．AbshireとKenetの報告や本調査から得られた多くの臨床使用実績の結果からみて血栓塞栓性事象の発生率はきわめて低く，rFVIIaの総合的な忍容性は良好であると考えられる．本調査では，本剤投与後の重篤な副作用は先天性血友病患者に比し，後天性血友病患者に比較的多い傾向があったが，AbshireとKenetの報告結果も総合すると，高齢あるいは血栓症の既往歴を有するなど血栓性リスクファクターを有する患者に本剤を投与する際には，先天性，後天性を問わず血栓塞栓性事象の発現に十分留意する必要があろう．

謝辞：使用成績調査にご協力いただきました各施設（**Table 7**）の先生方に紙面をおかりいたしまして深謝いたします．

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Table 7 市販後調査協力施設一覧

市立千歳市民病院	大阪市立総合医療センター
札幌徳洲会病院	兵庫県立こども病院
岩手県立大船渡病院	神戸市立西市民病院
独立行政法人国立病院機構西多賀病院	神戸大学医学部附属病院
三友堂病院	明石市立市民病院
群馬大学医学部附属病院	赤穂市民病院
埼玉県立小児医療センター	兵庫県立尼崎病院
三愛会総合病院	兵庫県立淡路病院
松戸市立病院	兵庫医科大学病院
日本大学医学部附属板橋病院	姫路医療生活協同組合共立病院
荻窪病院	奈良県立医科大学附属病院
駿河台日本大学病院	岡山市立市民病院
独立行政法人国立病院機構東京病院	独立行政法人国立病院機構岡山医療センター
東京医科大学病院	岡山赤十字病院
神奈川県立こども医療センター	川崎医科大学附属病院
昭和大学藤が丘病院	広島大学病院
独立行政法人国立病院機構福井病院	山口大学医学部附属病院
長野赤十字病院	徳山中央病院
諏訪赤十字病院	都志見病院
波田総合病院	徳島大学病院
岐阜市民病院	麻植協同病院
松波総合病院	香川大学医学部附属病院
西美濃厚生病院	松山赤十字病院
静岡県立こども病院	愛媛大学医学部附属病院
独立行政法人国立病院機構天竜病院	高知大学医学部附属病院
県西部浜松医療センター	産業医科大学病院
名古屋大学医学部附属病院	佐賀大学医学部附属病院
愛知三の丸病院	佐賀県立病院好生館
豊橋市民病院	熊本市立熊本市民病院
三重大学医学部附属病院	大分県立病院
公立甲賀病院	宮崎県立日南病院
京都府立医科大学附属病院	鹿児島大学医学部・歯学部附属病院
北野病院	鹿児島市立病院
関西医科大学附属病院	豊見城中央病院

Long-term safety and efficacy of recombinant activated factor VII (NovoSeven®) in haemophilia patients with inhibitors : interim post marketing study analysis of 5 patient exposure years

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Key words: rFVIIa, haemophilia, inhibitor, bleeding episode

This article reviews an interim analysis of the long-term safety and efficacy of recombinant activated factor VII (rFVIIa) over a 5-year period. Episodes of 1,580 bleedings were recorded in 102 patients with either congenital or acquired hemophilia. An overall efficacy rate in achieving excellent or good hemostasis within a 12-hour period was 69.6%. An excellent efficacy achieved within ≦ 8 hours was almost doubled as compared to the results in a previously reported Japanese clinical trial (60.9 vs. 31.2%).

Furthermore, the efficacy rate for rFVIIa was significantly higher in thepatient group, which met all three recommended practice requirements, i.e., initial dose ≧ 90 ug/kg; time from the onset of hemorrhage to initial dose ≦ 3 hours; and mean dosing interval ≦ 3 hours. This group of patients demonstrated an 82.4% efficacy rate compared with the non-optimal practice group, which had an efficacy rate of 44.4%. However, only 40% of bleeding episodes in the patients were treated according to the recommended practices. Compliance with treatment remains a challenge that needs to be addressed in order to achieve improved efficacy. Forty two adverse drug reactions were reported in 20 patients including 4 severe ones in 3 patients, but none of them were thought to be related to the treatment with rFVIIa.

原　著

循環抗凝血素を有する症例における凝固因子活性測定

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要　旨

循環抗凝血素を有する患者血漿を対象として，4種類のAPTT試薬における凝固因子活性（FVIII：CおよびFIX：C）を比較検討した．

健常人例では試薬較差は認められなかったが，インヒビター保有血友病A症例，および後天性第VIII因子インヒビター症例では希釈率の上昇に伴って活性値が高値化し（平均5.5倍～17.1倍），特に後者のFVIII：Cでは160倍希釈において著しい試薬較差（CV33.6％）が認められた．これに対し，3例のLA陽性症例での試薬較差は，いずれも希釈率の上昇に伴って縮小し，最も変化が大きかった症例では，FVIII：CでCV95.0％が10.6％，FIX：CではCV103.1％が1.9％まで縮小した（320倍希釈）．さらに，LAに対して感度が高いとされるPLAでは低値（$p<0.01$）を示し，リン脂質濃度の高いACTでは高値（$p<0.0001$）を示す傾向が認められた．

以上の成績は，使用するAPTT試薬の特異性や検体希釈率の違いによって，得られる凝固因子活性に差異が生じることを示唆している．特に，CAを有する症例では試薬の特異性を充分に認識した上で測定値を評価することが必要であると思われた．

緒　言

循環抗凝血素（CA）は凝固因子に対する抗体とループスアンチコアグラント（LA）に大別される．CAによって引き起こされる凝固異常は出血傾向と血栓傾向という全く正反対の臨床像を呈するにもかかわらず，日常の凝固系スクリーニング検査においては，「凝固時間の延長」という同様の結果として検出され，このことが本症の臨床診断をより複雑にしている要因となっている．

現在，内因系凝固因子活性測定法として，一般には活性化部分トロンボプラスチン時間（APTT）試薬と凝固因子欠乏血漿を用いた一段法が利用されている．APTT試薬はリン脂質と活性化剤との組み合わせで構成されるが，血小板第3因子（リン脂質）の代用として牛脳由来セファリン，ウサギ脳由来セファリン，卵黄由来セファリン，ヒト胎盤由来リン脂質，さらに近年開発された合成リン脂質[1,2]など，由来や性状が異なる数種類

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のリン脂質成分が用いられている．また，活性化剤もカオリン，コロイドシリカ，エラジン酸などが用いられており，実際には多様なAPTT試薬が日常検査において利用されている．

これまで，APTT試薬成分の組み合わせや濃度の違いによりヘパリン感受性や凝固因子活性に対する特異性が異なることが報告されている[3)4)]が，CA保有例での検討はなされていない．今回，われわれはCAを有する症例を対象とし，組成の異なる4種のAPTT試薬を用いて凝固第VIII，第IX因子活性（FVIII：C，FIX：C）を測定し，各種APTT試薬の凝固因子活性測定における差異について検討した．

対象および方法

1．対象

検討材料として，凝固検査依頼のあった3.2％クエン酸ナトリウム加血漿を用い，測定時まで－80℃に凍結保存した．対象は健常人1例，インヒビター保有血友病A症例（16BU/ml）1例，後天性第VIII因子インヒビター症例（50BU/ml）1例，およびLA陽性症例3例（LA-1，LA-2，LA-3）とした．なお，LA陽性症例3例は全例とも国際血栓止血学会の診断基準[5)]に従って，希釈APTTおよび希釈蛇毒試験の二法[6)7)]を用い，両系共に陽性と判定された症例で，さらに，抗β_2グリコプロテインI抗体についても全例が陽性であった．

2．凝固因子活性測定

凝固因子活性は，症例ごとの各測定に同一バイアルの第VIII，IX因子欠乏血漿（シスメックス社）を用いて測定した．検量線作成には－80℃に凍結保存した当院の健常職員（n=50）のプール血漿を正常ヒト血漿（NHP）として使用し，測定機器にはKC-10A micro（Amerung社）を用いた．

APTT試薬は，トロンボチェックAPTT SLA（シスメックス社）：SLA，データファイAPTT（シスメックス社）：ACT，プラテリンLS（ビオメリュー社）：LS，PTT-LA（スタゴ社）：PLAの4種類を用いた（表1）．

一段法によるFVIII：C，FIX：C定量で用いた検量線は，オーレン緩衝液（pH7.35）で希釈したNHP 5倍希釈検体を100％とし，段階希釈して作成した．凝固因子活性測定は5倍希釈を基準希釈とし，必要に応じて最大320倍まで希釈して凝固時間を測定し，作成した検量線より活性値を求め，希釈倍数を乗じてその凝固因子活性値とした．

なお，被検血漿量の都合により，後天性第VIII因子インヒビター症例とLA-3ではPLA試薬による測定を実施することはできなかった．

3．Western blot法による凝固因子に対する抗体の検出

インヒビター保有血友病A症例および後天性第VIII因子インヒビター症例での凝固因子に対する抗体確認法として，遺伝子組み換え第VIII，第IX因子製剤を抗原としたWestern blot法を行った．各抗原をSDS-ポリアクリルアミドゲル電気泳動してPVDF膜に転写後，プロテインAカラムにより抽出した患者IgG（約20 μg/ml）と室温で1時間反応させて，ペルオキシダーゼ標識—抗ヒト—ヤギ抗体による免疫染色法で抗原抗体複合体を検出した．

4．統計学的検定

4試薬の活性値比較は，各希釈倍数での測定値をその平均値で除した値をBonferroni/Dunn testにて比較検定し，得られたp値により判定した．

結　果

1．基準希釈での凝固因子活性値

基準希釈（5倍希釈）での4試薬における凝固因子活性を比較した．健常人ではFVIII：C，FIX：Cとも試薬による較差は小さく，FVIII：CでCV 9.4％（60.7％～74.1％，平均68.2％），FIX：CではCV 10.6％（71.8％～90.4％，平均79.6％）と，健常人血漿としてはやや低い活性値であったが，4試薬とも同様の結果を示した（表2）．インヒビター保有血友病A症例では，FVIII：CはCV 72.7％（0.3～1.3％，平均0.7％），FIX：CはCV 38.3％（4.5～13.1％，平均9.3％）で，FVIII：Cの方がより大きな試薬較差を示した．また，後天性第VIII因子インヒビター症例では，FVIII：CがCV 19.8％（3.0～4.5％，平

表1．APTT 試薬一覧

試薬名	略称	リン脂質	活性化剤
トロンボチェック APTT SLA	SLA	合成リン脂質	エラジン酸
データファイ APTT	ACT	ウサギ脳セファリン	エラジン酸
プラテリン LS	LS	卵黄・牛脳リン脂質	軽質無水ケイ酸
PTT LA	PLA	ウサギ脳セファリン	シリカ

表2．健常人

第Ⅷ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	72.9	74.1	64.9	60.7	68.2	9.4
10倍	68.1	84.0	68.0	65.3	71.4	12.0
20倍	67.4	89.0	76.5	67.1	75.0	13.7

第Ⅸ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	71.8	90.4	81.8	74.2	79.6	10.6
10倍	74.8	104.3	90.7	80.7	87.6	14.7
20倍	68.7	125.1	83.8	81.6	89.8	27.2

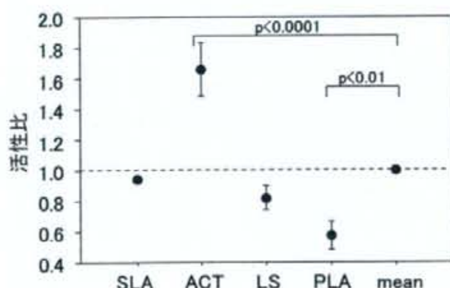

図1．4試薬の凝固因子活性値比較
各希釈倍率の凝固因子活性値を，4試薬で得た平均活性値で除した値を活性比として比較した．PLAでは平均値に対して低値を示し，ACTでは高値を示した．

均3.7%），FIX：CがCV 32.7%（20.2～37.8%，平均25.5%）と両因子とも4試薬の較差は小さかった（表4）．LA陽性症例では，LA-1でFVIII：CがCV 9.8%（80.9%～101.4%，平均94.4%），FIX：CがCV 16.9%（63.1%～93.9%，平均83.0%）と，FIX：Cの試薬較差の方がより大きく，PLAでやや低い活性値を示したが，その他は両因子とも正常基準値範囲内の活性値を示した．LA-2では，FVIII：CがCV 93.2%（4.7%～51.4%，平均22.1%），FIX：CがCV 107.6%（4.6%～59.4%，平均23.0%）と両因子ともに顕著な試薬較差が認められ，特に，ACTでの活性値は他に比べ高値を示した．LA-3ではFVIII：CがCV 95.0%（12.7%～75.3%，平均36.0%），FIX：CがCV 103.1%（9.1%～63.6%，平均29.1%）と，LA-2同様の大きな試薬較差が認められ，比較的低い活性値のSLAおよびLSと，高い活性値のACTとに二分されるような結果であった（表5）．

以上の結果より，LAに感度が高いとされるPLAは，4試薬の平均値に比べて低値を示す傾向にあり（$p < 0.01$），また，リン脂質濃度の高いACTは，高値（$p < 0.0001$）を示す傾向にあることが明らかとなった（図1）．

2．検体希釈率の上昇に伴う活性値の変動

検体希釈率の上昇に伴う活性値の変動は，各希釈倍率で得られた活性値を基準希釈の活性値で除した活性値比（10倍/5倍および20倍/5倍）とし

表3．インヒビター保有血友病A症例

第Ⅷ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	0.3	1.3	0.7	0.3	0.7	72.7
10倍	0.7	2.4	1.5	0.8	1.4	58.2
20倍	2.0	5.0	3.5	2.0	3.1	46.0

第Ⅸ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	9.6	13.1	9.9	4.5	9.3	38.3
10倍	31.4	40.2	35.7	20.2	31.9	26.9
20倍	48.8	81.1	58.5	46.1	58.6	27.1

表4．後天性第Ⅷ因子インヒビター症例

第Ⅷ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	4.2	3.2	4.5	3.0	3.7	19.8
10倍	4.6	5.3	6.8	3.7	5.1	25.7
20倍	3.5	5.2	6.8	2.9	4.6	38.3
40倍	10.3	29.5	26.1	—	22.0	46.5
80倍	21.6	47.6	52.5	—	40.6	40.8
160倍	40.2	77.4	79.2	—	65.6	33.6

第Ⅸ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	20.5	37.8	20.2	23.4	25.5	32.7
10倍	26.4	45.6	29.7	25.0	31.7	30.0
20倍	34.8	58.1	36.4	37.7	41.8	26.3
40倍	64.6	140.3	91.7	—	98.9	38.8
80倍	124.1	227.3	135.1	—	162.2	35.0
160倍	170.9	296.0	211.3	—	226.1	28.2

て比較検討した．

健常人では，FVIII：C，FIX：Cとも平均1.1倍と小さかった（表1）が，インヒビター保有血友病A症例では，FVIII：Cで平均5.5倍（20倍/5倍），FIX：Cでは平均6.9倍と，いずれの試薬も希釈率の上昇に伴って活性値は高値化した（表3）．また，後天性第VIII因子インヒビター症例では，FVIII：C，FIX：Cともに160倍までの検体希釈を必要とした例であったが，FVIII：Cで平均17.1倍（160倍/5倍），FIX：Cで平均8.9倍と，希釈に伴った著しい活性値の上昇が認められ，特に，FVIII：Cの試薬較差は，CV 19.8%（5倍希釈）から33.6%（160倍希釈）と増大した（表4）．LA陽性症例では，LA-1でFVIII：C，FIX：Cとも平均1.2倍（20倍/5倍）と軽度の上昇であったが，試薬較差が観察され，このうちPLAが最も大きな変化を示した．同様の結果はLA-2でも認められ，FVIII：C，FIX：Cとも平均2.9倍（20倍/5倍）と軽度であったが，PLAの変化は4.6倍，4.2倍と最も大きな変化を示した．

表5．LA陽性症例

LA-1

第Ⅷ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	98.6	96.7	101.4	80.9	94.4	9.8
10倍	107.0	112.9	115.1	95.8	107.7	8.0
20倍	101.1	117.2	116.6	102.1	109.3	8.1

第Ⅸ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	83.3	93.9	91.8	63.1	83.0	16.9
10倍	86.7	111.6	102.6	82.8	95.9	14.1
20倍	89.3	120.4	101.5	92.3	100.9	13.9

第Ⅷ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	20.6	51.4	11.7	4.7	22.1	93.2
10倍	34.2	72.3	18.8	8.1	33.4	84.2
20倍	50.7	88.6	32.6	21.8	48.4	60.5

第Ⅸ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	17.0	59.4	11.1	4.6	23.0	107.6
10倍	30.0	77.4	19.9	7.7	33.8	90.4
20倍	46.6	111.1	32.4	19.2	52.3	77.9

LA-3

第Ⅷ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	20.1	75.3	12.7	—	36.0	95.0
10倍	41.2	110.1	23.4	—	58.2	78.6
20倍	92.5	151.8	47.3	—	97.2	53.9
40倍	149.9	208.1	103.2	—	153.7	34.2
80倍	168.4	224.8	174.8	—	189.3	16.3
320倍	240.6	253.0	293.5	—	262.4	10.6

第Ⅸ因子活性（%）

希釈倍率	SLA	ACT	LS	PLA	mean	CV%
5倍	14.6	63.6	9.1	—	29.1	103.1
10倍	23.8	92.0	16.3	—	44.0	94.8
20倍	40.1	117.2	27.0	—	61.4	79.4
40倍	75.8	175.9	51.7	—	101.1	65.1
80倍	110.0	194.1	88.7	—	130.9	42.6
320倍	190.4	191.7	197.4	—	193.2	1.9

また，ACTでは10倍希釈以降において両因子とも正常範囲内の活性値となった．LA-3は320倍まで検体希釈を必要とした例であったが，FVIII：Cで平均12.8倍（320倍/5倍），FIX：Cで平均12.6倍と希釈に伴う高値化はいずれの試薬の場合も著しく，特に，SLAおよびLSでより大きな高値化を認めた．しかしながら，これら4試薬間の試薬較差は希釈率が上昇するに従って減少し，基準希釈時で認めたFVIII：Cの試薬較差CV 95.0%は，320倍希釈時で10.6%に，FIX：CでもCV 103.1%から1.9%と減少し一定値に収束した（表5）．

3. Western blot法による凝固因子に対する抗体の検出（図2）

インヒビター保有血友病A症例と後天性第VIII因子インヒビター症例について，凝固第VIII因子および第IX因子を抗原として行ったWest-

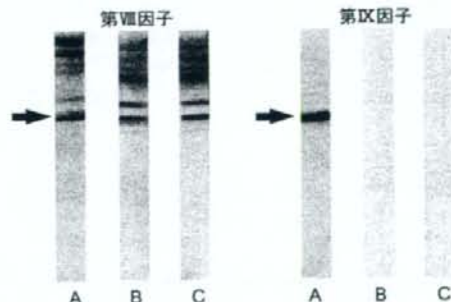

図2．Western blotによる抗体検索
第VIII因子，第IX因子を抗原とし，患者IgGを一次抗体としたWestern blot法を行った．
A：蛋白染色，B：インヒビター保有血友病A症例，C：後天性第VIII因子インヒビター症例．
両症例とも第VIII因子に対するIgG抗体を認めた．

ern blot法の結果，第VIII因子に対するIgG型の抗体が両症例において認められた．

考　察

これまでAPTT試薬については，ヘパリンやLAに対する感受性の差異[8〜10]について検討がなされており，また，凝固因子活性測定についても，使用する標準血漿や[11][12]欠乏血漿の性状についての検討がなされている．しかしながら，APTT試薬の試薬組成を考慮に入れた検討は少なく，不明な点も多い．このことは，未だにAPTT法がプロトロンビン時間（PT）法のように標準化できない理由の一つになっている．今回，われわれは日常の凝固スクリーニング検査において使用される4種（SLA，ACT，LSおよびPLA）の組成の異なるAPTT試薬について，各種CA陽性症例を対象として，欠乏血漿および健常人プール血漿による検量線用希釈系列を同一にした測定条件下でFVIII：CおよびFIX：Cを測定し比較検討した．

健常人例では，4種のAPTT試薬は同様の活性値を示し差異は認められず，また，希釈率を変えた場合でも得られる活性値は，ほぼ一致した．

インヒビター保有血友病A症例では，保有する第VIII因子インヒビターを反映した結果を示した．4種の試薬較差は，基準希釈（5倍希釈）ではFVIII：C，FIX：Cともに小さかったものの，希釈に伴って両活性値は変動し，全試薬でその活性値は高値化した．FVIII：CだけでなくFIX：Cも同様に変化したことについては，患者血漿中の抗第VIII因子抗体が第IX因子欠乏血漿中に含まれる第VIII因子に作用した結果と考えられた．

インヒビター値が50BU/mlと高力価であった後天性第VIII因子インヒビター症例でも，同様に，基準希釈での試薬較差は小さかったが，FVIII：Cでは希釈に伴って試薬較差が増大した．今回検討したインヒビター保有血友病A症例および後天性第VIII因子インヒビター症例は，各測定における活性値からそれぞれtypeIおよびtypeIIインヒビターと考えられ[13]，Western blotの結果より両者ともIgGタイプの第VIII因子インヒビターの存在が確認された．後天性第VIII因子インヒビター症例でFVIII：Cのみ検体希釈倍率の上昇に伴う試薬較差の増大を認めたことは，インヒビター保有血友病A症例と異なった現象であり，興味ある所見と考えられたがその機序については不明であった．インヒビター活性測定法であるBethesda法については，測定上の変動要因として正常血漿や緩衝液の問題点が指摘されているが[14]，今回の成績より，さらに，測定に用いるAPTT試薬による差異も今後の検討課題となると考えられた．

基準希釈で試薬較差が大きかったLA-2およびLA-3症例では，希釈倍率の上昇に伴う著しい高値化を認めたが4試薬の較差は徐々に縮小し，最終的にはほぼ同一の値を示す結果となった．この結果は，希釈操作がインヒビター作用を受けない条件にまで徐々に導くことを示した結果であり，本来の目的であるインヒビター作用を除いた活性値（抗原量）を表現し得る条件に達したことを示唆している．今回のLA症例での検討では，LAに感度が高い試薬とされるPLA[9]も検討した．PLAが示した活性値は4試薬中最も低く，次いでSLA，LSの順で，リン脂質濃度が高いACTは最も高い活性値を示した．この結果は，検量線などの正常血漿での凝固時間測定では影響を受けず，リン脂質依存性抗体が存在する場合においてのみ影響を受けるというPLAの特性（すなわち，PLA試薬のLAに対する高い感受性）を反映した結果であり，さらに，APTT試薬に含まれるリン脂質の濃度は，LAに対する感受性を左右する重要な試薬要素であることを示唆している．

今回実施したCAを有する症例での検討で，検量線用血漿と凝固因子欠乏血漿を同一にしても，用いるAPTT試薬の特異性の違いや測定時の希釈率によって，得られる活性値は著明に異なることが明らかとなった．健常人例での結果を踏まえると，このようなAPTT試薬の違いによる差異は，今回検討対象としたインヒビター保有症例やLA陽性症例などのCA症例での検討で初めて明らかとなる結果と思われる．LA陽性症例での凝固因子活性測定値や，凝固因子インヒビター症例での高希釈倍率で得られる高い活性値の臨床的意

義については未だ疑問が残るものの，検査上両者の明確な判別が困難な症例が存在するのも事実である．凝固スクリーニング検査をはじめとして，広く凝固因子活性測定に使用されているAPTT試薬であるが，今回の検討で示されたように，試薬によって活性値に差異を生じ得ることも念頭に置く必要があり，故に，用いたAPTT試薬の特異性を認識した上で活性値を評価することが重要と考えられた．

結　語

CAを有する症例を対象として，検量線用正常血漿と凝固因子欠乏血漿を同一にして，特異性の異なるAPTT試薬を用い凝固第VIII因子および第IX因子活性測定を行った結果，APTT試薬の特異性の違いや検体希釈率により得られる活性値に差異があることが明らかとなった．また，LA陽性症例においては，APTT試薬のLAに対する感度が測定上大きく影響すると考えられた．

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Abstract

Evaluation of coagulation factor activity of different APTT reagents using samples from patients with circulating anticoagulants

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To evaluate the coagulant specificity of different APTT reagents, we studied the level of coagulation factor activity obtained from four commercial APTT reagents using samples from patients with circulating anticoagulants (CA).

The mean values of FVIII: C and FIX: C using the four APTT reagents were 0.75% (CV 72.7%) and 9.3% (CV 38.3%), respectively, in a haemophilia A patient with inhibitor to factor VIII, and 3.7% (CV 19.8%) and 38.3% (CV 32.7%), respectively, in an acquired haemophilia A patient. Some discrepancies in activity between the four APTT reagents were observed. Specifically, in a sample from the acquired haemophilia A patient, a marked discrepancy of CV 33.6% (FVIII: C) was observed at 160-fold dilution of the sample. Moreover, 5.5 to 17.1-fold of the increased was found to accompany sample dilution.

On the other hand, in samples (LA-1, LA-2 and LA-3) from three patients with lupus anticoagulant (LA), the two coagulation factor activities varied, but the discrepancies between the four reagents decreased in line with sample dilution. The largest discrepancy of CV 95.0% (FVIII: C) and CV 103.1% (FIX: C) observed in LA-3 decreased to CV 10.6% and CV 1.9%, respectively, at ×320 dilution. The results from PLA reagent with high sensitivity to LA showed lower activities ($p<0.01$), while, ACT reagent containing a high phospholipid concentration showed higher activities ($p<0.0001$).

These results show that striking differences in coagulation factor activity resulted from the use of these APTT reagents depending on their coagulant specificity to CA. An understanding of the characteristics of the APTT reagent used would ensure precise evaluation of coagulation factor activity.

Key words: Circulating anticoagulants (CA), Lupus anticoagulant (LA), Inhibitors to factor VIII, Coagulation factor activity, Coagulant specificity

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Platelet-Directed Gene Therapy

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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 secretary 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 phänotyische 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.

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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) Ibα / 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 GPIbα/GPV/GPIX complex (CD42b+CD42c/CD42d/CD42a), but also serve as a strong stimulus for platelet activation through its collagen receptors GPVI and GPIa/IIa (also known as integrin α2ββ1, CD29/CD49b) [6, 7]. This results in signaling pathways that induce platelets to change their shape, spread along collagen fibrils and secrete thromboxane A2 (TxA2) and adenosine diphosphate (ADP) into circulation [4, 6, 7]. The released TxA2 and ADP stimulate neighboring platelets, causing them to become activated and in turn secrete additional TxA2 and ADP. Activated platelets directly bind to the abundant plasma protein fibrinogen, via the platelet receptor GPIIb/IIIa (also known as integrin αIIbβ3; CD41/CD61) [8]. This platelet-fibrinogen-platelet interaction initiates the process of platelet aggregation (fig. 1).

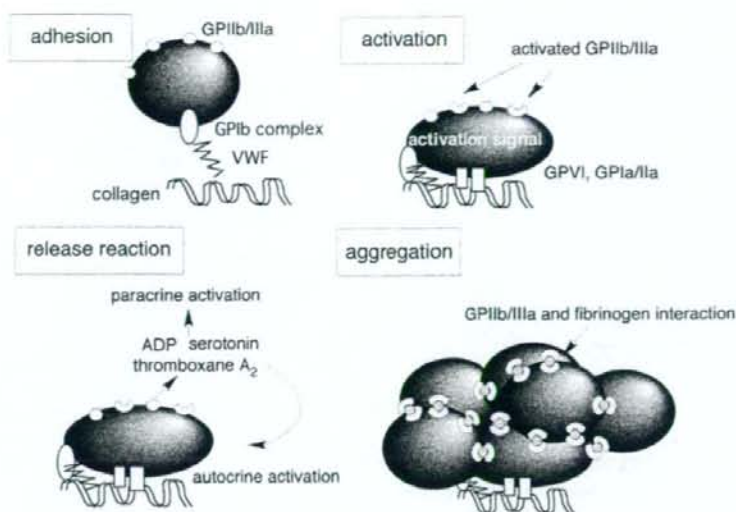
Another important function of platelets is the release of a variety of substances that modulate the coagulation cascade and/or functions of platelets and other cells [9, 10]. These can regulate thrombus formation and affect its mechanical properties as well as contribute to cell-adhesive events, immunity, 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.

Table 1. Major bioactive substances and glycoproteins within platelet granules

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

Platelets contain 3 types of granules within cytoplasm; lysosomes, dense granules, and α-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]. α-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]. α-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 α-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 GPIa/IIa (activation, top right). Collagen receptors mediate platelet activation signalling, which results in spreading and in the secretion and release of thromboxane A2 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)



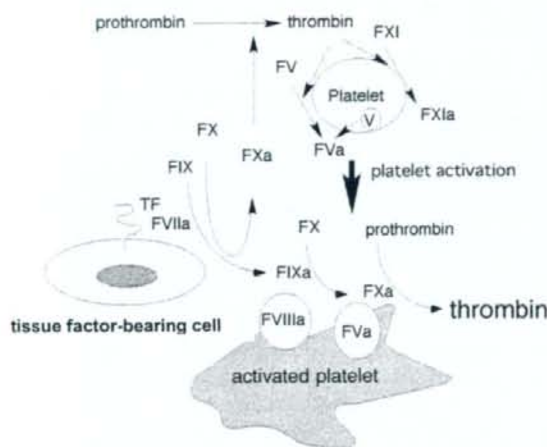


Fig. 2. Involvement of platelets in the coagulation cascade. Cofactors FVa (released from activated platelets) and FVIIIa are rapidly colocalized to the platelet membrane surface. FIXa formed by the FVIIa/TF complex binds to the surface of activated platelets. Activated platelets bind FIXa and promote 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.

tivation 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].

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 phosphotidyl 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

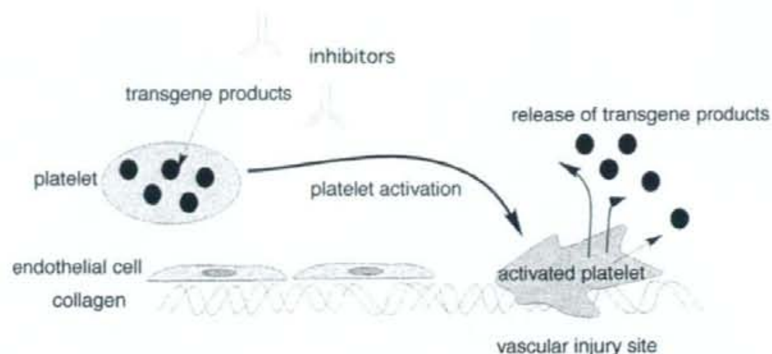


Fig. 3. Advantages of platelet-directed gene therapy. Platelets provide a way to enhance the local concentrations of target substances at sites of vascular injury while minimizing the influence of plasma proteins that may inhibit their activities. Platelet-mediated protein delivery may also inhibit the 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/doxycline 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.

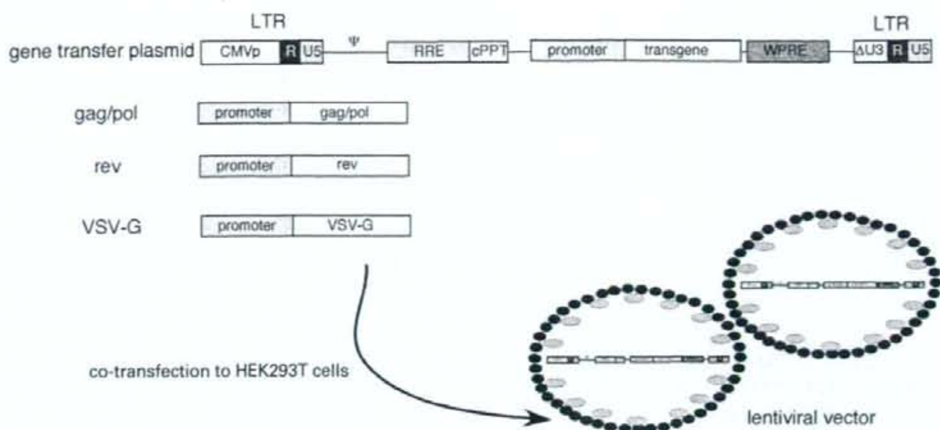


Fig. 4. Structure and production of lentiviral vector. A minimal lentiviral vector plasmid consisting of the CMV/LTR chimera promoter followed by the packaging signal (Ψ), rev-binding element RRE for cytoplasmic export of the RNA, the transgene expression consisting of internal promoter and transgene, 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 (Ψ), a heterogonous 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, flaviviridеae, 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+ cells very poorly [56]. On the other hand, SIV vectors efficiently transduce macaque CD34+ 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 transcription 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 β-globin locus control region [67, 68]. Insertion of cHS4 in SIN lentiviral vectors results in higher and less variable expressions of human β-globin, similar to observations with cHS4-containing retroviral vectors carrying the human γ-globin gene [65, 66]. The levels of β-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+ 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-

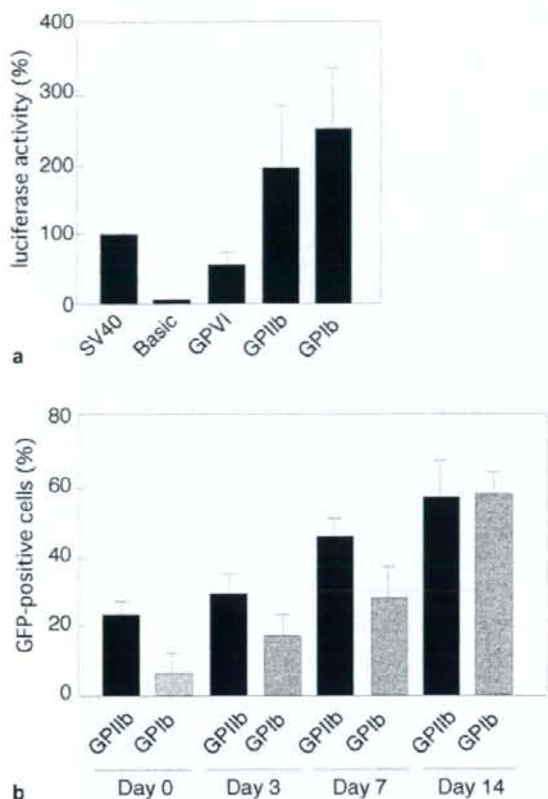


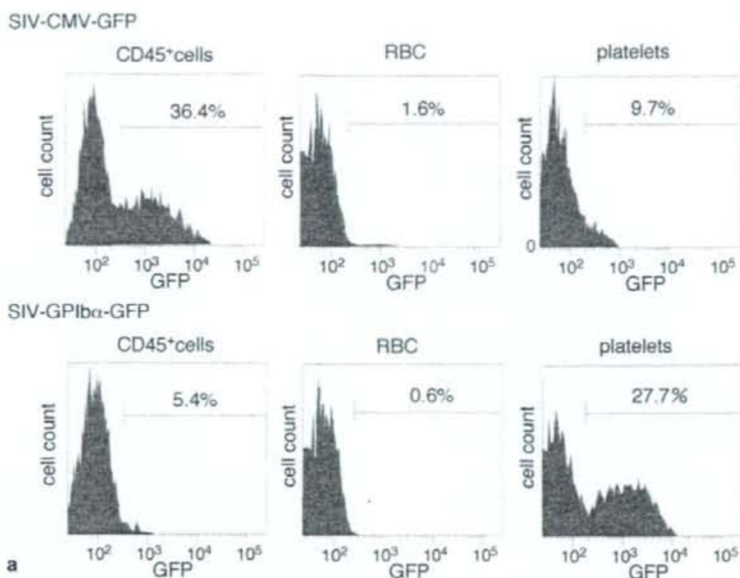
Fig. 5. Advantage of GPIbα promoter as a platelet specific promoter. **a** Comparison of platelet-specific promoter activities. Each construct along with a promoter-less vector (basic) or a positive control vector (SV40) was transfected into the UT-7/TPO megakaryocytoblastic cell line. Luciferase activities were measured 48 h after transfection and are shown relative to the activity driven by the SV40 promoter (SV40/Enhancer) (n = 5). **b** CD34+ 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 GPIbα promoter or GPIIb promoter at a MOI of 30. Columns and error bars represent the percentage of GFP-positive cells (mean ± SD; n = 3).

ciencies of CD45+ 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, pf4, 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 GPIbα promoter as a platelet-specific promoter because the promoter activity of GPIbα is more potent than that of GPIIb in UT-7/TPO and CD34+ cell-derived megakaryocytes [73] (fig. 5a). Another reason is that the GPIbα 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+ 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 GPIbα 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 colleges [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 β-galactosidase gene driven by GPIIb promoter and found that the transduced CD34+ 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+ 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



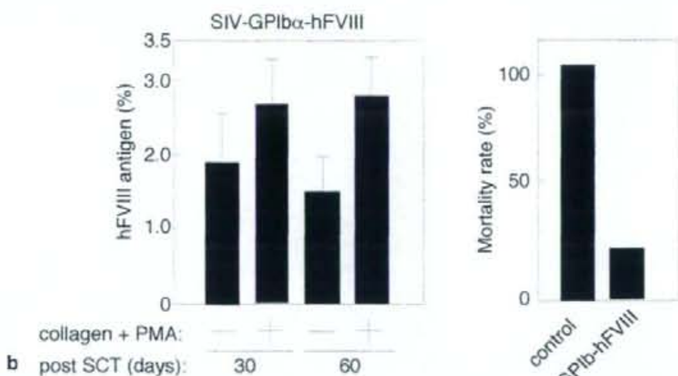


Fig. 6. Efficient expression of transgene in platelets using lentiviral vector haboring GPIbα promoter. **a** KSL cells were transduced with a SIV lentiviral vector equipped with eGFP driven by a CMV (SIV-CMV-eGFP) or GPIbα (SIV-GPIbα-eGFP) promoter at a MOI of 30. Representative flow cytometric analyses of eGFP-positive cells in CD45+ 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-GPIbα-hFVIII was stimulated in the presence or absence of 50 μg/ml of collagen and 1 μmol/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-GPIbα-hFVIII-transduced KSL cells (n = 10 for control, n = 8 for GPIbα) (right panel).

lentivirus vector equipped with the human integrin β3 gene under control of the GPIIb promoter resulted in phenotypic correction of a mouse model of GT, integrin β3-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 antibody 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+ HSCs enables FVIII-transduced megakaryocytes to store human FVIII with VWF within α-granules [91]. This is a very important finding because VWF is the natural carrier protein of FVIII, and FVIII interaction with VWF within platelet α-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 β-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.

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