(2) 血中投与可能なナノ粒子の調製

われわれはこれまで2種類の血中投与可能なナノ粒子(アルブミン重合体, リポソーム)を提案してきた. それらの調製法を以下に紹介する. アルブミンは血漿中で最も多く存在する蛋白質(約5 g/dL)であり, 膠質浸透圧の調節や栄養物や代謝物などの運搬を担っている. これから成る微粒子, 例えばアルブミン凝集体, マイクロスフェア, マイクロカプセル等は生体適合性を有するため, 1950年代から静注用製剤として血管造影剤, 超音波診断用増感剤や徐放性薬物担体等に臨床応用されている. しかし, これらは高温加熱処理を施すスプレードライ法や有機溶媒による不可逆な変性あるいは界面活性剤や架橋剤を用いるため, 粒子径の制御や添加物の除去操作が煩雑であった. そこでわれわれは, 遺伝子組換えヒト血清アルブミン(rHSA)をモノマーと見なし, ジスルフィド縮合重合を誘起させてアルブミンナノ粒子を調製する方法を確立した[27]. これはrHSA水溶液のpHと温度を制御するだけでナノ粒子を調製できるクリーンな方法であり, 親水性の表面をもつナノ粒子となる(図1B左). 架橋剤を用いた表面修飾技術を利用すれば, アルブミンナノ粒子表面にH12を水溶液中で担持させることができる. ここではナノ粒子の血中滞留性を考慮して, 末端にN-ヒドロキシスクシンイミド基とマレイミド基を有する二官能性ポリエチレングリコール(PEG)誘導体を使用した[28, 29]. 前者の官能基は粒子表面のリジン残基のアミノ基と反応し, 結果としてマレイミド基含有PEGが粒子表面に固定される. 遠心分離にて精製後, システイン残基をN末端に導入したH12を混合するだけで, 水溶液中でH12を定量的に結合可能となる.

他方, リン脂質を水中に分散させると自己集合し, 多重層のリポソームが形成する[30]. これを多孔質フィルターに透過させるエクストルージョン法によって, 粒子径と被覆層数の制御を簡便に制御できる(図1B右). リポソームには, ①膜成分であるリン脂質やコレステロールは生体適合性に優れる, ②水溶性や脂溶性の低分子薬物, 蛋白質や遺伝子などの水溶性高分子を内水相あるいは脂質膜内に封入できる, ③リポソームの表面に蛋白質やペプチドを担持させて特定領域への指向性やターゲティング能を付与できる[31], 等の長所が挙げられる. われわれのグループは, グルタミン酸に炭素数18のアシル鎖を2本結合させた合成脂質(Glu2C18)

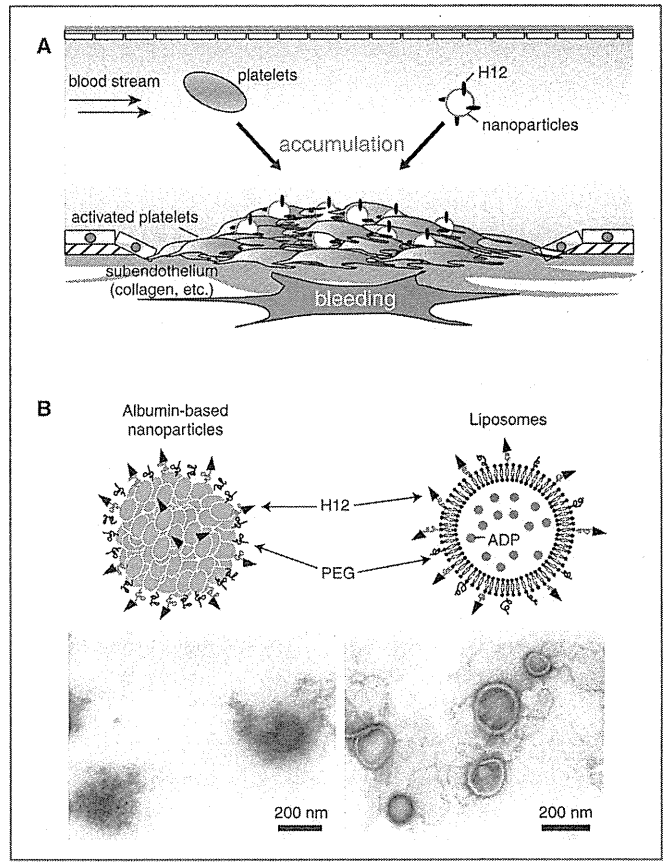

図1
A. 血小板代替物の設計方針
活性化血小板を認識する分子(フィブリノーゲンγ鎖C末端ドデカペプチド, HHLGGAKQAGDV:H12)を担持させたナノ粒子を設計している. このナノ粒子が血管損傷部位へ特異的に集積して血栓形成を誘導する起点となり, 集積したナノ粒子を利用して出血部位を充填し止血能を補助させるとの発想に基づいて血小板代替物を設計している.
B. 生体投与可能なナノ粒子(アルブミン重合体とリポソーム)の模式図と透過型電子顕微鏡像

に, PEG鎖を介してH12を結合させたH12-PEG-Glu2C18を新規に合成した[32]. これをジパルミトイルホスファチジルコリンとコレステロールを主成分とする粒径250 nm程度のリポソームに導入し, 血小板代替物として開発してきた. さらにリポソームの止血効果を増幅させるために, 内水相に血小板凝集を惹起させるADPを封入している点も新しい試みである(図1B右)[33]. 次項では誌面の関係上, リポソームの評価に絞って解説する.

(3) 機能評価

設計したH12-リポソームが出血部位のみに特異的に集積することが必須条件であり, この実証実験を行った. リポ

ソームを可視化するために，ここでは水溶性造影剤であるイオパミドールを内包させた[34]．これをWister系雄性ラットに投与し，投与5分後に尾静脈に沿って1 cmの傷をつけ，出血が止まるまで生理食塩水に浸漬させた．実験小動物用のX線コンピュータ断層撮影法にて出血部位周辺を撮影したところ，出血部位に対応する領域が白く造影された．他方，H12未結合のイオパミドール内包リポソーム群やイオパミドール水溶液投与群では造影されなかった．従って，H12-リポソームが出血部位に対して特異的に集積する能力を持つことを*in vivo*試験で実証できた．

続いて，止血能を増幅させるためにADPを内水相に封入したリポソーム（H12-(ADP)-リポソーム）を調製し，その放出特性を検討した[33]．まず，H12を担持させていないADPリポソームを多血小板血漿に添加した後，コラーゲンにて血小板凝集を惹起させ，その透過率変化を血小板凝集計にて計測した．コラーゲン添加約1分後に血小板形態変化に伴う緩やかな透過率の減少が見られ，その後安定な血小板凝集を示すコラーゲン凝集特有の透過率曲線を描いた（最大透過率：42±5%）．これはコントロールリポソーム（H12未結合，ADP未内包）と同等の挙動であり，本リポソームは血小板凝集に何ら寄与しないことが分かった．次いで，ADPを内包させていないH12-リポソームの最大透過率は49±6%と僅かに増大した．これは，リポソーム表面のH12によって血小板と多点結合し，血小板凝集塊形成を促進させたためと考えられる．最後に，H12-(ADP)-リポソームを添加して同様に評価した．コラーゲン添加直後から透過率の上昇が見られ，最大透過率は65±6%に達した．コラーゲンを添加しなければ血小板凝集は全く起こらないことから，血小板凝集の刺激を受けてH12-(ADP)-リポソームからADPが放出されたことを示唆する興味深い結果である．測定終了後の血小板凝集塊を電子顕微鏡にて観察したところ，血小板間に多数のリポソームが存在しており，その形状は変形していた．従って，H12-(ADP)-リポソームは，H12を介して多点結合して血小板凝集塊に巻き込まれ，その際に血小板間で受ける物理的な刺激によって変形してADPを放出することで血小板凝集を顕著に促進させるリポソームと言える（図2A）[35]．これは，ドラッグデリバリーとコントロールドリリースを同時に実現させた薬物運搬体としての可能性を秘める新しい知見である．

最後に，H12-(ADP)-リポソームの*in vivo*止血能評価の結果をまとめる[33]．まず，ニュージーランドホワイトウサギに抗がん剤のブスルファンを30 mg/kgを2回に分けて投与すると，その副作用によって，投与15日後には血小板数のみが正常ウサギ（血小板数：37±8×10^4/μL）の1/15程度（血小板数：2.4±1.3×10^4/μL）まで再現性よく減少する．この重篤な血小板減少症モデルウサギの耳にメスで6 mmの長さに切傷し，生理食塩水中に浸し出血が止まるまでの時間（出血時間）を測定したところ1,695±197秒となり，正常ウサギ（112±24秒）と比較して約15倍も延長した（図2B）．まず，陽性対照群として30分前にウサギ血小板を輸血したところ，投与量依存的に血小板数が増大するとともに出血時間を顕著に短縮でき，血小板輸血の効果を再確認した．そこで，血小板輸血の代わりにH12-(ADP)-リポソームを投与したところ，出血時間が投与量依存的に有意に短縮され，かつ血小板輸血に匹敵する程の止血効果が発現した（図2B）．これは，H12によってリポソームが特異的に出血部位に集積し，内包したADPが効率よくその場で放出されて安定な血小板凝集塊を形成させたためと考えられる．さらに，ごく最近，西川らはH12-(ADP)-リポソームの外傷性大量出血に対する止血治療の有効性を実証している[36]．具体的には，大量の赤血球輸血で血小板が減少したウサギの肝臓に傷をつけるとほとんどが出血死するが，H12-(ADP)-リポソームの投与によって血小板輸血と同様に全例の止血救命が可能となることを明らかにした（図3）．これは大震災等で大怪我し大量出血を来たした患者の止血治療に大いに役立つことが期待できる成果である．現在のところ，H12-(ADP)-リポソームを投与しても，血球成分，血液凝固系には何ら影響しないことを確認しているが[33]，今後は，臨床試験を見据えながら本リポソーム製剤の安全性に関する詳細な知見を集積する必要があると考えられる．

まとめ

血小板代替物の研究開発を期待するニーズが高いにも関わらず，未だ実用化には至っていない．しかしわれわれのグループでは，血小板減少動物モデルや外傷性大量出

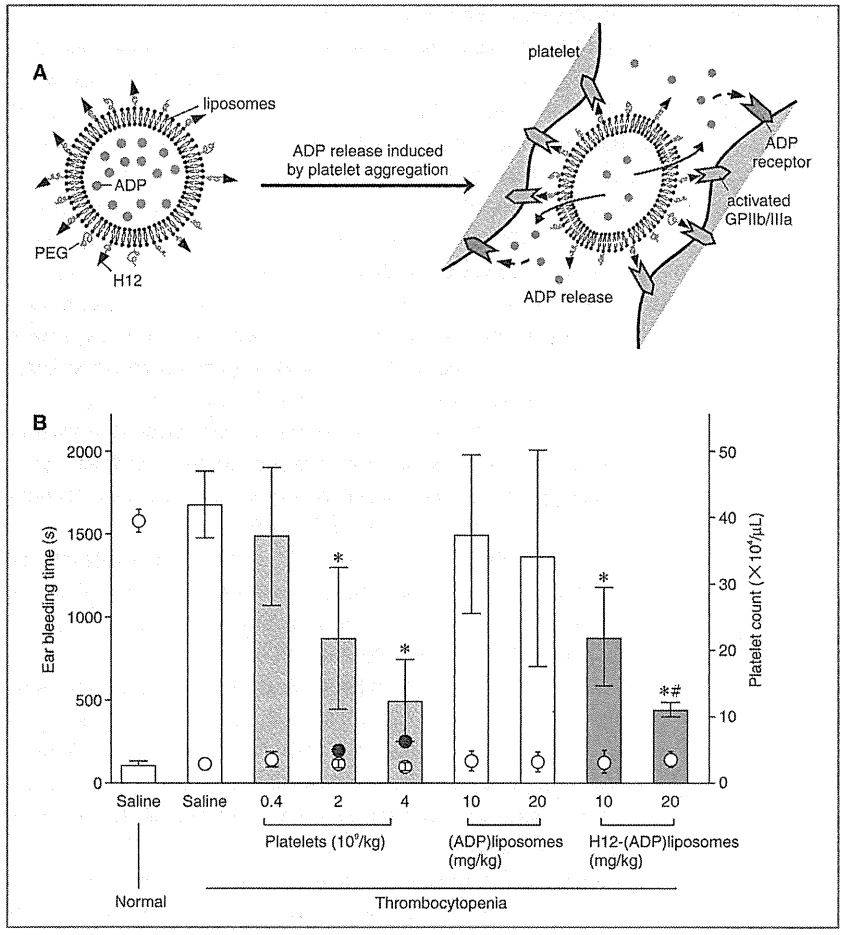

図2
A. H12-(ADP)-リポソームからのADP放出機構
H12-(ADP)-リポソームはH12を介して血小板凝集塊に特異的に巻き込まれ，その際に血小板間で受ける物理的な刺激によって変形してADPを放出し，血小板凝集を亢進させる．
B. H12-(ADP)-リポソームの出血時間短縮効果
血小板減少ウサギに生理食塩水，ウサギ血小板，(ADP)-リポソーム，H12-(ADP)-リポソームを投与し，30分後に耳介周囲に6 mmの傷をつけ，生理食塩水に浸して出血時間を測定．
○：ウサギの血小板数
●：ウサギ血小板を輸血した後の血小板数．
* P＜0.05 vs. 生理食塩水群
# P＜0.05 vs. (ADP)-リポソーム群

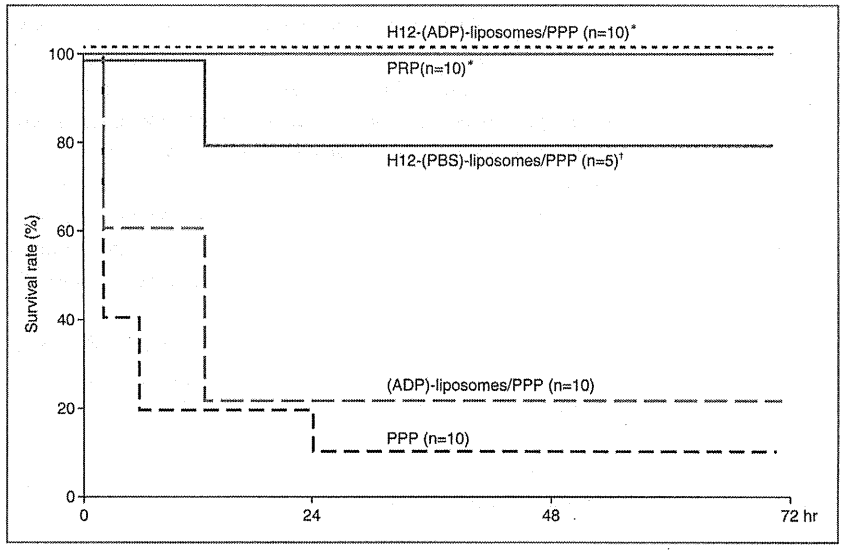

図3　肝臓外傷による大量出血ウサギモデルにおけるH12-(ADP)-リポソームの救命効果
全血脱血と洗浄赤血球の返血を8回繰り返し作製した稀釈性血小板減少ウサギにH12-(ADP)-リポソーム(20 mg/kg)と乏血小板血漿(PPP)を投与し，15分後に肝臓に貫通性損傷を与え，輸液のみの処置にて動物の予後を観察．比較対照として，多血小板血漿(PRP)，PPPのみ，H12未結合リポソーム((ADP)-リポソーム)およびADP未内包リポソーム(H12-(PBS)-リポソーム)を用いた．
* P＜0.01 vs. PPPあるいは(ADP)-リポソーム群
† P＜0.01 vs. PPPあるいはP＜0.05 vs. (ADP)-リポソーム群

血モデルを用いた止血効果に関する知見を確実に得ており，完全人工系の血小板代替物の設計方針の妥当性は実証された．近年，海外の研究グループも完全人工型血小板代替物の開発に移行してきており，研究の方向性は正しいと信じている．今後は，ナノ粒子の安全性試験，例えば，ナノ粒子を巻き込んだ血栓の線溶系に及ぼす影響，反復投

与に対する代謝系，細網内皮系，免疫系に及ぼす影響や抗原性の検討を詳細に行う必要がある．同時に，臨床試験に向けた適応法（出血症状を予防するのか，治療するのか）を明確にし，臨床試験のプロトコールを作成していく計画を立てている．近い将来に実用可能な血小板代替物が創製されるであろうし，それに限らずドラッグデリバリーシステムの基盤技術の発展にも貢献できる革新的技術になるであろう．

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ORIGINAL ARTICLE

Fibrinogen γ-chain peptide-coated, ADP-encapsulated liposomes rescue thrombocytopenic rabbits from non-compressible liver hemorrhage

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Summary. *Background:* We developed a fibrinogen γ-chain (dodecapeptide HHLGGAKQAGDV [H12])-coated, ADP-encapsulated liposome (H12-[ADP]-liposome) that accumulates at bleeding sites via interaction with activated platelets via glycoprotein IIb–IIIa and augments platelet aggregation by releasing ADP. *Objective:* To evaluate the efficacy of H12-(ADP)-liposomes for treating liver hemorrhage in rabbits with acute thrombocytopenia. *Methods:* Thrombocytopenia (platelets < 50 000 μL^{-1}) was induced in rabbits by repeated blood withdrawal (100 mL kg^{-1} in total) and isovolemic transfusion of autologous washed red blood cells. H12-(ADP)-liposomes with platelet-poor plasma (PPP), platelet-rich plasma (PRP), PPP, ADP liposomes with PPP or H12-(PBS)-liposomes/PPP, were administered to the thrombocytopenic rabbits, and liver hemorrhage was induced by penetrating liver injury. *Results:* Administration of H12-(ADP)-liposomes and of PRP rescued all thrombocytopenic rabbits from liver hemorrhage as a result of potent hemostasis at the liver bleeding site, although rabbits receiving PPP or ADP liposomes showed 20% survival in the first 24 h. Administration of H12-(ADP)-liposomes and of PRP suppressed both bleeding volume and time from the site of liver injury. H12-(phosphate-buffered saline)-liposomes lacking ADP also improved rabbit survival after liver hemorrhage, although their hemostatic effect was weaker. In rabbits with severe thrombocytopenia (25 000 platelets μL^{-1}), the hemostatic effects of H12-(ADP)-liposomes tended to be attenuated as compared with those of PRP treatment. Histologic examination revealed that H12-(ADP)-liposomes accumulated at the bleeding site in the liver. Notably, neither macrothombi nor microthrombi were detected in the lung, kidney or liver in rabbits treated with H12-(ADP)-liposomes. *Conclusions:* H12-(ADP)-liposomes appear to be a safe and effective therapeutic tool for acute thrombocytopenic trauma patients with massive bleeding.

Keywords: hemostasis, liver hemorrhage, nanotechnology, platelet substitute, rabbits, thrombocytopenia.

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Introduction

Hemorrhage is a major cause of preventable death in trauma victims and combat casualties [1,2]. In particular, non-compressible thoracoabdominal hemorrhage accounts for half of these deaths [2]. Therefore, control of exsanguinating hemorrhage is crucial for reducing mortality in severe trauma victims. Excess blood loss due to uncontrollable hemorrhage often requires massive blood transfusion, which also increases patient mortality [3]. Coagulopathy and thrombocytopenia following massive blood transfusion are frequently observed in such critical patients, resulting from hypothermia, acidosis, and dilution, as well as consumption of hemostatic elements, such as platelets and coagulation factors. Coagulopathy and thrombocytopenia are considered to be the major reasons for the high mortality [4,5].

Transfusion of platelet concentrates and plasma products is effective in attenuating coagulopathy/thrombocytopenia in hemorrhagic patients receiving massive red blood cell (RBC) transfusions, resulting in reduced mortality [6,7]. Replenish-

ment of coagulation factors after massive transfusion can be accomplished by administration of fresh frozen plasma (FFP) containing a normal amount of coagulation factors such as fibrinogen, and this approach is particularly recommended for combat casualties [8,9]. Nevertheless, restoration of coagulation factors by FFP may not terminate the clinical coagulopathy in patients showing severe thrombocytopenia (platelets < 50 000 μL^{-1}) [10]. In these critical cases, platelet therapy is required [11]. However, platelet concentrates are not usually available for emergency use, owing to their short-term viability, which requires stringent storage conditions [12,13]. The utility of platelet concentrates is very limited in difficult-to-reach rural areas, such as remote islands or mountain ranges. Furthermore, the populations of several countries, including Japan, are rapidly aging, and there is concern that a shortage of blood donors will become a serious problem in the near future.

The application of effective blood substitutes, such as oxygen-carrying blood substitutes or platelet substitutes, is therefore expected. It has obvious advantages, particularly in the care of large-scale disaster victims or combat casualties. Although oxygen-carrying blood substitutes appear to be effective in resuscitating patients with hemorrhagic shock [14], they have no hemostatic effect and are therefore ineffective against thrombocytopenic bleeding. Novel infusible hemostatic agents, namely platelet substitutes, are expected to rescue such critical patients [15,16].

We have developed liposome-based artificial platelet substitutes (mean diameter of 210 nm) bearing synthetic HHLGGAKQAGDV (H12) peptides corresponding to the C-terminus of the fibrinogen γ-chain on the surface, and with the physiologic platelet agonist ADP inside [17]. In primary hemostasis, platelet plug formation is mediated by fibrinogen through the bridging of adjacent platelets via integrin $\alpha_{IIb}\beta_3$ (glycoprotein [GP]IIb–IIIa) in an activation-dependent manner [18,19]. With the H12 sequence, a primary recognition site for the GPIIb–IIIa receptor, the synthetic nanocarriers, HHLGGAKQAGDV-coated, ADP-encapsulated liposome (H12-[ADP]-liposomes), specifically target the sites of vascular injury where platelets have been activated [19,20]. H12-(ADP)-liposomes then reinforce platelet aggregation by releasing encapsulated ADP (Fig. 1). H12-(ADP)-liposomes thus work as platelet crosslinkers, and ADP release may be induced by liposomal membrane perturbation and/or destruction elicited by aggregation-driven sheared forces [21,22]. In our previous

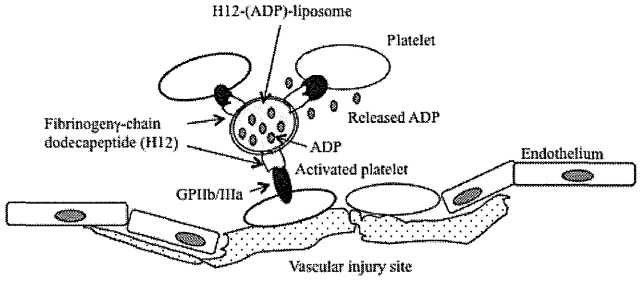

Fig. 1. Platelet aggregation mediated by H12-(ADP)-liposomes at the injured vessel site. GP, glycoprotein.

study, we found that H12-(ADP)-liposomes significantly shortened bleeding time in rats and rabbits with busulfan-induced thrombocytopenia, thus suggesting that they may be able to replace platelet products in prophylactic platelet transfusion [21]. We herein investigated the therapeutic effects of H12-(ADP)-liposomes on non-compressible intra-abdominal hemorrhage resulting from liver injury, using a rabbit model with massive transfusion-induced acute thrombocytopenia.

Materials and methods

This study was conducted according to the guidelines of the Institutional Review Board for the Care of Animal Subjects of the National Defense Medical College.

Rabbits and reagents

New Zealand White rabbits (2 kg, male; Japan SLC, Hamamatsu, Japan) were used. Cholesterol and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) were from Nippon Fine Chemical (Osaka, Japan), 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-(monomethoxypoly[ethylene glycol]) (PEG-DSPE; 5.1 kDa) was from NOF (Tokyo, Japan), and ADP was from Sigma-Aldrich (St Louis, MO, USA). We synthesized 1,5-dihexadecyl-*N*-succinyl-L-glutamine (DHSG) and H12-PEG-Glu2C18, where fibrinogen γ-chain dodecapeptide (C-HHLGGAKQAGDV [Cys-H12]) was conjugated to the end of the PEG–lipids, as described elsewhere [21].

Preparation of H12-(ADP)-liposomes, ADP liposomes, and H12-(phosphate-buffered saline [PBS])-liposomes

H12-(ADP)-liposomes were prepared as described elsewhere [21]. Briefly, DPPC (1 g, 1.36 mmol), cholesterol (527 mg, 1.36 mmol), DHSG (189 mg, 272 μmol), PEG-DSPE (52 mg, 9 μmol) and H12-PEG-Glu2C18 (47 mg, 9 μmol) were dissolved in benzene and freeze-dried. The resulting mixture of lipids was hydrated with PBS containing 1 mmol L^{-1} ADP or PBS alone, and was filtered twice with Durapore (pore size, 0.45 μm; first and 0.22 μm; second; Millipore, Tokyo, Japan) to prepare H12-(ADP)-liposomes or H12-(PBS)-liposomes, respectively. After washing of liposomes with PBS followed by centrifugation (100 000 × *g*, 30 min, 4 °C), the remaining ADP was removed with Sephadex G25. ADP liposomes without H12 dodecapeptide were also prepared by skipping the conjugation of fibrinogen γ-chain dodecapeptide to PEG–lipid.

Acute thrombocytopenic rabbit model

Rabbits were anesthetized with intramuscular injections of ketamine (25 mg kg^{-1}) and xylazine (10 mg kg^{-1}), and this was followed by maintenance of anesthesia with intravenous injections of pentobarbital (15 mg kg^{-1}) every 30 min during the experiment. Surgical catheters (polyethylene indwelling needle 22G; Terumo Co., Tokyo, Japan) were inserted into the femoral artery and vein in each rabbit (Fig. S1). Thereafter,

12.5 mL kg^{-1} blood (sample 1) was drawn from the femoral artery, and the same volume of lactated Ringer's solution containing 5% human serum albumin (HSA) (Kaketsuken, Kumamoto, Japan) was simultaneously transfused via the femoral vein. Forty minutes later, the next blood sample (12.5 mL kg^{-1}, sample 2) was withdrawn, and the same volume of washed RBCs as prepared with sample 1 was transfused. This isovolemic blood exchange was repeated seven times, and the last transfusion of washed RBCs was performed without simultaneous blood withdrawal (Fig. 2). Thereafter, platelet counts decreased to $< 5 \times 10^4$ μL^{-1}. Furthermore, we repeated this isovolemic blood exchange 14 times in order to ensure a severe thrombocytopenic condition. After blood exchange, platelet counts decreased to $(25 \pm 1) \times 10^3$ μL^{-1}. Body temperature was maintained at 37–38 °C in rabbits, with a heating pad. Arterial blood pH was maintained at 7.35–7.45.

Preparation of washed RBCs, platelet-rich plasma (PRP), and platelet-poor plasma (PPP)

Blood samples drawn with a 10% volume of 3.8% (w/v) sodium citrate were centrifuged at $100 \times g$ for 15 min, and the supernatant was used as PRP (Fig. S1). The remaining sample was further centrifuged at $500 \times g$ for 10 min, and the supernatant was used as PPP. Thereafter, the remaining cells were washed with saline, diluted in 25 mL of lactated Ringer's solution containing 5% HSA, and transfused into the rabbit as washed RBCs (Fig. S1).

Administration of H12-(ADP)-liposomes, PRP, PPP, ADP liposomes and H12-(PBS)-liposomes

We administered the PRP or PPP that was prepared from the blood taken at the first and second phlebotomies. These PRP and PPP samples showed similar coagulation activity (fibrinogen, ~ 150 mg dL^{-1}; antithrombin [AT]III activity, 99%; prothrombin time [PT], 12 s; activated partial thromboplastin time [APTT], 32 s). After the last transfusion of washed RBCs, H12-(ADP)-liposomes (20 mg per 4 mL kg^{-1}) were intravenously administered to the rabbits, and this was followed by administration of 11 mL kg^{-1} PPP (n = 10; Fig. 2). ADP liposomes (without H12) (20 mg per 4 mL kg^{-1}, n = 10) or H12-(PBS)-liposomes (lacking ADP) (20 mg per 4 mL kg^{-1}, n = 5) were also administered to the rabbits, and this was followed by PPP transfusion in the same manner. Similarly, 15 mL kg^{-1} PRP or PPP was administered to the rabbits (n = 10 in each group, Fig. 1). In addition, another three rabbits were prepared in each group for electron microscopic examination. In the severe thrombocytopenic model (blood exchange repeated 14 times), H12-(ADP)-liposomes/PPP, PRP or PPP were similarly administered to the rabbits.

Liver hemorrhage in thrombocytopenic rabbits

Thirty minutes after administration of H12-(ADP)-liposomes/PPP, PRP, PPP, ADP liposomes/PPP, or H12-(PBS)-liposomes/PPP, rabbits underwent laparotomy, and the liver was penetrated with a DermaPunc (5 mm in diameter; Nipro Medical Industries, Tokyo, Japan) (Fig. S2A,B). To precisely evaluate bleeding volume, we cut a hole in a surgical glove and passed the injured hepatic lobe through the hole to collect exsanguinating blood in the glove (Fig. S2C,D). We measured the bleeding volume from the liver during the initial 5 min and the following 5 min (5–10 min). Bleeding time from the liver-penetrating injury was measured for 20 min. The rabbit abdomen was then closed to monitor survival for 72 h under ad libitum feeding with laboratory diet and water. Postoperative analgesia was performed with two intramuscular injections of buprenorphine (0.02 mg kg^{-1}): immediately after wound closure and after 12 h.

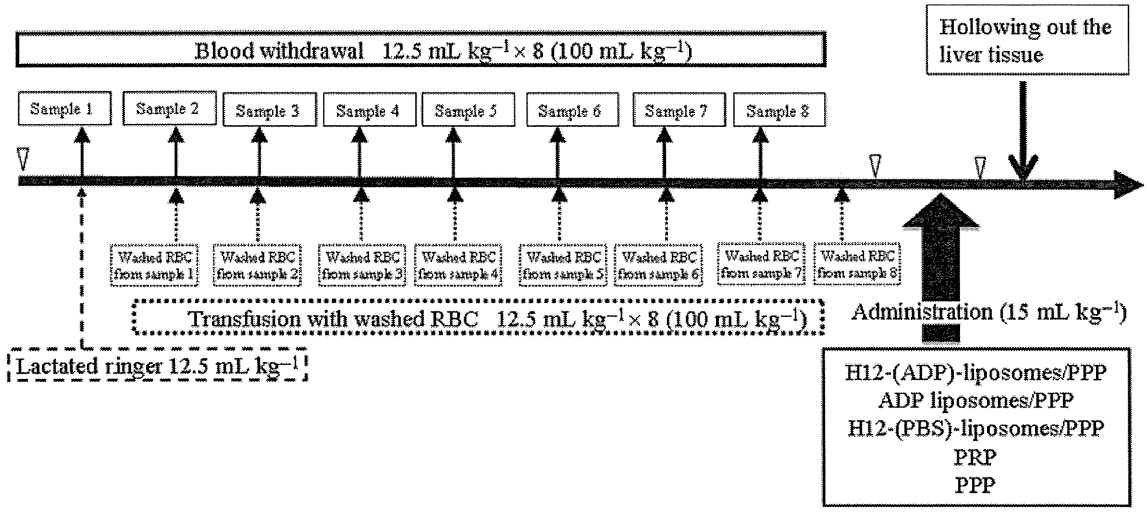

Fig. 2. Experimental design for acute thrombocytopenia and subsequent non-compressible liver hemorrhage in rabbits. PPP, platelet-poor plasma; PRP, platelet-rich plasma; RBC, red blood cell.

Analyses of whole blood coagulation activity

The coagulation activity of whole blood was examined with a Sonoclot Coagulation & Platelet Function Analyzer (Sienco, Morrison, CO, USA). Briefly, a tubular probe mounted on an ultrasonic transducer and vibrating vertically with a distance of 1 μm and a frequency of 200 Hz is immersed to a fixed depth in a cuvette containing 400 μL of whole blood obtained from the femoral artery without anticoagulant. As the sample clots, the increasing impedance to the vibration of probe is detected by the sensor and converted to an output signal, which reflects the viscoelastic properties of the developing clot. The signal typically describes coagulation parameters including clotting time (CT), which indicates the period up to the beginning of fibrin formation, and clot rate (CR), which indicates the slope of fibrin gel formation, and which is affected by both the rate of the fibrinogen to fibrin conversion and the amount of fibrinogen (Fig. 6A).

Measurements of ear bleeding time, mean arterial pressure, hematologic parameters, and coagulation factors

Ten minutes after administration of H12-(ADP)-liposomes/PPP, PRP, PPP, ADP liposomes/PPP, or H12-(PBS)-liposomes/PPP, the auricle was cut with a 6-mm incision where no vessels were visible, and the ear was immersed in a saline bath. The time required for bleeding to stop was then measured for 20 min. Mean arterial pressure was measured from the cannulated femoral artery with a polygraph recording system (RM-6000; Nihon Kohden, Tokyo, Japan). Blood samples were also collected from the femoral artery. Platelet count, hemoglobin concentration and white blood cell (WBC) count were measured with a hematology analyzer (PEC 170; Erma, Tokyo, Japan). The plasma concentration of fibrinogen, ATIII activity, PT and APTT were measured at the SRL Laboratory (Tokyo, Japan).

Histologic examination

Rabbits treated with H12-(ADP)-liposomes, PPP or PRP were killed 24 h after liver hemorrhage ($n = 3$ in each group). One rabbit treated with PPP that died after ~ 24 h was also examined. Three rabbits receiving H12-(ADP)-liposomes were also killed at 2 weeks after hemorrhage. Liver (uninjured lobe), spleen, lung and kidney were extracted from all subject rabbits, fixed in 20% formalin for 2 days, and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin.

Electron microscopic examinations

Three rabbits were prepared for each group. Liver specimens were obtained from the injury site at 1 h after injury. These were prefixed with a fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 mol L^{-1} phosphate buffer (pH 7.4) for 3 h at 4 °C, and this was followed by postfixing in 1% osmium tetroxide in 0.1 mol L^{-1} phosphate buffer (pH 7.4) for 2 h at 4 °C, dehydration, and embedding in epoxy resin. For selection of the bleeding site lesion, semithin sections were stained with toluidine blue. Ultrathin sections stained with uranyl acetate and lead citrate were then examined under an electron microscope (JEM 1030; JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

For immunoelectron microscopy, samples were processed as described elsewhere [23,24]. Briefly, fixed liver specimens were rinsed with 0.1 mol L^{-1} phosphate buffer and PBS at 4 °C, and then infused with 1 mol L^{-1} sucrose in PBS for 1 h, 1.84 mol L^{-1} sucrose in PBS for 2 h, and 1.84 mol L^{-1} sucrose containing 20% poly(vinylpyrrolidone) in PBS overnight at 4 °C. After being frozen in liquid nitrogen, ultrathin frozen sections were cut and incubated with rabbit anti-H12 (1 : 5000 dilution) in PBS overnight at 4 °C [20]. After being rinsed with PBS five times, sections were incubated with goat anti-rabbit IgG coupled to 15-nm colloidal gold at a dilution of 1 : 100 for 60 min at room temperature. After being rinsed with PBS three times and with distilled water (DW) five times, sections were stained with 1% uranyl acetate and washed with DW, and then adsorption-stained with a mixture of 3% poly(vinyl alcohol) and 0.3% uranyl acetate. Stained sections were examined under a JEM 1230 electron microscope as described above.

Statistical analyses

Statistical analyses were performed with the STAT VIEW 4.02J software package (Abacus Concepts, Berkeley, CA, USA). Survival rates were compared by use of the Wilcoxon signed rank test. Statistical evaluations were compared by use of one-way analysis of variance, followed by Bonferroni post hoc tests. Data are presented as means ± standard errors, with $P < 0.05$ considered to be statistically significant.

Results

Acute thrombocytopenia in rabbits

Platelet counts decreased gradually in rabbits after repeated blood withdrawal and washed RBC transfusion, reaching 5×10^4 μL^{-1} ($n = 40$) at the end of blood exchange (Fig. S3A). Ear bleeding time was prolonged markedly by blood exchange (Fig. 3). Nevertheless, mean arterial blood pressure was maintained as a result of isovolemic exchange (Fig. S3B). The hemoglobin concentration was also maintained at ~ 8 g dL^{-1} (Fig. S3C), and the WBC count was minimally altered (Fig. S3D). Coagulation factor levels were, however, markedly decreased (Table S1), probably because of substantial plasma loss.

Ear bleeding time after administration of H12-(ADP)-liposomes in thrombocytopenic rabbits

In our previous studies on rabbits with busulfan-induced thrombocytopenia, H12-(ADP)-liposomes administered at a

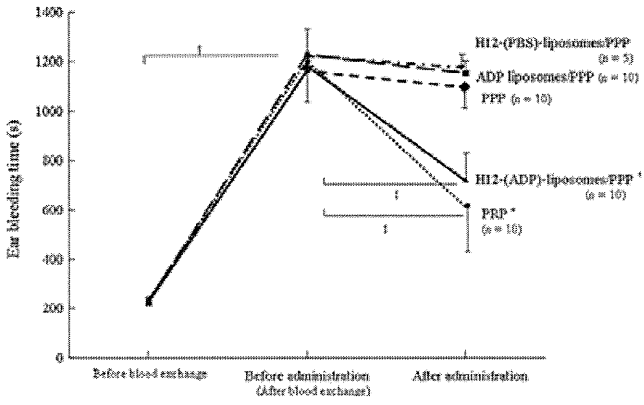

Fig. 3. Ear bleeding time in thrombocytopenic rabbits. Ear bleeding time was examined in rabbits before blood exchange, after blood exchange (before administration), and after administration of H12-(ADP)-liposomes/platelet-poor plasma (PPP), platelet-rich plasma (PRP), PPP, or ADP liposomes/PPP. Data are means ± standard errors. *$P < 0.01$ vs. PPP, ADP liposomes/PPP, or H12-(phosphate-buffered saline [PBS])-liposomes/PPP, †$P < 0.01$, ‡$P < 0.05$.

dose of 20 mg kg^{-1} gave optimal bleeding time-shortening effects, similar to those of PRP [21]. Therefore, this liposome dose was selected for all of the present experiments. Administration of H12-(ADP)-liposomes/PPP and of PRP efficiently corrected bleeding time in thrombocytopenic rabbits (Fig. 3), although administration of H12-(PBS)-liposomes/PPP, ADP liposomes/PPP or PPP alone did not.

Changes in hematologic parameters after administration of H12-(ADP)-liposomes

Mean arterial blood pressure was not significantly altered after administration of H12-(ADP)-liposomes/PPP, PRP, PPP or ADP liposomes/PPP in all groups (Table 1). Platelet count was markedly increased after PRP administration, but was not affected by other treatments (Table 1). Neither hemoglobin concentration nor WBC count significantly changed in any of the four groups, whereas coagulation factor levels were corrected in all groups (Table 1). Hematologic parameters were also unaffected by administration of H12-(PBS)-liposomes/PPP (data not shown).

Survival after liver hemorrhage in rabbits receiving H12-(ADP)-liposomes

After 72 h of observation, administration of H12-(ADP)-liposomes/PPP and of PRP rescued all rabbits from lethal liver hemorrhage. H12-(PBS)-liposome/PPP treatment also showed significant therapeutic effects on survival (4/5; 80%). In contrast, rabbits with PPP and ADP liposome treatment showed significantly poorer prognoses, with survival rates of 20% in the first 24 h (Fig. 4). Administration of H12-(PBS)-

Table 1 Changes in hematologic parameters and coagulation factors in rabbits after administration of H12-(ADP)-liposomes/platelet-poor plasma (PPP), platelet-rich plasma (PRP), PPP, or ADP liposomes/PPP

	H12-(ADP)-liposomes/PPP	PRP	PPP	ADP liposomes/PPP
Mean blood pressure (mmHg)				
Before administration	90 ± 7	91 ± 7	89 ± 7	88 ± 4
After administration	96 ± 10	98 ± 7	87 ± 8	90 ± 7
Hemoglobin concentration (g dL^{-1})				
Before administration	8.3 ± 0.6	7.9 ± 0.5	8.6 ± 1.3	8.1 ± 0.4
After administration	6.4 ± 0.5	6.2 ± 0.6	6.0 ± 0.2	6.1 ± 0.6
Platelet count ($\times 10^3$ μL^{-1})				
Before administration	50 ± 5	50 ± 6	46 ± 7	50 ± 6
After administration	47 ± 5	108 ± 12*	42 ± 4	42 ± 10
WBC count ($\times 10^3$ μL^{-1})				
Before administration	3.2 ± 0.4	3.5 ± 0.6	3.6 ± 0.3	3.0 ± 0.5
After administration	3.3 ± 0.4	4.1 ± 0.8	4.0 ± 0.7	2.8 ± 0.2
Fibrinogen concentration (mg dL^{-1})				
Before administration	50 ± 1	53 ± 3	54 ± 3	51 ± 1
After administration	69 ± 6*	73 ± 7*	73 ± 7*	65 ± 8*
ATIII activity (%)				
Before administration	38 ± 4	40 ± 5	37 ± 4	39 ± 5
After administration	52 ± 2†	57 ± 2†	53 ± 4†	53 ± 2†
PT (s)				
Before administration	29 ± 6	30 ± 5	25 ± 3	27 ± 2
After administration	18 ± 2†	16 ± 1†	15 ± 1†	19 ± 2†
APTT (s)				
Before administration	37 ± 4	39 ± 8	40 ± 4	37 ± 4
After administration	34 ± 10	35 ± 1	28 ± 4	29 ± 6

APTT, activated partial thromboplastin time; ATII, antithrombin II; PT, prothrombin time; WBC, white blood cell. Each parameter was measured in rabbits before and after administration of H12-(ADP)-liposomes/PPP, PRP, PPP, or ADP liposomes/PPP. Data are mean ± standard error from 10 rabbits in each group, *$P < 0.01$ and †$P < 0.05$ vs. before administration.

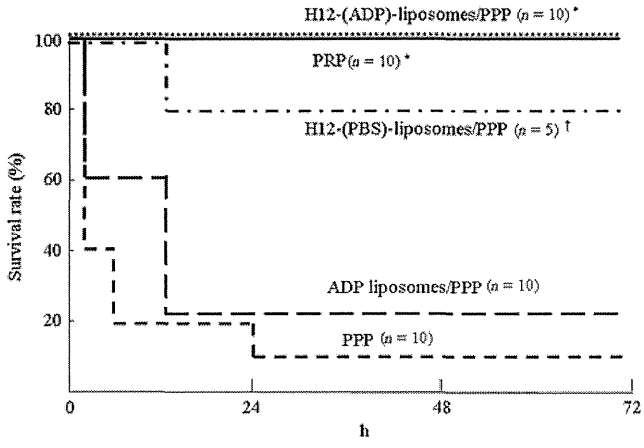

Fig. 4. Survival rates of thrombocytopenic rabbits after non-compressible liver hemorrhage. After administration of H12-(ADP)-liposomes/platelet-poor plasma (PPP), platelet-rich plasma (PRP), PPP, ADP liposomes/PPP or H12-(phosphate-buffered saline [PBS])-liposomes/PPP, thrombocytopenic rabbits were subjected to penetrating liver injury. *$P < 0.01$ vs. PPP or ADP liposomes/PPP. †$P < 0.01$ vs. PPP. $P < 0.05$ vs. ADP liposomes/PPP.

liposomes/PPP also did not rescue any rabbits (data not shown).

Hemostatic effect of H12-(ADP)-liposomes on liver hemorrhage in rabbits

Administration of H12-(ADP)-liposomes/PPP tended to decrease bleeding volume from the liver injury site in the initial 5-min period as compared with PPP or ADP liposome/PPP treatment (difference not statistically significant, Fig. 5A). Interestingly, H12-(ADP)-liposome/PPP administration significantly reduced bleeding volume in the subsequent 5 min as compared with that in the initial 5 min (Fig. 5A). In contrast, PRP administration significantly reduced liver hemorrhage even in the initial 5 min, but did not further augment hemostatic effects in the subsequent period (Fig. 5A). Administration of H12-(PBS)-liposomes/PPP did not reduce bleeding volume in the initial 5 min, but tended to reduce it in the subsequent 5 min as compared with that in the initial 5 min (difference not significant) (Fig. 5A). Bleeding time from the penetrating liver wound was significantly shortened by administration of H12-(ADP)-liposomes/PPP, as well as of PRP, as compared with PPP, ADP liposome/PPP or H12-(PBS)-liposome/PPP administration (Fig. 5B). Liver hemorrhage was almost fully stopped at 10 min after administration of H12-(ADP)-liposomes/PPP (Fig. S2C), whereas a substantial amount of hemorrhaging was observed at 10 min after PPP administration (Fig. S2D). Administration of ADP liposomes/PPP did not show any obvious hemostatic effects at the liver injury sites (Fig. 5A,B). Administration of H12-(PBS)-liposomes/PPP also showed no significant hemostatic effects (Fig. 5A,B), although it significantly improved recovery from liver hemorrhage in thrombocytopenic rabbits (Fig. 4).

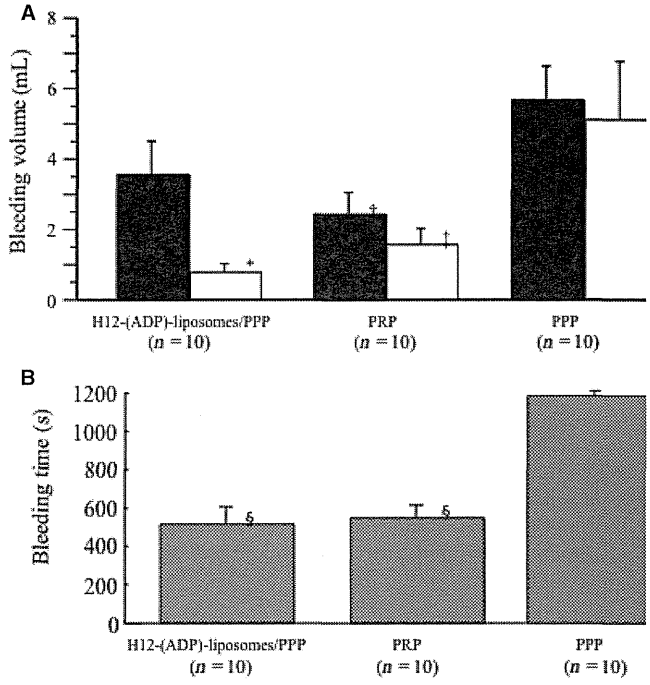

Fig. 5. Liver hemorrhage in thrombocytopenic rabbits after administration of H12-(ADP)-liposomes/ platelet-poor plasma (PPP), platelet-rich plasma (PRP), PPP, ADP liposomes/PPP or H12-(phosphate-buffered saline [PBS])-liposomes/PPP. (A) Bleeding volumes from the penetrating liver wound. (B) Bleeding time from liver injury. Data are means ± standard errors. *$P < 0.05$ vs. H12-(ADP)-liposomes (0–5 min). $P < 0.01$ vs. PPP, ADP liposomes, or H12-(PBS)-liposomes (5–10 min). †$P < 0.05$ vs. PPP (0–5 min). ‡$P < 0.05$ vs. PPP or ADP liposomes/PPP (5–10 min). §$P < 0.01$ vs. PPP, ADP liposomes, or H12-(PBS)-liposomes.

Platelet-based blood coagulation activity in rabbits receiving H12-(ADP)-liposomes

Blood samples obtained after repeated blood withdrawal/RBC transfusion were not coagulated (Fig. 6A). However, supplementation of coagulation factors with PPP administration corrected the clotting parameters CT and CR to some extent, although correction of both parameters was not sufficient to restore normal clotting activity (Fig. 6B). Administration of PRP to supplement platelets, in addition to coagulation factors, further shortened CT and increased CR (Fig. 6C,D), indicating that both parameters measured on the Sonoclot apparatus depended on *ex vivo* platelet-based coagulation (platelet procoagulant) activity. Notably, H12-(ADP)-liposomes/PPP significantly corrected both parameters to a similar level as PRP (Fig. 6C,D). In contrast, ADP liposomes/PPP showed no significant correcting activity, as compared with that obtained with PRP or H12-(ADP)-liposomes/PPP. H12-(PBS)-liposomes also gave no significant corrections of these parameters. These results demonstrate that H12-(ADP)-liposomes possess the ability to replace platelets in accelerating the coagulation pathway towards fibrin clot formation.

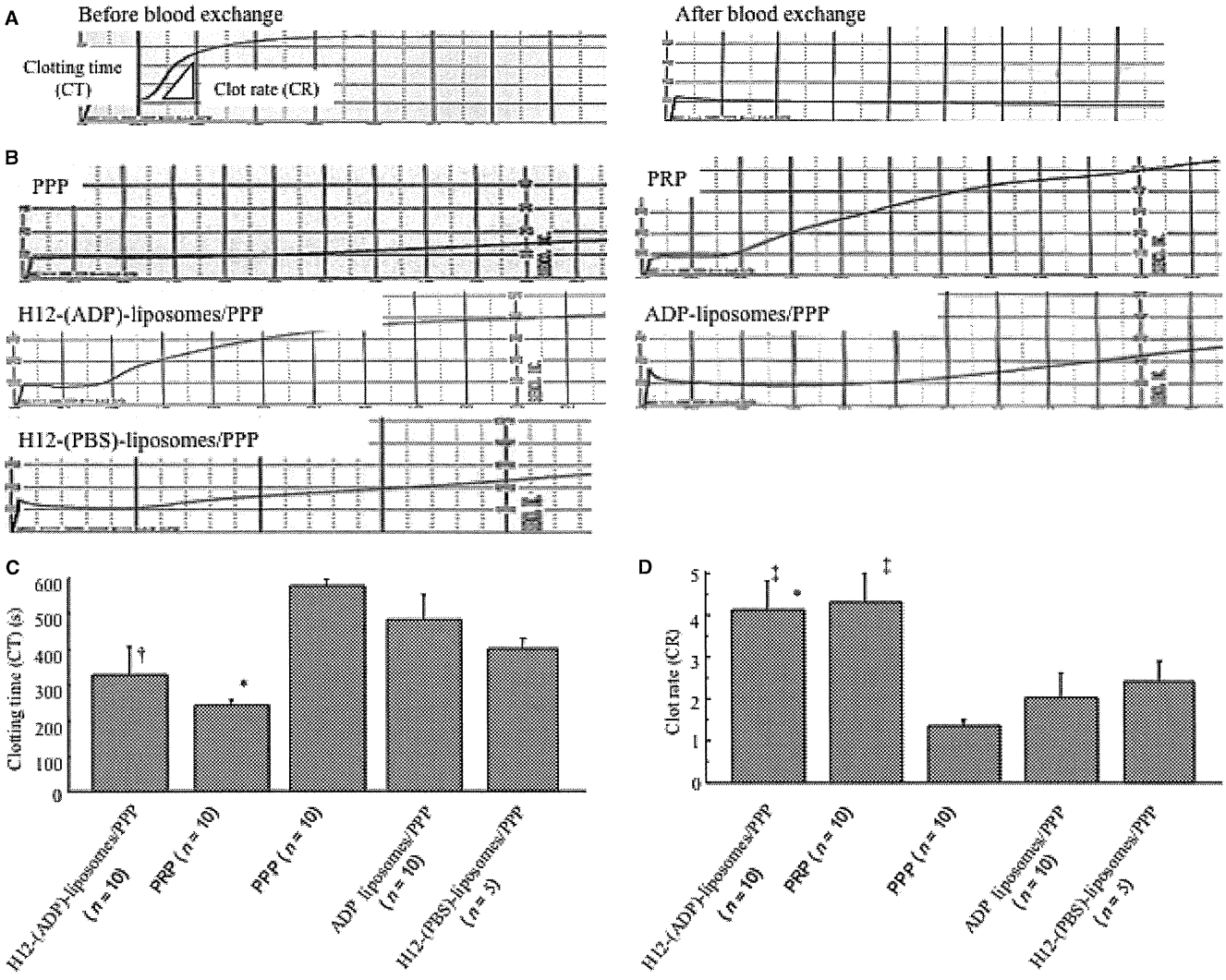

Fig. 6. Analyses of whole blood coagulation activity in rabbits with Sonoclot. (A) Whole blood coagulation activities in rabbits before and after blood exchange. Clotting time (CT) and clot rate (CR) are indicated by the arrow and the triangle, respectively. (B) Blood coagulation activities in thrombocytopenic rabbits after administration of H-12-(ADP)-liposomes/platelet-poor plasma (PPP), platelet-rich plasma (PRP), PPP, ADP liposomes/PPP, or H12-(phosphate-buffered saline [PBS])-liposomes/PPP. Representative data with similar results from 10 rabbits (H12-[PBS]-liposomes; five rabbits) in each group are shown. (C, D) CT (C) and CR (D) were analyzed in rabbits after administration of H12-(ADP)-liposomes/PPP, PRP, PPP, ADP liposomes/PPP, or H12-(PBS)-liposomes/PPP. Data are means ± standard errors. $*P < 0.01$, $\dagger P < 0.05$ vs. PPP or ADP liposomes/PPP. $\ddagger P < 0.01$ vs. PPP or ADP liposomes/PPP. $P < 0.05$ vs. H12-(PBS)-liposomes/PPP.

Electron microscopic examination of clot at the site of liver injury

Dense coagulation clots adjacent to the injured site in the liver were observed after PRP administration (Fig. 7A, indicated by circles). These clots had substantial involvement of platelets and fibrin (Fig. 7B). H12-(ADP)-liposome-treated rabbits also showed coagulation clots adjacent to the injured tissue, whereas these appeared to be sparse as compared with those in PRP-treated rabbits (Fig. 7C, indicated by circles). Anhistous particles approximately 0.2–0.4 μm in diameter were observed around the RBC or fibrin deposits of H12-(ADP)-liposome-treated rabbits (Fig. 7D,E, indicated by arrowheads), although such particles were not observed in PRP-treated rabbits (Fig. 7B). On immunoelectron microscopy with anti-H12 polyclonal antibody, gold-labeled liposomes with unit membranes ~ 0.3 μm in diameter were identified between the coagulation clots in the H12-(ADP)-liposome-treated rabbits (Fig. 7F, indicated by arrows), suggesting that some anhistous particles were H12-(ADP)-liposomes. In fact, anhistous particles were not seen at the liver injury sites of ADP liposome-treated rabbits (Fig. 7G), suggesting that H12-(ADP)-liposomes exert local hemostatic activity at sites of vascular injury through the specific binding of H12 to activated platelets.

Pathologic examination of lung, liver (uninjured lobe), and kidney

Neither macrothombi nor microthrombi were observed in the lung, liver (uninjured lobe) or kidney in the rabbits at 24 h after

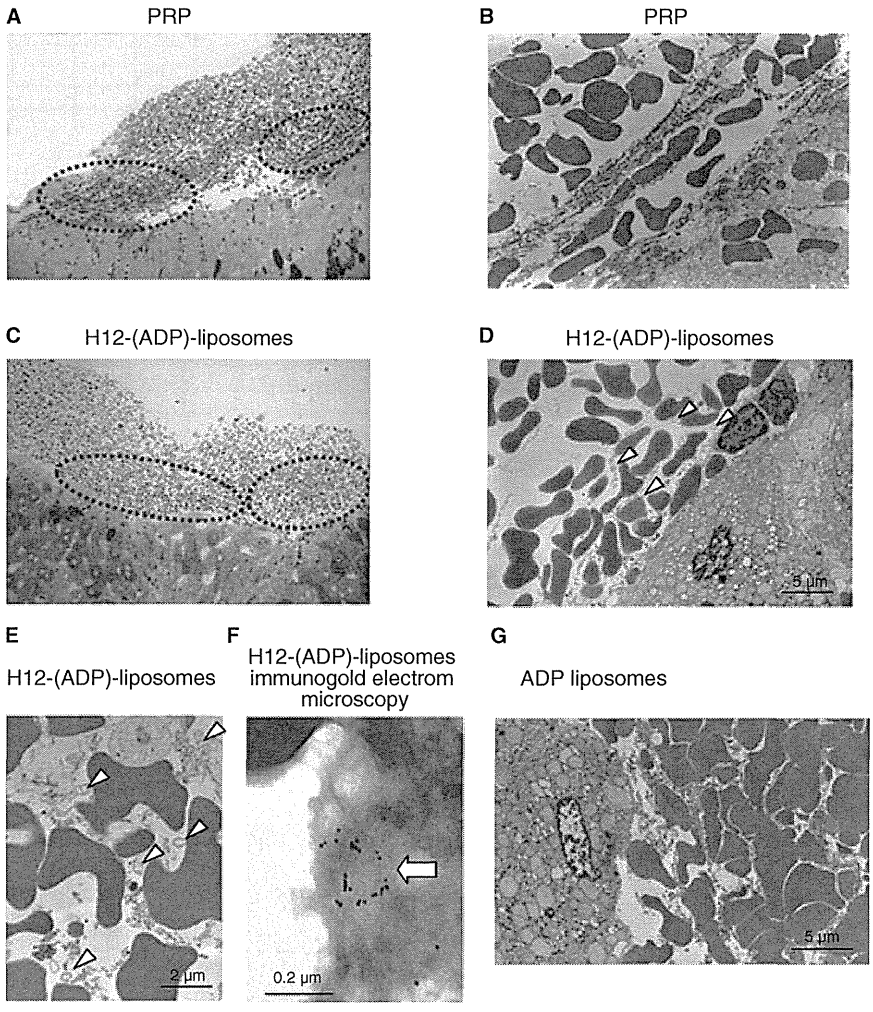

Fig. 7. Microscopic and electron microscopic observation of the penetrating liver in thrombocytopenic rabbits. (A) Microscopic and (B) electron microscopic observation of the liver injury site after administration platelet-rich plasma (PRP). (C) Microscopic and (D, E) electron microscopic observation of liver injury site after administration of H12-(ADP)-liposomes/platelet-poor plasma (PPP) ([E] is a magnification of [D]). Coagulation clots are indicated by dotted circles in A and C. Anhistous particles are indicated by arrowheads in D and E. (F) Immunoelectron microscopic observation of liver injury site after administration of H12-(ADP)-liposomes/PPP. H12 peptides are labeled with gold (indicated by arrows). (G) Electron microscopic observation in ADP liposome/PPP-transfused rabbits. Representative data with similar results from three rabbits in each group are shown.

administration of H12-(ADP)-liposomes, PRP, or PPP (Fig. 8). Although severe pulmonary edema was observed after PPP transfusion, no such lesions were observed after administration of H12-(ADP)-liposomes or PRP (Fig. 8). Severe hepatocyte degeneration was also observed in PPP-treated rabbits, but not in H12-(ADP)-liposome-treated or PRP-treated rabbits (Fig. 8, indicated by arrows). However, some lymphocyte infiltration was observed around the central vein in the liver after administration of H12-(ADP)-liposomes (Fig. 8, indicated by arrowhead), suggesting an immune response to foreign bodies. Remarkable proliferation of neither Kupffer cells (Fig. 8) nor splenic macrophages (not shown) was observed in the rabbits after administration of H12-(ADP)-liposomes, suggesting that no serious damage occurs in the reticuloendothelial system. No significant changes were observed in the kidney in any groups (Fig. 8). We also histologically examined the liver, spleen, lung and kidney at 2 weeks after administration of H12-(ADP)-liposomes/PPP. Neither thrombi nor significant changes indicating immune reactions were observed in any organs. Lymphocyte infiltration around the central vein in the liver was not significant (Fig. S4).

Hemostatic effects of H12-(ADP)-liposomes on liver hemorrhage in the severe thrombocytopenic model

After 14 repeated isovolemic blood exchanges, platelet counts in rabbits decreased to $(25 \pm 1) \times 10^3\ \mu L^{-1}$. Administration of H12-(ADP)-liposomes/PPP rescued three of five rabbits from lethal liver hemorrhage in this severe thrombocytopenic model (60% survival). In contrast, PRP treatment that

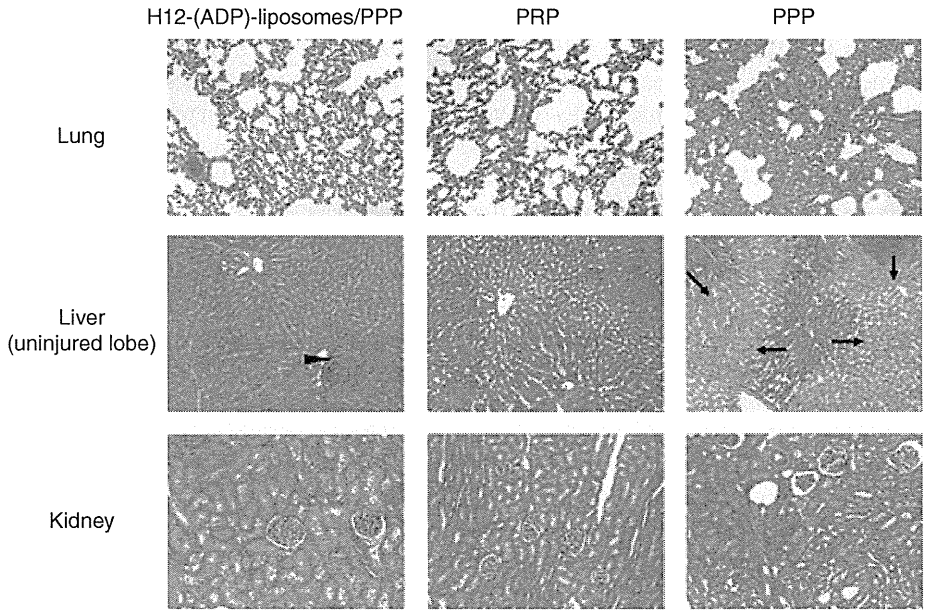

Fig. 8. Histological findings 24 h after liver hemorrhage in thrombocytopenic rabbits. Lung, liver and kidney samples were obtained from the rabbits 24 h after liver injury. Lymphocyte infiltration in the liver is indicated by an arrowhead (H12-[ADP]-liposomes/platelet-poor plasma [PPP]), and hepatocyte degeneration is indicated by arrows (PPP). Representative data with similar results from three rabbits in the H12-(ADP)-liposomes/PPP and platelet-rich plasma (PRP) groups and one rabbit in the PPP group are shown.

increased platelet counts to approximately 86×10^3 μL^{-1} (average) rescued four of five rabbits (80% survival). PPP treatment rescued no rabbits (Table 2). Although the rabbits given H12-(ADP)-liposomes/PPP showed a large amount of liver hemorrhage in the initial 5 min, their bleeding volume was clearly reduced in the subsequent 5 min (9.8 ± 4.4 to 3.1 ± 1.3 mL). In contrast, PRP treatment did not give such an obvious reduction in bleeding volume (average of 6.8–5.7 mL). Bleeding time from the liver injury tended to be prolonged (but not significantly) in rabbits receiving H12-(ADP)-liposomes/PPP as compared with those receiving PRP (1144 ± 50 vs. 875 ± 173 s; PPP treatment, over 1200 s).

Table 2 Survival after non-compressible liver hemorrhage in rabbits with severe thrombocytopenia

	H12-(ADP)-liposomes	PRP	PPP
12 h	5/5 (100)	5/5 (100)	0/3
72 h	3/5 (60)	4/5 (80)	0/3

PPP, platelet-poor plasma; PRP, platelet-rich plasma. Data are given as no. (%). Severe thrombocytopenia was induced by 14 blood exchanges (12.5 mL kg^{-1}) in rabbits, and their platelet counts decreased to $(25 \pm 1) \times 10^3$ μL^{-1}.

Discussion

In this study, we demonstrated the feasibility of H12-(ADP)-liposomes as a safe and efficacious synthetic platelet substitute applicable to uncontrollable traumatic bleeding confounded by acute thrombocytopenia after massive RBC transfusion and fluid resuscitation.

The current rabbit model did not exactly reflect a clinical situation with penetrating injuries, as acute thrombocytopenia usually occurs following injury. During the bleeding process, several interventions are essential for life-saving, including volume resuscitation, damage control surgery, and replacement of deficient coagulation factors and platelets. H12-(ADP)-liposomes were able to act as a substitute for platelets to rescue acute thrombocytopenic rabbits from lethal liver hemorrhage by controlling bleeding. When major surgical blood loss is replaced with plasma/platelet-poor packed RBCs, patients show deficiencies in both platelets and coagulation factors [11,25]. Our thrombocytopenic rabbits also showed severe deficiency in coagulation factors. Therefore, on the basis of current hemostatic resuscitation protocols for trauma patients, we administered H12-(ADP)-liposomes together with PPP to treat bleeding [7,26]. In fact, similarly to platelet transfusion with PRP, administration of H12-(ADP)-liposomes with PPP rescued all thrombocytopenic rabbits, although most did not survive when treated with PPP alone (Fig. 4).

Replenishment of coagulation factors by FFP should not be a major therapeutic choice for transfusion-induced coagulopathy [10,11]. Instead, dilutional thrombocytopenia may be a crucial target for coping with coagulopathy, as fibrin clot formation leading to secondary hemostasis through the coagulation pathway is not adequately completed without initial platelet thrombi being generated by platelets [10,11]. This hypothesis was supported by the finding that the levels of Sonoclot clotting activity in whole blood from thrombocytopenic rabbits rescued by treatment were higher with PRP

(platelets/PPP) or H12-(ADP)-liposomes/PPP than with PPP alone or ADP liposomes/PPP (Fig. 6).

H12-(PBS)-liposomes potently increased the survival of thrombocytopenic rabbits after liver hemorrhage, whereas ADP liposomes without H12 did not (Fig. 4). Crosslinking platelets by H12 may be crucial for hemostatic resuscitation in this particular lethal liver hemorrhage animal model. However, direct hemostatic effects of H12-(PBS)-liposomes on bleeding time and bleeding volume from the injured liver were not observed (Fig. 5). At present, there is no convincing explanation for the discrepancy between the effects of H12-(PBS)-liposomes on direct hemostatic activity and those on animal survival. However, consistent with our previous observation of the effects of these H12 liposomes with or without ADP on prolonged tail bleeding time in rats with busulfan-induced thrombocytopenia [21], crosslinking of residual platelets by H12 may be a prerequisite for the hemostatic activity of the liposomes, and local release of ADP appears to have a crucial role in their optimal therapeutic function.

H12-(ADP)-liposomes have several advantages over platelet concentrates in the treatment of acute thrombocytopenia. First, they can be used in emergencies, as they can be stored for long periods of time under simple conditions. In addition, they are entirely synthetic, and as such are free from the risks associated with blood-borne infections and allogeneic host immune reactions. Currently, recombinant factor VIIa (rFVIIa) is widely used as a hemostatic agent to treat massive bleeding in emergency settings, such as the battlefield [27]. However, the effects of rFVIIa on the lifetime prognosis in treated patients remain elusive [28]. Under severe platelet-deficient conditions, rFVIIa may not exert an effective hemostatic function. Combination therapy with rFVIIa and H12-(ADP)-liposomes may be useful for the treatment of thrombocytopenic bleeding in trauma patients.

The most important safety issue for our synthetic platelet substitutes concerns thrombotic complications [15,29]. Our findings to date suggest that this is not a problem with H12-(ADP)-liposomes. In healthy rats and rabbits, there were no changes in coagulation and platelet activation parameters after liposome administration at doses up to 40 mg kg^{-1} [21]. Pathologic examination revealed that rabbits with acute thrombocytopenia treated with H12-(ADP)-liposomes at a therapeutic dose of 20 mg kg^{-1} were free of thrombosis in tissues from lung, kidney, and intact liver. This relative safety may stem from the specificity of H12 ligand for GPIIb-IIIa receptor on the activated platelets. Histologic studies have demonstrated that H12-(ADP)-liposomes, but not ADP liposomes, accumulated in clots formed during liver injury. Nevertheless, it is premature to draw conclusions on safety with respect to the induction of intravascular thrombosis, as clinical conditions of patients receiving synthetic platelets may be complex, owing to the hypercoagulable state resulting from activating platelets in the circulation. Therefore, further study is needed to confirm the safety issues related to synthetic platelet substitutes.

As compared with PRP, the hemostatic effects of H12-(ADP)-liposomes on traumatic liver hemorrhage are somewhat slow, but were equally potent after 10 min of observation (Fig. 5). This mode of activity of liposomes may be related to their dependence on residual platelets to exert hemostatic activity in thrombocytopenic animals. Therefore, it is possible that there will be a thrombocytopenic concentration of platelets at which H12-(ADP)-liposomes will be unable to rescue the animals. When tested under severe thrombocytopenic conditions ($[25 \pm 1] \times 10^3$ platelets μL^{-1}), they still showed significant effects on survival, similar to those of PRP, and the mode of their hemostatic effect on liver bleeding was similar to that obtained with $(50 \pm 1) \times 10^3$ platelets μL^{-1} (Table 2). Thus, H12-(ADP)-liposomes are capable of exerting their hemostatic function through residual platelets ranging from $(25 \pm 1) \times 10^3$ to $(50 \pm 1) \times 10^3$ μL^{-1} in this particular rabbit model. Another concern is that H12-(ADP)-liposomes will behave like platelet inhibitors in blocking fibrinogen-mediated platelet thrombus formation. In fact, H12-(ADP)-liposomes competed with fibrinogen to bind to activated platelets through GPIIb–IIIa, as determined with *in vitro* experiments under non-stirring conditions; 50% inhibition of liposome binding was obtained in the presence of a concentration of fibrinogen $\sim$ 4.3-fold higher than its average blood level (200 mg dL^{-1}) [30]. This dilutional thrombocytopenic animal model was associated with severe hypofibrinogenemia at levels of 50–70 mg dL^{-1}. Nevertheless, H12-(ADP)-liposomes showed potent hemostatic activity, suggesting that crosslinking of the remaining platelets by H12-(ADP)-liposomes is indispensable for platelet thrombus formation. Platelets are first activated at the site of vascular injury, and thereafter, liposomes become vital. Therefore, further careful dose and administration studies are needed to optimize the indications for our platelet substitutes in clinical settings.

Addendum

K. Nishikawa, K. Hagisawa, M. Kinoshita, D. Saitoh, and M. Handa: conception and design; K. Nishikawa, K. Hagisawa, M. Kinoshita, S. Shono, S. Katsuno, M. Doi, R. Yanagawa, H. Suzuki, and K. Iwaya: acquisition of data; K. Nishikawa, K. Hagisawa, M. Kinoshita, T. Sakamoto, and M. Handa: analysis and interpretation of data; M. Kinoshita and M. Handa: drafting of the manuscript; S. Takeoka and S. Seki: supervision.

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Disclosure of Conflict of Interests

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Scheme of blood withdrawal from rabbits.
Fig. S2. Non-compressible liver hemorrhage in thrombocytopenic rabbits.
Fig. S3. Changes in hematologic parameters after blood exchange.
Fig. S4. Histologic findings of the lung, liver, kidney and spleen 2 weeks after administration of H12-(ADP)-liposomes and liver hemorrhage in the thrombocytopenic rabbits.
Table S1. Changes in coagulation factors in rabbits after blood exchange.

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Pharmaceutical Nanotechnology

Ability of fibrinogen γ-derived dodecapeptides with different sequences to bind to rat platelets

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ABSTRACT

A dodecapeptide (γ400–411) derived from a fibrinogen γ-chain carboxyl-terminal sequence recognizes specifically the active form of GPIIb/IIIa on the surface of activated platelets. For the purpose of efficient hemostasis, we previously developed ADP-encapsulated liposomes modified with human-dodecapeptide (HHLGGAKQAGDV, human-H12). On the other hand, the amino-acid sequence of H12 from rats is HHMG-GSKQVGDM, having only 67% homology to that from humans. Here, we investigated the ability of rat-H12 in comparison with human-H12 to bind to platelets. Firstly, rat platelets were activated with phorbol-12-myristate-13-acetate (PMA), and the activation was confirmed by flow cytometry. Next, we evaluated the dissociation constant (K_d) of human-H12 and rat-H12 for dissociation from rat platelets by using FACS. As a result, the K_d of human-H12 and rat-H12 with respect to rat platelets was 2.78 ± 0.21 and 2.91 ± 0.22 μM, respectively. Furthermore, H12 from both species inhibited quite similarly the aggregation of rat platelets in platelet-rich plasma (PRP). These results suggest that H12 from different species with different amino acid sequences interacts similarly with GPIIb/IIIa on platelets.

1. Introduction

Platelet transfusion has an important function in supportive therapy for patients with thrombocytopenia caused by hematological malignancies, or as a result of intensive chemotherapy and radiation therapy for cancer. However, the shortage of platelet concentrates has always been a serious issue because of their short shelf-life (4 days in Japan), insufficient donation, the risk of viral and bacterial infections, as well as alloimmunization associated with transfusion. For these reasons, several trials have been conducted to develop platelet substitutes (artificial platelets) reproducing platelet functions. These substitutes include infusible platelet membranes (Graham et al., 2001) solubilized platelet membrane protein-conjugated liposomes (plateletsomes; Rybak and Renzulli, 1993), fibrinogen-bonded red blood cells (Agam and Livine, 1992), fibrinogen-bearing liposomes (Casals et al., 2003), fibrinogen-coated albumin microcapsules (synthocytes; Levi et al., 1999), and arginine-glycine-aspartate (RGD) peptide-bound red blood cells (thromboerythrocytes; Coller et al., 1992). All of these platelet substitutes consist of materials derived from blood components.

Integrin αIIbβIII (GPIIb/IIIa) on the platelet surface changes from its inactive to its active form when platelets adhere to collagen exposed at the sites of vascular injury (Takagi et al., 2002; Xiao et al., 2004). The activated GPIIb/IIIa acts as a receptor for fibrinogen and von Willebrand factor (Mustard et al., 1978; Ruggeri et al., 1983), which ligand/receptor interaction leads to platelet aggregation (De Marco et al., 1986). H12, a dodecapeptide (γ400–411) derived from the fibrinogen γ-chain carboxyl-terminal sequence, recognizes specifically the active form of GPIIb/IIIa on the surface of activated platelets (Kloczewiak et al., 1982; Andrieux et al., 1989; Ruoslahti, 1996; Taub et al., 1989). We previously developed a novel hemostatic agent with a human-dodecapeptide (HHLGGAKQAGDV, human-H12): H12-coated polymerized albumin and H12-coated polyethylene glycol-modified liposomes show specific interaction with activated platelets, increased platelet-mediated thrombus formation on collagen-immobilized surfaces under flow conditions *in vitro*, and prolonged hemostatic ability *in vivo* to correct

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bleeding time in a dose-dependent manner in thrombocytopenic rats and rabbit models (Okamura et al., 2005, 2007, 2008, 2010a,b).

Recently, we developed human H12-decorated liposomes encapsulating ADP, H12-(ADP)Lipo, which show enhanced hemostatic efficacy (Okamura et al., 2009, 2010a,b). The concept of the hemostatic effect of H12-(ADP)Lipo is based on the specific binding of human-H12 to GPIIb/IIIa on the surface of activated platelets. These *in vivo*, as well as some *in vitro*, data were obtained from animal experiments using rats and rabbits. Thereafter, we found that the multivalent and cooperative binding between H12 peptides and GPIIb/IIIa strengthened the binding of the liposomes to activated platelets (Tokutomi et al., 2011). However, it is known that the amino acid sequences of rat-H12 (HHMGGSKQVGDM; Andrieux et al., 1989) and rabbit-H12 (FHMGGAKQAGDV; Gene Bank, XM_002716891.1) are different from the human-H12 sequence: Four out of the 12 amino acid residues in rat-H12 and 2 of those in rabbit-H12 are different from those in human-H12 at the same positions. Since the species specificity with respect to the ability of the H12 peptide to bind to activated platelets is largely unknown, in the present study, we compared this ability of rat and human H12 peptides. This information seems to be important to predict the practical use of H12 as a platelet-aggregation enhancer for humans based on the data obtained from rat experiments.

2. Materials and methods

2.1. Materials

9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619,thromboxane A2 analogue) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Adenosine 5′-diphosphate sodium salt (ADP), phorbol-12-myristate-13-acetate (PMA), thrombin receptor-activating peptide (PAR4 agonistic peptide, AYPGKF-NH_2 trifluoroacetate salt, TRAP), prostaglandin E1 (PGE_1), and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) were purchased from Sigma (St. Louis, MO, USA). Anti-human fibrinogen, FITC-conjugated, was purchased from Millipore (Victoria, Australia). FITC-conjugated rat-H12 fibrinogen γ-chain dodecapeptide (FITC-PEG-HHMGGSKQVGDM) and FITC-conjugated human-H12 (FITC-PEG-HHLGGAKQAGDV) were synthesized by GL Biochem (Shanghai, China) on consignment contract.

2.2. Animals

F344/N male rats were purchased from Charles River Japan Inc. (Atsugi, Japan). The animals were housed in a temperature (23 ± 2 °C)-controlled room in our institute for at least 2 weeks before the experiments were started.

2.3. Activation of rat platelets

Blood withdrawn from F344/N male rats by cardiac puncture was mixed with a 10% volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood (160 × *g* for 10 min at 25 °C). PRP was mixed with a 15% volume of acid-citrate-dextrose solution composed of 2.2% sodium citrate, 0.8% citric acid, and 2.2% glucose (ACD) and containing 1 μM PGE_1. The suspension was centrifuged (1000 × *g* for 5 min at 25 °C), and the plasma was replaced with a Ringer's-citrate-dextrose solution (RCD solution, composition: 0.76% citric acid, 0.090% glucose, 0.043% $MgCl_2$, 0.038% KCl, 0.60% NaCl, pH 6.5) containing 1 μM PGE_1. After the pellets had been resuspended in the RCD solution, the suspension was centrifuged (1000 × *g* for 8 min at 25 °C); and the concentrated platelets were then resuspended at 1.0×10^5 cells/μL in a modified HEPES-Tyrode buffer (137 mM NaCl, 0.42 mM NaH_2PO_4, 2.7 mM KCl, 12 mM $NaHCO_3$, 2 mM $MgCl_2$, 10 mM HEPES, and 0.1% glucose; pH 7.4). The platelet count was determined by using an automated hematology analyzer (K-4500, Sysmex Co., Kobe, Japan).

Prior to platelet activation, FITC-conjugated anti-human fibrinogen (FITC-human-Fbg) was added (final concentration, 0.1 μM) to the washed platelets (1.0×10^5 cells/μL). A given activation agent (final concentration of 100 or 200 μM) was added to the washed platelets to activate them. Of these activating agents, U46619, ADP, and TRAP were dissolved in phosphate-buffered saline (PBS, pH 7.4), whereas PMA was dissolved in ethanol, beforehand and adjusted to the concentration of 1 mM. The suspension was incubated at 37 °C for 10 min before fixation with formaldehyde (final concentration, 1.8%, v/v). The mixture was then incubated with FITC-human-Fbg in the dark (15 min at 25 °C), after which HEPES-Tyrode buffer (1 mL) was added to it. The platelets were gated to their characteristic forward *versus* side scatter, and 10,000 platelets were analyzed by using a FACSCalibur flow cytometer (Nippon Becton Dickinson, Co., Tokyo, Japan). The number of platelets binding with the fibrinogen was quantified as the fraction of the fluorescence-positive platelets.

The mean fluorescence intensity (MFI) was calculated by Eq. (1), and served as an indicator of the relative amount of FITC-human-Fbg bound to platelet cells. For the comparison of the platelet GPIIb/IIIa-activating ability of each activation agent, the ratio of the mean fluorescence intensity (RMFI), which represents MFI of stimulated rat platelets against to non-stimulated rat platelets, was also determined ($n = 3$).

$$\mathrm{MFI} = \frac{\sum(\mathrm{Fl\text{-}H} \times \mathrm{Count})}{\mathrm{Total\ count}} \quad (1)$$

Fl-H: fluorescent intensity with detection channel of FITC; Count: number of cells with each fluorescence intensity; Total count: number of all cells detected.

2.4. Flow cytometric analysis of the binding of rat-H12 and human-H12 to rat platelets

Washed platelets were prepared as described in Section 2.3. FITC-conjugated rat-H12 or human-H12 (final concentration, 0.1–330 μM) was added to the washed platelets (1.0×10^5 cells/μL). The ethanol solution of PMA (final concentration, 200 μM) was added to the suspension to activate the rat platelets, and the suspension was then incubated at 37 °C for 10 min before fixation with formaldehyde (final concentration, 1.8%, v/v). The mixture was incubated in the dark (15 min at 25 °C), after which HEPES-Tyrode buffer (1 mL) was added. Rat-H12 or human-H12 bound to the rat platelets was determined as described in Section 2.3. The average ($n = 3$) MFI of the non-stimulated group of each H12 concentration was subtracted from each MFI as background. Then, the dissociation constants (K_d) were determined by using analytical software, Graph Pad Prism 5J. "Non-liner fitting" and "One site specific binding with Hill slope" were used as analytical conditions.

2.5. Inhibitory effect of rat-H12 and human-H12 on the aggregation of rat PRP

Rat PRP was prepared as described in Section 2.3. The platelet count was adjusted to 2.0×10^5 cells/μL by using platelet-poor plasma (PPP) prepared by centrifugation (1500 × *g* for 10 min at 25 °C). ADP solution (final concentration, 20 μM) was added to the PRP containing rat-H12 or human-H12 (final concentration, 0.1–1.0 mM), and light transmittance was measured with an aggregometer (Hema Tracer T-638; Nico Bioscience, Tokyo, Japan). The light transmittance of PPP was assumed to be 100%, and that of PRP before H12 and ADP were added was assumed to be 0%. The 50%

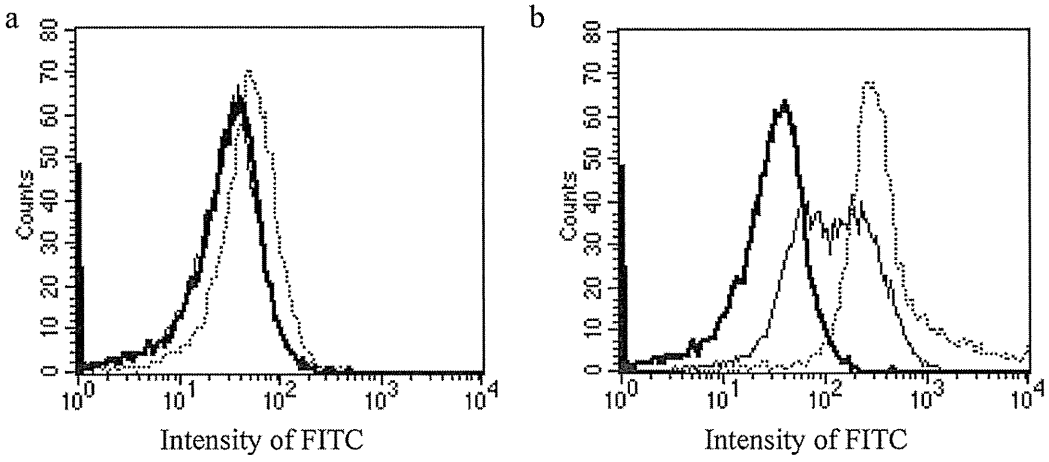

Fig. 1. Flow cytometric analysis of the binding of FITC-human-Fbg to activated GPIIb/IIIa on rat platelets. (a) The thick-lined histogram represents the binding of FITC-human-Fbg to the non-stimulated rat platelets. The thin-line and dotted-line histograms represent the binding of FITC-human-Fbg to rat platelets stimulated by 100 μM and 200 μM TRAP, respectively. (b) Same as (a) except PMA was used instead of TRAP.

inhibition concentrations of each H12 for aggregation of rat PRP (IC_{50}) were determined by using the Graph Pad Prism 5J analytical software.

3. Results

3.1. Comparison of K_d of human-H12 and rat-H12 for activated GPIIb/IIIa on rat platelets

Before obtaining the H12 binding affinity with respect to activated GPIIb/IIIa on rat platelets, we firstly examined the activation of washed rat platelets by various platelet-activating agents, namely, U46619, ADP, PMA, and TRAP. Since human fibrinogen is known to bind to activated GPIIb/IIIa on rat platelets (Peter and Verhallen, 1991; Cox et al., 1992), the activation of GPIIb/IIIa on the platelets was detected with FITC-labeled anti-human fibrinogen (FITC-human-Fbg). As shown in Fig. 1, 200 μM TRAP activated GPIIb/IIIa on rat platelets to some extent. In contrast, PMA remarkably activated GPIIb/IIIa on rat platelets in a dose-dependent manner. RMFIs for 100 and 200 μM TRAP were 1.11 ± 0.31 and 1.80 ± 0.16, respectively; and those for 100 and 200 μM PMA were 4.17 ± 0.50 and 12.71 ± 2.27, respectively. On the other hand, U46619 and ADP at the concentration of 200 μM showed little increase in RMFI (data not shown). Therefore, we decided to use PMA at the final concentration of 200 μM for the activation of GPIIb/IIIa on rat platelets in subsequent experiments.

To evaluate the binding affinity of human-H12 and rat-H12 with respect to activated GPIIb/IIIa on rat platelets, we investigated the K_d of each H12 peptide for activated GPIIb/IIIa on the platelets. Fig. 2 shows the binding curve of each FITC-H12 as determined by FACS analysis. The amount of H12 peptide that bound increased in an H12 concentration-dependent manner, reaching a plateau at 10 μM H12. The K_d's of human-H12 and rat-H12 for dissociation from GPIIb/IIIa on the platelets, determined by using the analytical software Graph Pad Prism 5J, were 2.78 ± 0.21 μM and 2.91 ± 0.22 μM, respectively. As a result, no significant difference between human-H12 and rat-H12 in affinity of binding to platelets was observed.

3.2. Inhibitory effect of rat-H12 and human-H12 on the aggregation of platelets in rat PRP

Platelets in PRP activated by ADP are known to form aggregates by the bridging effect of Fbg and von Willebrand factor (vWF). Fbg contributes to platelet aggregation because it has 2 H12 sites. But human- or rat-H12 binds only to a single site on platelets. Therefore, human- and rat-H12 can act as an inhibitor of platelet aggregation caused by Fbg. In the present study, we evaluated the binding of H12 to rat platelets, which binding should inhibit the binding of Fbg to

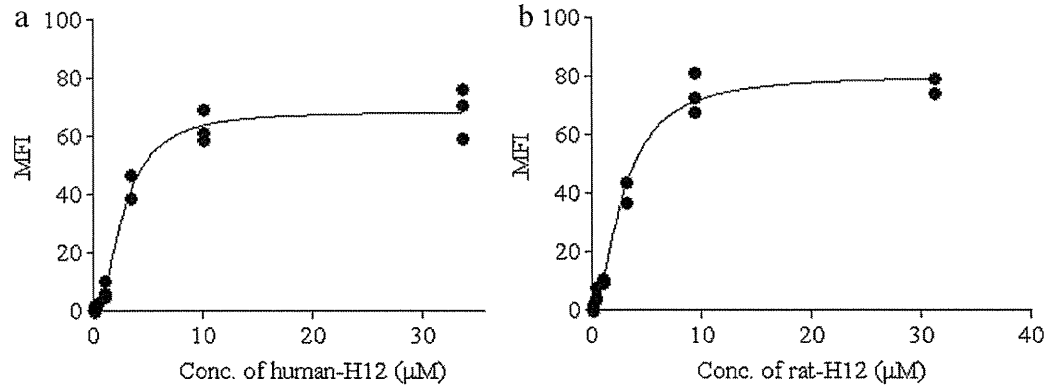

Fig. 2. Binding of H12 peptide to GPIIb/IIIa on rat platelets activated by PMA. FITC-conjugated human-H12 (a) or rat-H12 (b) was added (final concentration of 0.1–330 μM) to washed platelets (1.0×10^5 cells/μL). The suspension was incubated at 37 °C for 10 min in the presence of 200 μM PMA for activation of the platelets. Then the platelets were fixed with formaldehyde (1.8%, v/v), incubated in the darkness at 25 °C for 15 min, and mixed with HEPES-Tyrode buffer (1 mL). Then the samples were examined by a flow cytometer. H12 bound to the rat platelets was quantified as the fraction of fluorescence-positive platelets. The average ($n = 3$) MFI of the non-stimulated group of each H12 concentration was subtracted from each MFI as background. The dissociation constants (K_d) were calculated by using the analytical software Graph Pad Prism 5J.