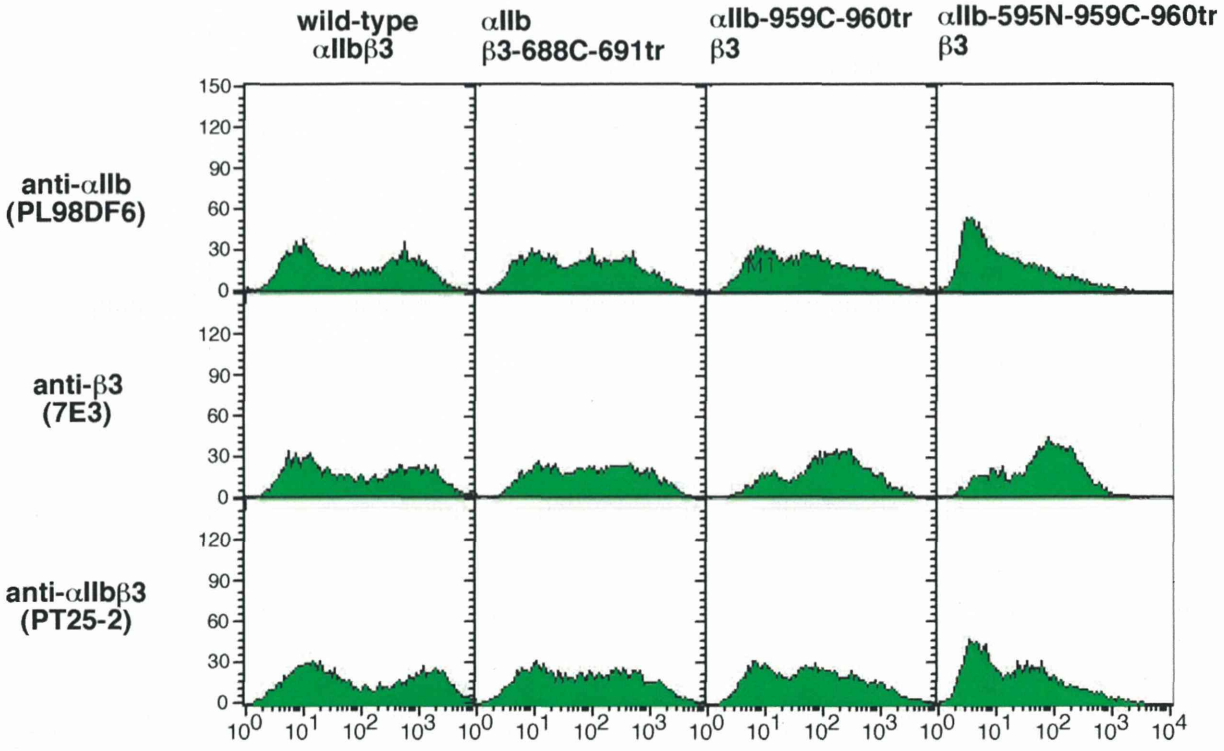

図３．膜貫通ドメイン欠損と Cys 変異がαIIbβ3 発現に与える影響

III. 可溶性αIIbβ3 の発現

αIIb とβ3 の双方の膜貫通ドメイン以降を欠損した可溶性αIIbβ3 を CHO 細胞に発現し、ウエスタンブロット法にて発現量の比較を行った。方法は、αIIb cDNA とβ3 cDNA を電気穿孔法にて CHO 細胞に導入し、４８時間後培養上清を回収した。図４は培養上清 10μl を SDS-PAGE で展開した後 PVDF 膜に転写し、anti-β3 mAb の VNR5-2 を用いて上清中のβ3 を可視化したものである。SDS-PAGE ではαIIb とβ3 は解離して泳動するため、β3 は分子量約 85kDa の単一のバンドとして検出された。使用した cDNA クローンによる差はあるものの、野生型αIIbβ3 （sol-wt ; αIIb-960tr/β3-691tr）に比べ Q595NTT 変異体（sol-Q595NTT ; αIIb-595N-960tr/β3-691tr）の発現量は若干低下していた。一方、αIIb とβ3 の間に人工的なジスルフィド結合を導入したものでは、αIIb とβ3 が共有結合で結合しているため SDS-PAGE 上でも解離しない。このため大部分は分子量約 250kDa のバンドとして検出されたが、一部は 85kDa のバンドとして検出された。発現量ではジスルフィド結合をもたないものと同様の傾向がみられ、野生型αIIbβ3 (sol-wt-cl ; αIIb-959C-960tr/β3-688C-691tr) に比べ Q595NTT 変異体 (sol-Q595NTT-cl ; αIIb-595N-959C-960tr/β3-688C-691tr) の発現は低下していた。

次に、培養時間が可溶性αIIbβ3 の産生量に与える影響を検討した。図５左は CHO 細胞に可溶性αIIbβ3 遺伝子を導入した後の培養時間と産生量の変化を表したものである。導入した遺伝子は、人工的なジスルフィド結合をもつ sol-Q595NTT-cl (αIIb-595N-959C-960tr/β3-688C-691tr) である。培養上清 10μl を SDS-PAGE で展開した後 PVDF 膜に転写し、anti-β3 mAb の VNR5-2 を用いて培養上清中のβ3 を可視化した。遺伝子導入後 48 時間から 5 日

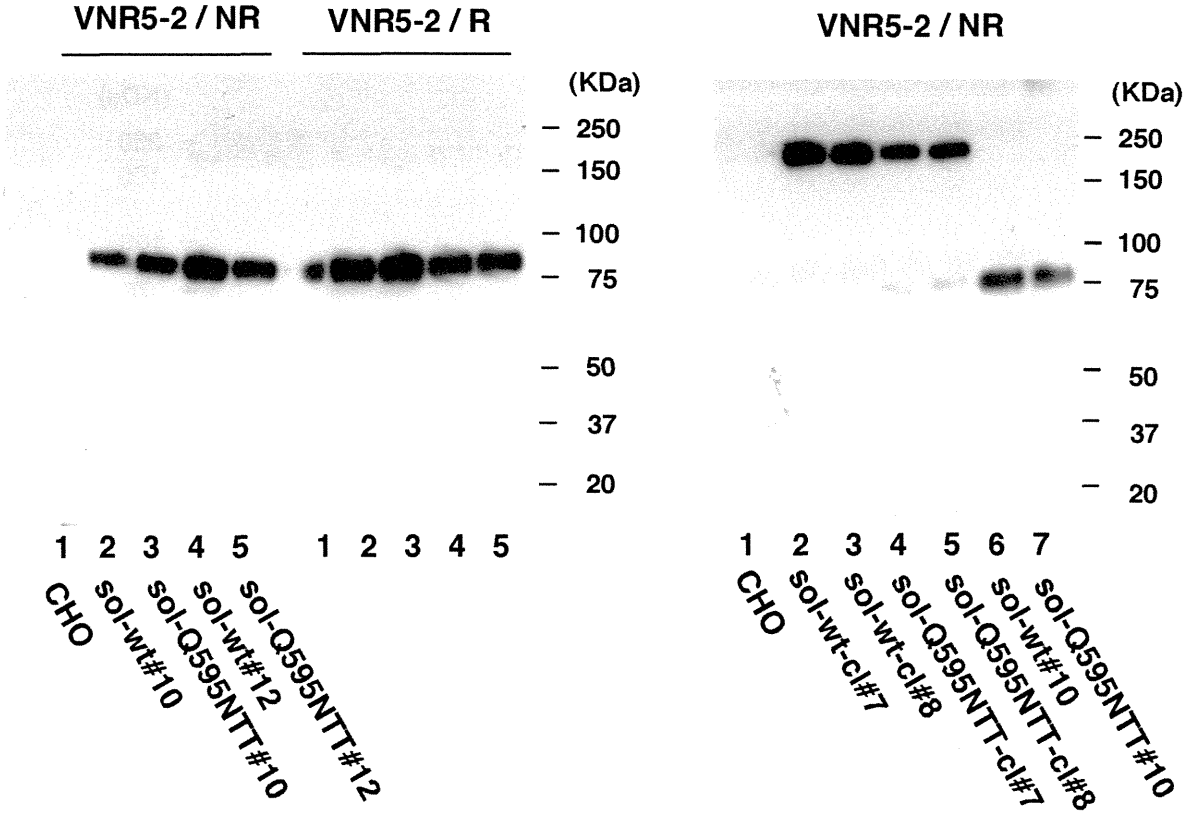

図４．可溶性αIIbβ3 の発現

まで産生量は増加するが、その後は変化しないことがわかった。また、αIIb とジスルフィド結合を形成していないβ3（分子量 90kDa）も、培養時間に依存して増加する傾向がみられた。図５右は可溶性αIIbβ3 産生量がほぼピークに達する培養４日目の培養上清中のβ3 を可視化したものである。人工的に導入したジスルフィド結合の有無は、タンパク産生量に大きな影響は与えなかった。しかし、Q595NTT 変異の有無は、産生量に大きく影響することがわかった。これらの結果は II の FACS を用いた解析結果と一致しており、Q595NTT 変異が可溶性αIIbβ3 産生に悪影響を及ぼすことが確認された。

IV. 可溶性αIIbβ3 の精製

III で産生量の違いは有るものの、可溶性αIIbβ3 が産生可能であることがわかった。次のステップでは、可溶性αIIbβ3 を培養上清中から精製し、濃縮することを試みた。遺伝子導入後 5 日を経過した培養上清 50ml を回収し、RGD-Sepharose Beads 5 ml と４℃にて一晩転倒混和した。遠心して培養上清をとり除き、Beads を洗浄した後、5mM EDTA を含んだバッファー5ml と混和して結合したタンパクを溶出した。同様の操作を４回繰り返し行った。図６は培養上清、素通り、溶出液の各分画 10μl 中のαIIbβ3 をウエスタンブロット法で調べたものである。培養上清からは分子量 90kDa のバンドが検出された。素通り分画からも同じ分子量のバンドが検出されたが、培養上清に比べその量はほとんど減少していなかった。溶出液分画からも 85kDa のバンドが検出されたものの、その量は極めて少なかった。

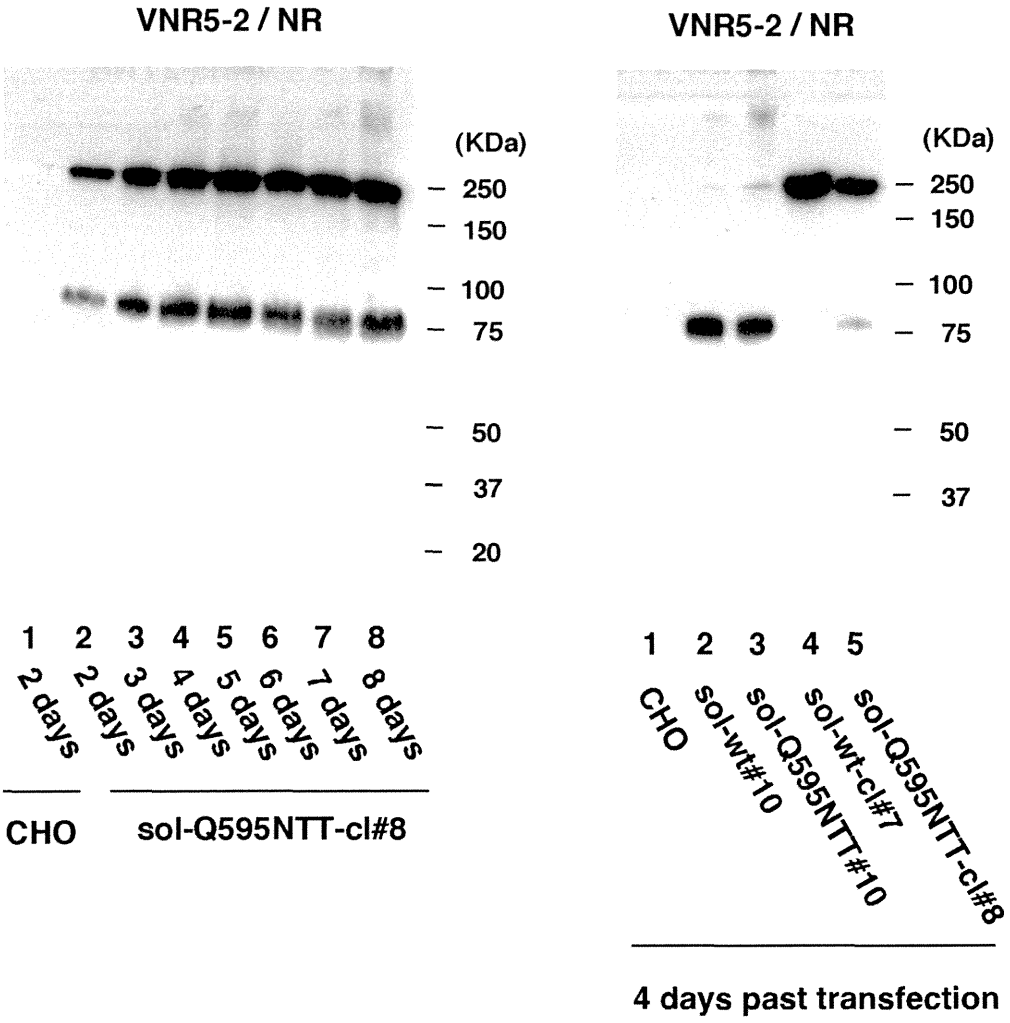

図５．可溶性αIIbβ3 発現量の経時的変化

D. 考察

本研究の目的は、H12V 製剤の機能をモニターする上で、信頼性の高い評価法を確立することである。これまでの研究から、血小板あるいはそれに代わる培養細胞を用いた測定法は、結果が細胞の状態に大きく依存するため、再現性に問題があることが判明した。これらの生きた細胞による不確定要素を排除するため、前年度の研究では無細胞系の測定法、特に SPR 法による H12V とαIIbβ3 の直接結合の測定法の検討を行った。この方法では、αIIbβ3 を CM5 センサーチップに固定化し、その上に H12V を注入して結合する際に生じる質量変化（SPR の変化）を経時的に測定する。これにより、結合速度定数、解離速度定数の算出が可能となる。当初、センサーチップに固定化するαIIbβ3 のソースとしてαIIbβ3 発現細胞株を用いた。界面活性剤で細胞膜を可溶化し、溶解液中から RGD-Sepharose Beads を用いてαIIbβ3 をアフィニティー精製した。この精製αIIbβ3 を抗αIIbβ3 モノクローナル抗体を用いて間接的にセンサーチップに固定化する方法を試みたが、ほとんど固定化されなかった。そこで、直接センサーチップに精製αIIbβ3 を固定化し H12V の結合を調べた。固定化はされたものの、H12V の結合は全く観察されなかった。また、チップに固定化したαIIbβ3 に対する抗αIIbβ3 モノクローナル

抗体の結合を調べたところ全く結合しなかった。これらの結果から、αIIbβ3 が精製の途中で変性したと考えられた。第一の原因として界面活性剤の使用が考えられた。

そこで今年度は、界面活性剤を使用せずにαIIbβ3 を精製する方法を試みた。αIIbβ3 は細胞膜を一回貫通するⅠ型膜タンパク質である。このためαIIbβ3 を精製するためには、界面活性剤を用いて細胞膜を可溶化しなければならない。この過程を回避するため、予め膜貫通ドメイン以降のアミノ酸配列を欠損し、細胞外に分泌される可溶性αIIbβ3 の作成を行った。この目的のために、遺伝子組み換え技術を用いてαIIb 鎖、β3 鎖のそれぞれの膜貫通ドメイン手前のアミノ酸残基に停止コドンを導入し、全部で4種類の可溶性αIIbβ3 を設計した（図1）。一つは膜貫通ドメイン以降を欠損するのみで、それ以外は野生型と同じ配列をもつ sol-wt（αIIb-960tr/β3-691tr）、もう一つはαIIb 鎖の細胞外ドメインに高活性型の Q595NTT 変異をもつ sol-Q595NTT（αIIb-595N-960tr/β3-691tr）である。そして、これらのαIIb 鎖とβ3 鎖の C 末端が互いにジスルフィド結合で結合した sol-wt-cl（αIIb-959C-960tr/β3-688C-691tr）、sol-Q595NTT-cl（αIIb-595N-959C-960tr/β3-688C-691tr）である。

これらの可溶性αIIbβ3 は細胞膜に留まらないため FACS による発現量解析は不可能である。そこでαIIb 鎖とβ3 鎖のどちらか片方を野生型とすることで細胞膜表面での発現を可能とし、FACS で変異を導入した鎖の発現量の比較を行った（図2、図3）。β3 鎖の膜貫通ドメイン以降を欠損したβ3-691tr、β3-688C-691tr は、欠損しない野生型β3 に比べ発現量は低下していた。αIIb 鎖の膜貫通ドメイン以降を欠損したαIIb-960tr、αIIb-959C-960tr でも、欠損しない野生型αIIb に比べて発現量低下がみられた。細胞外ドメインに Q595NTT 変異をもつαIIb-595N-960tr、αIIb-595N-959C-960tr では、更なる発現量の低下が認められた。これらの結果は、膜貫通ドメイン以降の欠損、C 末端の Cys 変異は、αIIb 鎖とβ3 鎖の発現に軽度ではあるが悪影響を及ぼすことがわかった。さらに、Q595NTT 変異はこれらの変異と組み合わせることによりαIIb 鎖の発現に大きな影響を与えることが判明した。本研究で使用するαIIbβ3 では、構造変化に起因する活性変化を可能な限り抑制しなければならない。Q595NTT はαIIb 鎖の高次構造を extended form に固定することにより、αIIbβ3 を高活性に維持する変異である。従って、本研究で用いるαIIbβ3 には無くてはならない変異であるため、以後もこの変異を含めた検討を行った。

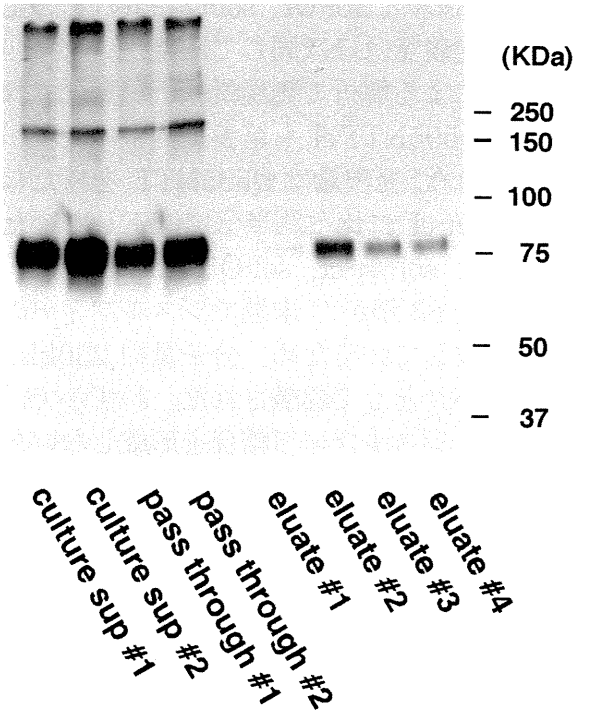

図6．可溶性αIIbβ3 のアフィニティー精製

次に、αIIb 鎖とβ3 鎖の双方の膜貫通ドメイン以降を欠損した変異体を CHO 細胞に発現し、培養上清中に分泌されたαIIbβ3 の分析を行った。ウエスタンブロット法による解析では、使用したクローンによる差

はあるものの、sol-wt、sol-Q595NTT の上清中の発現量はほぼ同じであった。一方、FACS による解析で発現量低下が懸念された sol-Q595NTT-cl も培養上清中の発現が確認された。発現量は Q595NTT を持たない sol-wt-cl に比べ約半分に低下していた（図４）。sol-wt-cl、sol-Q595NTT-cl は、αIIb 鎖とβ3 鎖が C 末端でジスルフィド結合を形成しているため、分子量約 250kDa のタンパクとして認識された。わずかではあるが、ジスルフィド結合を形成しない分子量約 85kDa のバンドも確認された。これらの発現はトランスフェクションしてから 48 時間後の培養上清中のものである。そこでトランスフェクション後、何日目に培養上清中の発現が最大になるかを調べるため、経時的な発現量の変化を調べた（図５）。その結果、トランスフェクション後４日から５日まで発現量は増加し、その後は変化しないことが判明した。トランスフェクション後４日目の発現を比べた結果では、sol-wt と sol-Q595NTT の発現量はほぼ同じであった。sol-wt-cl と sol-Q595NTT-cl の比較では、sol-wt-cl に比べて sol-Q595NTT-cl の発現量は約半分に低下していた。しかし、sol-Q595NTT-cl の発現は sol-wt や sol-Q595NTT とほぼ同等であり、実用可能と考えられた。

最後に、培養上清中の可溶性αIIbβ3 の精製を試みた。sol-Q595NTT をトランスフェクションし、５日後の培養上清 50ml を前年度に作成した RGD-Sepharose Beads 5ml と一晩振盪混和した。Beads を緩衝液で洗浄した後、5 mM EDTA を含む緩衝液 5ml と混和して結合したαIIbβ3 を溶出した。この溶出を四回繰り返した。図６は培養上清、素通り分画、溶出液分画の各 10μl をイムノブロット法で解析したものである。培養日時の異なる培養上清 culture sup #1、#2 とそれぞれの素通り分画である pass through #1、pass through #2 を比較すると、RGD カラム前後でαIIbβ3 量の変化はほとんど無く、大部分が RGD-Sepharose Beads に結合せず素通り分画に残存していることが判明した。溶出液分画の分析では、一回目の分画には、ごく僅かのαIIbβ3 しか存在せず、二回目で最大となり、以降は減少していた。このことは、5 mM EDTA では培養液中の二価イオンを除去しきれなかったことを示唆している。Bead に結合したαIIbβ3 を溶出するためには、より高濃度の EDTA を使用する必要があると考えられる。また、溶出したαIIbβ3 は培養上清中に存在したものに比べきわめて少量であった。このことは RGD-Sepharose Beads の RGD ペプチド結合量に問題があったことを示唆している。十分な量の RGD ペプチドを結合したカラムを使用することにより、可溶性αIIbβ3 の精製は可能と考えられる。

E. 結論

SPR 法を用いて H12V とαIIbβ3 の結合を測定する方法は、測定に伴う不確定要因が最も少なく再現性に優れた方法と考えられる。これを可能にするため、可溶性αIIbβ3 の作成を行った。高活性型αIIbβ3 は野生型に比べ発現量が有意に低いものの、可溶性タンパクとして培養上清中に分泌されることがわかった。アフィニティー精製法と溶出条件を改善することにより、可溶性高活性型αIIbβ3 の作成は十分に可能と考えられた。

F. 研究発表

1. 論文発表：無し。
2. 学会発表：無し。

G. 知的財産権の出願・登録：無し。

別添５

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻(号)	ページ	出版年
Hagisawa K, Nishikawa K, Yanagawa R, Kinoshita M, Doi M, Suzuki H, Iwaya K, Saitoh D, Seki S, Takeoka S, Handa M, Nishida Y.	Treatment with fibrinogen γ-chain peptide-coated, ADP-encapsulated liposomes as an infusible haemostatic agent against active liver bleeding in acute thrombocytopenic rabbits	*Transfusion*	55 (2)	314-25	2015
Taguchi K, Hashimoto M, Ogaki S, Watanabe H, Takeoka S, Ikeda Y, Handa M, Otagiri M, Maruyama T	Effect of repeated injections of adenosine diphosphate-encapsulated liposomes coated with a fibrinogen γ-chain dodecapeptide developed as a synthetic platelet substitute on accelerated blood clearance in a healthy and an anticancer drug-induced thrombocytopenia rat model.	*J Pharm Sci*	On line	doi: 10.1002 /jps.24 418.	2015, Mar 9
木下 学	人工赤血球、人工血小板の臨床応用	*A net*	18 (1)	14-7	2014
萩沢康介, 木下 学, 宮脇博基, 佐藤俊一, 鈴木英紀, 土井麻実, 武岡真司, 小野聡, 齋藤大蔵, 西田育弘	衝撃波による致死的肺出血マウスに対する人工血小板(H12(ADP)liposome)の救命効果	*Shock*	29 (2)	1-8	2014

Ⅳ. 研究成果の刊行物・別冊

Treatment with fibrinogen γ-chain peptide-coated, adenosine 5′-diphosphate–encapsulated liposomes as an infusible hemostatic agent against active liver bleeding in rabbits with acute thrombocytopenia

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BACKGROUND: We evaluated the hemostatic efficacy of H12-(adenosine 5′-diphosphate [ADP])-liposomes in the setting of active liver bleeding in rabbits with dilutional thrombocytopenia after massive transfusion.
STUDY DESIGN AND METHODS: Acute thrombocytopenia (platelet [PLT] count < 50×10^9/L) was induced in rabbits by repeated blood withdrawal and isovolemic transfusion of autologous washed red blood cells. Liver hemorrhage was initiated by a penetrating liver injury. Subsequently, the animals received tamponade treatment for the liver hemorrhage for 5 minutes and were intravenously administered H12-(ADP)-liposomes with PLT-poor plasma (PPP), PLT-rich plasma (PRP), PPP alone, H12-(phosphate-buffered saline [PBS])-liposome/PPP, or H12-(ADP)-liposomes/PPP plus fibrinogen concentrate during the tamponade.
RESULTS: Administration of H12-(ADP)-liposomes/PPP rescued 60% of the rabbits from the liver hemorrhage; PRP administration rescued 50%. In contrast, rabbits receiving PPP or H12-(PBS)-liposome/PPP achieved only 10 or 17% survival, respectively, for the first 24 hours. H12-(ADP)-liposomes/PPP as well as PRP consistently reduced bleeding volumes and shortened clotting times (CTs) in comparison to PPP administration. Specifically, bleeding volumes in the initial 5 minutes averaged 11 mL (H12-(ADP)-liposomes/PPP) and 17 mL (PRP) versus 30 mL (PPP; $p < 0.05$); CTs averaged 270 and 306 seconds versus 401 seconds ($p < 0.05$). H12-(ADP)-liposomes were observed at the bleeding site with thrombus formation, suggesting an induction of thrombi. Neither macro- nor microthrombi were detected in the lung, kidney, spleen, or liver in rabbits treated with H12-(ADP)-liposomes. Supplementation of fibrinogen to H12-(ADP)-liposomes/PPP did not significantly improve rabbit survival.
CONCLUSIONS: H12-(ADP)-liposomes might be a safe and effective therapeutic tool during damage control surgery for trauma patients with acute thrombocytopenia and massive bleeding.

Fluid resuscitation after massive hemorrhage in severe trauma might result in extensive hemodilution and coagulopathy.[1] Coagulopathy, hypothermia, and acidosis are identified as a lethal triad for patients presenting with exsanguinating

ABBREVIATIONS: APTT = activated partial thromboplastin time; AT = antithrombin; CR(s) = clotting rate(s); CT(s) = clotting time(s); MAP = mean arterial pressure; PPP = platelet-poor plasma; PRP = platelet-rich plasma; PT(s) = prothrombin time(s).

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TRANSFUSION 2015;55:314–326.

hemorrhage.[2] Platelet (PLT) transfusion is more effective for management of actual coagulopathy involved in trauma injury or surgery than the transfusion with fresh-frozen plasma (FFP), cryoprecipitate, desmopressin, or recombinant activated Factor VII.[3-5] The current US military resuscitation practice is to use a balanced approach, using FFP, PLTs, and red blood cells (RBCs; 1:1:1) as the primary resuscitation fluid for the most seriously injured casualties.[5,6] Nevertheless, FFP is usually preferred over PLTs for the treatment of coagulopathy after massive transfusion because, from a logistic point of view, the availability of PLT concentrates is restricted due to their short shelf life.[7] PLT concentrates expire within several days in spite of shaking preservation at 22°C.

We have developed liposome-based artificial PLTs bearing synthetic HHLGGAKQAGDV (H12) peptides corresponding to the carboxyl terminal of the fibrinogen γ-chain on the surface. The liposomes also contain the physiologic PLT agonist adenosine 5′-diphosphate (ADP) inside.[8] Preliminary observations indicate that this compound can be stored for at least 6 months at 4°C without shaking (S. Takeoka et al., unpublished observation, 2014). We previously demonstrated that H12-(ADP)-liposomes were a synthetic PLT substitute preventing uncontrollable traumatic hemorrhage confounded by acute thrombocytopenia after massive RBC transfusion.[9] However, the administration of H12-(ADP)-liposomes preceded liver injury by 30 minutes in that study. Thus it did not precisely simulate a clinical situation in which PLT transfusion starts after active bleeding. In this study, we examined the hemostatic effects of H12-(ADP)-liposomes after the occurrence of acute bleeding from liver injury.

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. The institutional review board approved this study and the ethical approval number was Number 13042.

Rabbits and reagents

A total of 68 New Zealand white rabbits (2.0 ± 0.2 kg, male; Japan SLC, Hamamatsu, Japan) were used in this study as follows: 52 rabbits for monitoring survival, 12 for pathologic examination, and four for electron microscopic observation. Cholesterol and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine were purchased from Nippon Fine Chemical (Osaka, Japan), 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[monomethoxypoly(ethylenglycol)] (PEG-DSPE, 5.1 kDa) was obtained from NOF (Tokyo, Japan), and adenosine 5′-diphosphate (ADP) was from Sigma-Aldrich (St Louis, MO). We synthesized 1,5-dihexadecyl-*N*-succinyl-L-glutamine (DHSG) and H12-PEG-Glu2C18. The fibrinogen γ-chain dodecapeptide (C-HHLGGAKQAGDV, Cys-H12) was conjugated to the end of the PEG-lipids, as described elsewhere.[10]

Preparation of H12-(ADP)-liposomes

H12-(ADP)-liposomes were prepared as described elsewhere.[10] Briefly, 1,2-dipalmitoyl-*sn*-glycero-3 -phosphatidylcholine (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 mixed lipids were hydrated with phosphate-buffered saline (PBS) containing 1 mmol/L ADP with filter membranes (pore size, 0.45, 0.22 μm; Millipore, Tokyo, Japan) to prepare H12-(ADP)-liposomes. After liposomes were washed with PBS followed by centrifugation (100,000 × *g*, 30 min, 4°C), the remaining ADP was removed using gel filtration medium (Sephadex G25, GE Healthcare, Tokyo, Japan). H12 liposomes without ADP (H12-(PBS)-liposome) were also prepared by skipping ADP encapsulation.

Acute thrombocytopenic rabbit model

Acute thrombocytopenia was induced in rabbits, as described elsewhere.[9] Briefly, rabbits were anesthetized using intramuscular injections of ketamine (25 mg/kg) and xylazine (10 mg/kg), followed by maintaining anesthesia with intravenous (IV) injections of pentobarbital (15 mg/kg) every 30 minutes during the experiment. The adequacy of anesthesia was monitored by the loss of the ear pinch reflex. Anaesthetized rabbits were placed on a warming plate to maintain the body temperature at 37°C. Aseptic techniques were adopted for all surgical procedures. Surgical catheters (polyethylene indwelling 20-gauge needle; Terumo Co., Tokyo, Japan) were inserted into the femoral artery and vein in each rabbit (Fig. 1). Thereafter, 12.5 mL/kg blood (Sample 1) was drawn from the femoral artery, and the same volume of dextran 40 (308 mOsm/L, Otsuka, Tokushima, Japan) was simultaneously transfused via the femoral vein (Fig. 2). Forty minutes later, the next blood sample (12.5 mL/kg, Sample 2) was withdrawn and the same volume of washed RBCs prepared using Sample 1 was transfused. This isovolemic blood exchange was repeated eight times, and the PLT counts were approximately fewer than 50×10^9/L. The last transfusion of washed RBCs was performed without simultaneous blood withdrawal (Fig. 2). Arterial pH was spontaneously maintained at 7.35 to 7.45 in rabbits.

Preparation of washed RBCs, PLT-rich plasma, and PLT-poor plasma

Blood samples drawn with a 10% volume of 3.8% (wt/vol) sodium citrate were centrifuged at 100 × *g* for 15 minutes,

and the supernatant was used as PLT-rich plasma (PRP; Fig. 1). The remaining sample was further centrifuged at 500 × *g* for 10 minutes and the supernatant was used as PLT-poor plasma (PPP). Thereafter, remaining cells were washed with saline, diluted in 12.5 mL/kg lactated Ringer's solution containing 5% human serum albumin, and transfused into the rabbit as washed RBCs (Fig. 1).

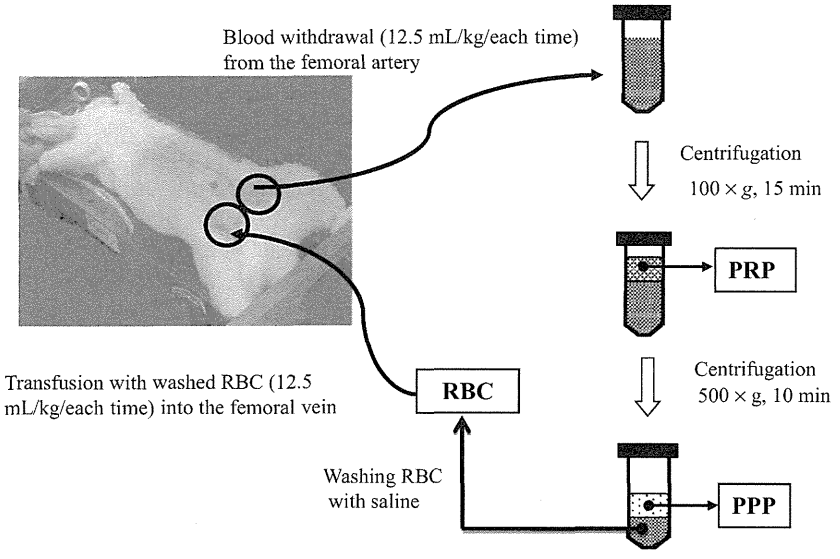

Fig. 1. Scheme of blood withdrawal from rabbits: preparation of PRP, PPP, and washed RBCs and transfusion of washed RBCs into rabbits.

Penetrating liver injury followed by balloon compression in the bleeding site

After it was confirmed that the PLT count was fewer than 50×10^9/L, rabbits underwent laparotomy to expose a liver lobe. We cut a hole in a surgical glove and passed the lobe through the hole to collect exsanguinating blood in the glove and precisely evaluate bleeding volume. Thereafter, the liver was penetrated using a disposable punch biopsy apparatus (5 mm in diameter; DermaPunch, Nipro Medical Industries Ltd., Tokyo, Japan; Figs. 3A and 3B). Immediately after the injury was induced, a 4-Fr urologic catheter (Bardex Biocath, 0165PL 8 ch/Fr 2.7 mm, C. R. Bard, Inc., Murray Hill, NJ) was inserted into the lesion and the balloon was inflated to compress the lesion for 5 minutes, simulating manual compression as damage control surgery[11] (Figs. 3C and 3D). During the balloon compression, H12-(ADP)-liposomes, PRP, PPP, H12-(PBS)-liposomes, or fibrinogen concentrates were administered to the rabbits. After deflating and removing the balloon catheter, we then measured the bleeding volume from the site of the liver injury for the initial 5-minute period (0 to 5 min) and the following 5 minutes (5 to 10 min). Bleeding time from the

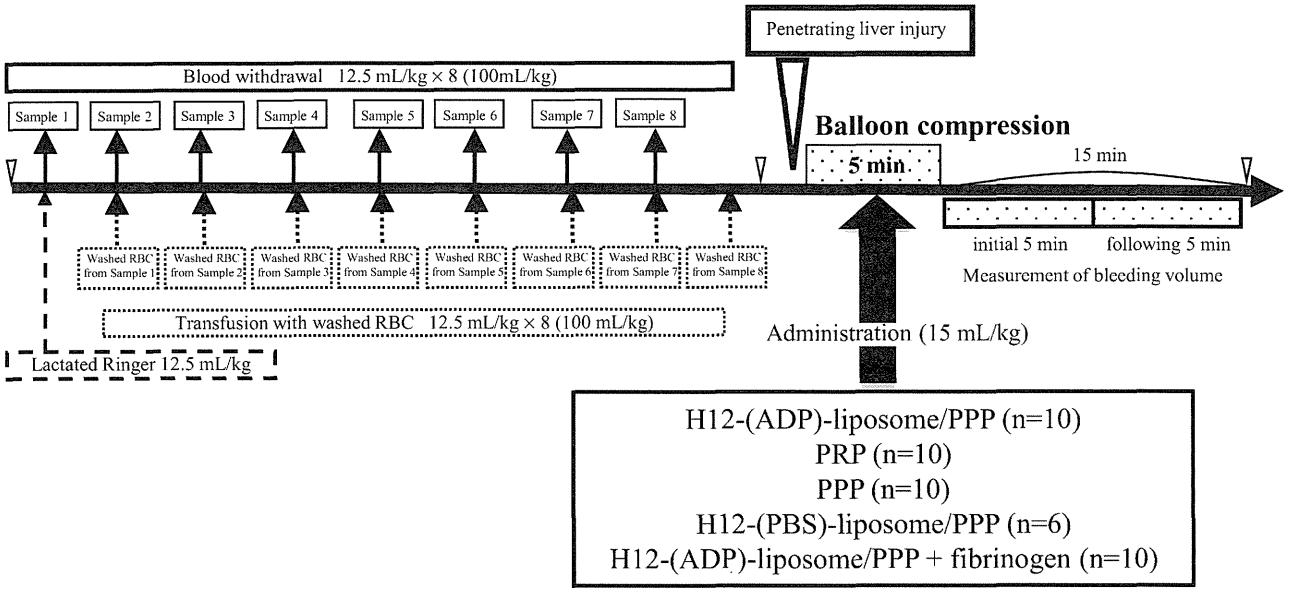

Fig. 2. Experimental design for acute thrombocytopenia and liver hemorrhage in rabbits, followed by balloon compression and infusion of hemostatic agents.

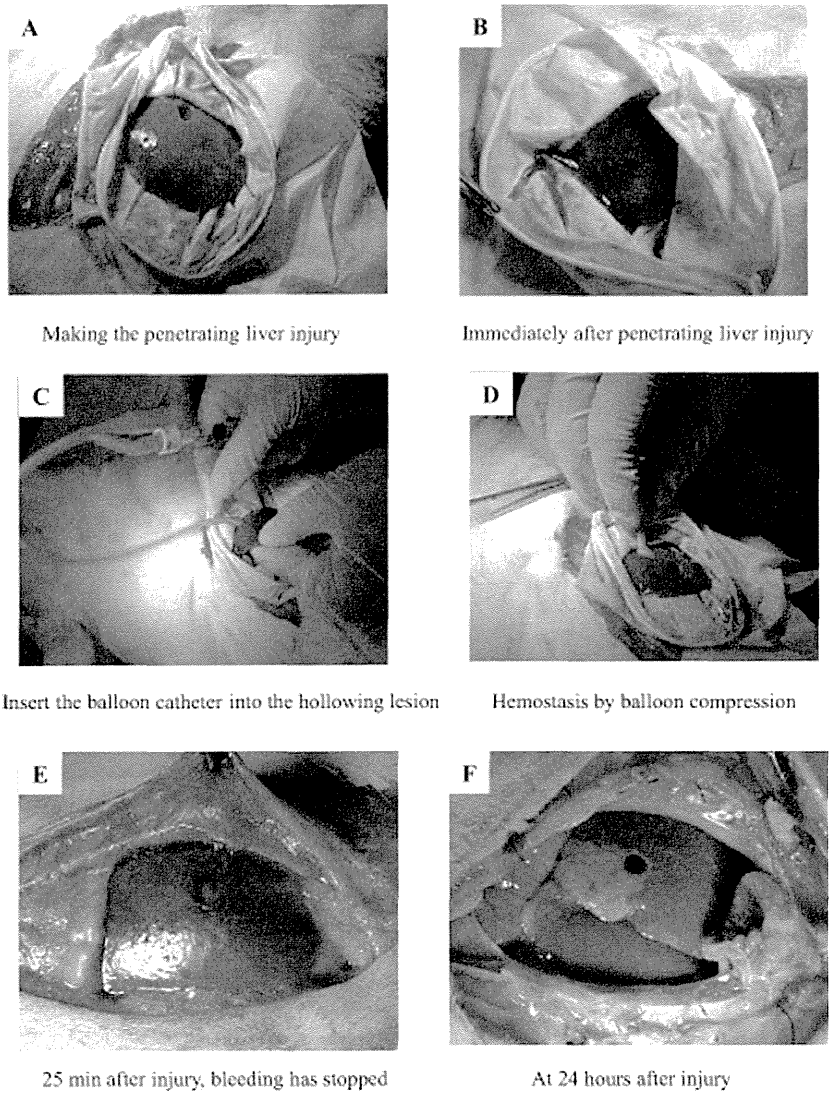

Fig. 3. Hemostasis after balloon compression. (A, B) Making a penetrating liver injury. (C, D) Balloon compression against penetrating liver injury by indwelling urethral catheter. (E) Hemostasis was achieved after administration of H12-(ADP)-liposomes/PPP following balloon compression. (F) Complete hemostasis was observed with ischemic change in the lesion 24 hours later.

penetrating liver injury was monitored for 60 minutes. Thereafter, the rabbit's abdomen was closed to monitor survival for 72 hours under ad libitum feeding with laboratory diet and water. Postoperative analgesia was performed with two intramuscular injections of buprenorphine (0.02 mg/kg), immediately after wound closure and 12 hours later.

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

PRP or PPP was prepared from the blood taken at the first and second phlebotomy. These PRP and PPP samples showed similar coagulation activities (fibrinogen, approximately 150 mg/dL; antithrombin [AT] III activity, 99%; prothrombin time [PT], 12 sec; activated partial thromboplastin time [APTT], 32 sec).[10] During balloon compression against liver bleeding, H12-(ADP)-liposomes (20 mg/4 mL/kg) were administered IV into the rabbits, followed by administration of 11 mL/kg PPP (n = 10, Fig. 2). In our previous studies of rabbits with busulfan-induced thrombocytopenia, H12-(ADP)-liposomes administered at a dose of 20 mg/kg gave optimal bleeding time-shortening effects, similar to those of PRP.[9,10] Therefore, this dose was selected for all of the present experiments. Similarly, 15 mL/kg PRP or PPP was administered to the rabbits during the balloon compression (n = 10 in each group, Fig. 2). Also, H12-(PBS)-liposomes that contain no ADP were administered IV to the rabbits, followed by administration of 11 mL/kg PPP (n = 6). To supplement an adequate amount of fibrinogen, 70 mg/kg fibrinogen concentrate (Haemocompletan, CSL Behring, Marburg, Germany) was injected into the rabbits, after the administration of H12-(ADP)-liposomes (20 mg/4 mL/kg) and PPP (11 mL/kg, n = 10; Fig. 2). For negative controls, two rabbits with thrombocytopenia treated with balloon compression alone (without PLT and/or plasma replacement) were evaluated. Also, the rabbits with thrombocytopenia treated with H12-(ADP)-liposome or PRP alone (without balloon compression) were evaluated (n = 2 in each group).

Analyses of whole blood coagulation activity

Whole blood samples were analyzed for hemostatic function three times: before and after blood exchange and 20 minutes after liver injury. The coagulation activity of whole blood was examined using the a coagulation and PLT function analyzer (Sonoclot, Sienco, Morrison, CO).[9] 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 probe vibration is

detected by the sensor and converted to an output signal that reflects the viscoelastic properties of the developing clot. The signal typically describes coagulation variables including "clotting time (CT)," which indicates the period up to the beginning of fibrin formation, and "clotting rate (CR)," which indicates the slope of fibrin gel formation that is affected by both the rate of the fibrinogen to fibrin conversion and the amount of fibrinogen (Fig. 4A).

Measurements of mean arterial pressure, hematologic variables, and coagulation factors

Mean arterial pressure (MAP) 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. PLT count, hemoglobin (Hb) concentration, and white blood cell count were measured using a hematology analyzer (PEC 170, Erma, Inc., Tokyo, Japan). Plasma concentration of fibrinogen, AT III activity, PT, and APTT were measured at the BML Laboratory (Tokyo, Japan).

Electron microscopic examinations

For electron microscopy, four rabbits treated with H12-(ADP)-liposomes were prepared. Liver specimens were obtained at 1 hour after liver hemorrhage. These were fixed with a fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 3 hours at 4°C, followed by postfixing in 1% osmium tetroxide in 0.1 mol/L phosphate buffer (pH 7.4) for 2 hours at 4°C, dehydration, and embedding in epoxy resin. To select 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 1010, JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.[12]

Histopathologic examinations

For histopathologic examinations, the rabbits treated with H12-(ADP)-liposomes, PRP, or H12-(ADP)-liposomes/PPP plus fibrinogen were euthanized 24 hours after liver hemorrhage (n = 3 in each group). Three rabbits with PPP that died after several minutes were also examined. The liver (uninjured lobe), lung, spleen, and kidney were removed from the subject rabbits. Excised organs were fixed by 20% formalin for 2 days and processed to paraffin embedding blocks to stain with hematoxylin and eosin.

Statistical analyses

Statistical analyses were performed with a software package (Stat View 4.02J, Abacus Concepts, Berkeley, CA). Survival rates were compared by Wilcoxon signed rank test. Statistical evaluations between two groups were compared using the t test, and any other statistical evaluations were compared using the one-way analysis of variance, followed by Bonferroni post hoc test. Data are presented as means ± standard deviation (SD), with p values of less than 0.05 considered to be significant.

RESULTS

Acute thrombocytopenia in rabbits

After isovolemic blood exchange and plateletpheresis, PLT counts in rabbits were decreased to $45 \times 10^9 \pm 8 \times 10^9$/L, indicating acute thrombocytopenia (Table 1). Although the subject rabbits maintained MAP as a result of isovolemic exchanges, their Hb concentrations were decreased to approximately 6 g/dL due to inevitable loss of RBCs during plateletpheresis, which we attribute to mechanical destruction of RBCs in the processes of centrifugation and washing with saline. However, apparent hemolysis was not observed in the blood samples obtained from rabbits with thrombocytopenia (Table 1). Their coagulation factors were also decreased to very low levels (Table 1), because they had not yet received PPP or PRP administration. CT and CR were also markedly worsened by the blood exchange (Table 1).

Survival from liver hemorrhage in the rabbits with acute thrombocytopenia

Balloon compression alone did not rescue any of the rabbits with thrombocytopenia from hemorrhage induced by the penetrating liver injury, as the treatment did not stop the bleeding and death occurred within 1 hour. However, administration of H12-(ADP)-liposomes/PPP as well as PRP after balloon compression significantly increased the survivals from liver hemorrhage in the rabbits with thrombocytopenia in comparison to administration of PPP (Fig. 5). Unlike H12-(ADP)-liposomes/PPP, administration of H12-(PBS)-liposomes/PPP after balloon compression was not effective (Fig. 5). Supplementation with fibrinogen concentrate did not increase the survival of the rabbits with thrombocytopenia treated with H12-(ADP)-liposomes/PPP (Fig. 5). We also treated the rabbits with thrombocytopenia with H12-(ADP)-liposome/PPP or PRP alone (without balloon compression, n = 2 in each group). However, neither treatment with H12-(ADP)-liposomes nor treatment with PRP rescued any rabbits from hemorrhage. Specifically, after treatment with H12-(ADP)-liposomes, two animals failed to stop bleeding and died within 12 hours; after treatment with PRP, bleeding stopped in one of two animals but both died within 6 hours. These outcomes suggested the importance of damage control intervention.

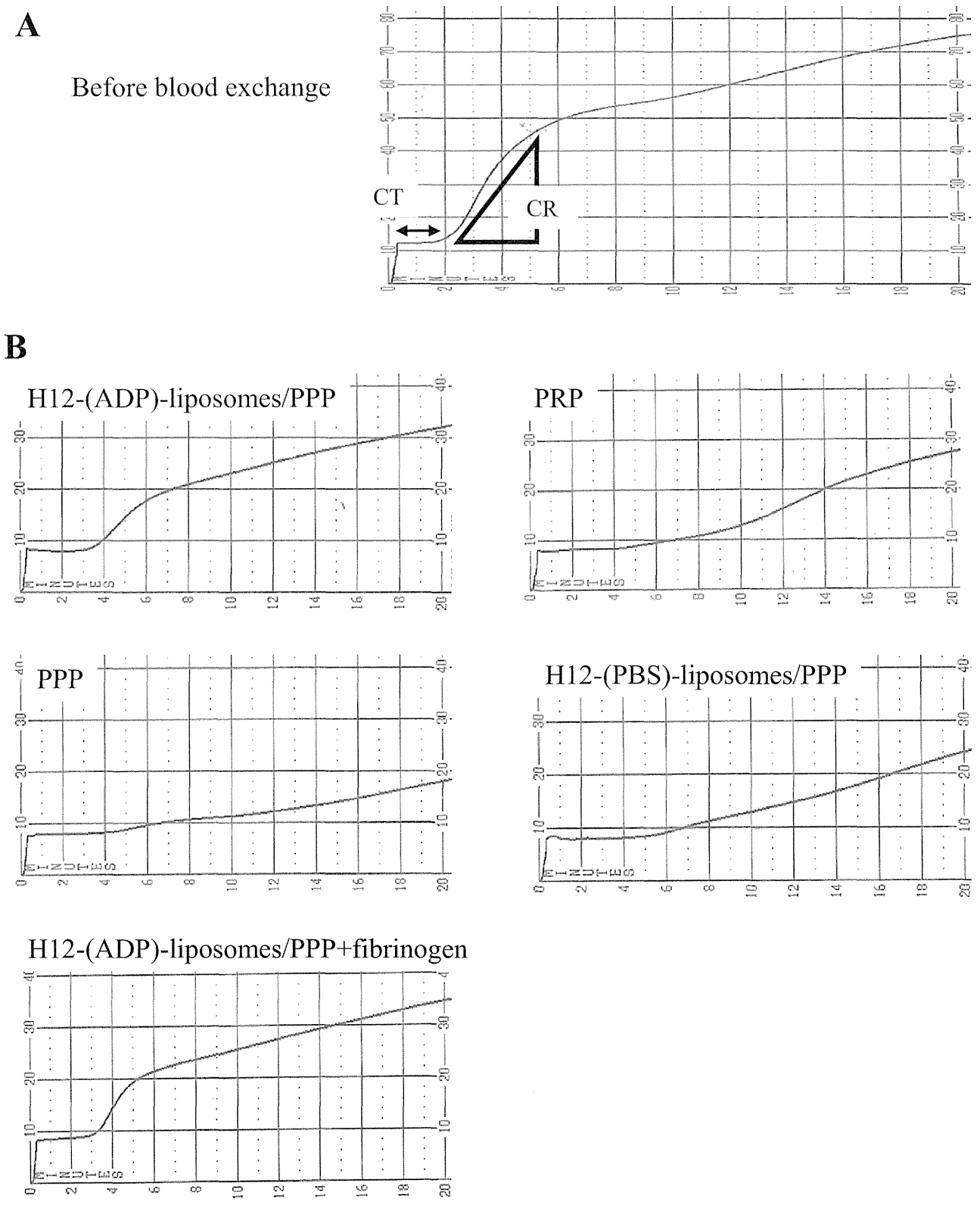

Fig. 4. Analyses of whole blood coagulation activities. (A) Whole blood coagulation activities in rabbits before blood exchanges. CT and CR are indicated by arrow and triangle, respectively. (B) Blood coagulation activities in each group 20 minutes after liver injury. Representative data with similar results are shown.

Bleeding time of the liver injury site

Administration of H12-(ADP)-liposomes/PPP as well as PRP achieved significant hemostasis in nine of 10 rabbits in both groups, while administration of PPP stopped bleeding in only two of 10 rabbits (90% vs. 20%, $p < 0.01$; Fig. 6A). Administration of H12-(PBS)-liposomes/PPP stopped bleeding in four of six rabbits; however, this treatment only rescued one rabbit, suggesting that H12-(PBS)-liposomes induced a weak (ineffective) hemostasis (Fig. 6A). Interestingly, although supplementation of fibrinogen to the H12-(ADP)-liposomes/PPP achieved hemostasis in all rabbits, six of 10 rabbits eventually died (Fig. 6A). We further analyzed the bleeding time in rabbits showing hemostasis. All treated groups showed significantly shorter bleeding times than those receiving the PPP group (Fig. 6A).

TABLE 1. Changes in the hematologic variables and coagulation factors in rabbits before and after blood exchange*

Variable	Before blood exchange (n = 68)	After blood exchange (n = 68)
MAP (mmHg)	71 ± 12	67 ± 14
Hb concentrations (g/dL)	11.3 ± 2.1	6.3 ± 2.3†
PLT counts ($\times 10^9$/L)	217 ± 66	45 ± 8†
Fibrinogen concentration (mg/dL)	186 ± 38	<55
AT III activity (%)	115 ± 18	35 ± 11†
PT (sec)	10 ± 1	>30
APTT (sec)	24 ± 8	>75
CT (sec)	114 ± 16	387 ± 185†
CR (sec)	17 ± 5	2.1 ± 1.3†

* Hematologic variables and coagulation factors were measured in rabbits before and after blood exchange. Data are reported as mean ± SD.
† $p < 0.01$ versus before blood exchange.

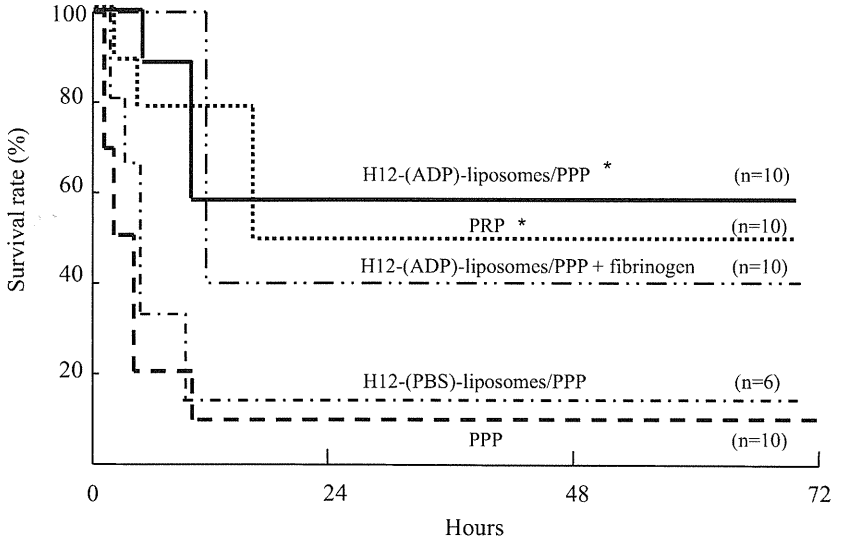

Fig. 5. Survival rates of rabbits with acute thrombocytopenia after liver hemorrhage. Balloon compression and administration of H12-(ADP)-liposomes/PPP, PRP, PPP, H12-(PBS)-liposome/PPP, or H12-(ADP)-liposomes/PPP with fibrinogen were used to treat liver hemorrhage in rabbits with acute thrombocytopenia. *$p < 0.01$ versus PPP, $p < 0.05$ versus H12-(PBS)-liposome.

Bleeding volume from the liver injury site

Administration of H12-(ADP)-liposomes/PPP as well as PRP significantly reduced the bleeding volume from the site of the liver injury in the initial 5 minutes compared to that of PPP (Fig. 6B). However, administration of H12-(PBS)-liposomes/PPP resulted in a substantial amount of bleeding in the rabbits during the initial 5 minutes compared to treatment with H12-(ADP)-liposomes/PPP or PRP alone (Fig. 6B), suggesting that H12-(PBS)-liposome did not promptly exert a hemostatic effect. Supplementation of fibrinogen to H12-(ADP)-liposomes/PPP resulted in a significant reduction of bleeding volume as did administration of H12-(ADP)-liposomes/PPP alone. H12-(ADP)-liposomes/PPP and PRP markedly reduced the bleeding volume in the subsequent 5 minutes (5 to 10 min) in comparison to those in the initial 5 minutes, suggesting achievement of effective hemostasis (Fig. 6B). PPP and H12-(PBS)-liposomes/PPP groups also showed a marked reduction of bleeding volume in the subsequent 5 minutes in comparison to those in the initial 5 minutes. However, both groups remarkably decreased their blood pressure 20 minutes after liver injury (Table 2), presumably owing to their substantial hemorrhage in the initial 5 minutes. In turn, this severe hypotension (shock) might have affected reducing the hemorrhagic volume in the next 5 minutes and caused subsequent death.

Immediately after making the penetrating liver injury, rabbits showed exsanguinating hemorrhage at the injured site (Figs. 3A and 3B). The rabbits received balloon compression for 5 minutes and were administered H12-(ADP)-liposomes/PPP (in this case) during balloon compression (Figs. 3C and 3D). Twenty-five minutes after penetrating liver injury, blood coagulation or hemostasis was observed in the rabbits, and 24 hours later, complete hemostasis in the liver's site of injury was confirmed (Figs. 3E and 3F).

Changes in hemodynamics, hematologic variables, and coagulation factors

Twenty minutes after liver injury, rabbits in the PPP group and the H12-(PBS)-liposomes/PPP group showed

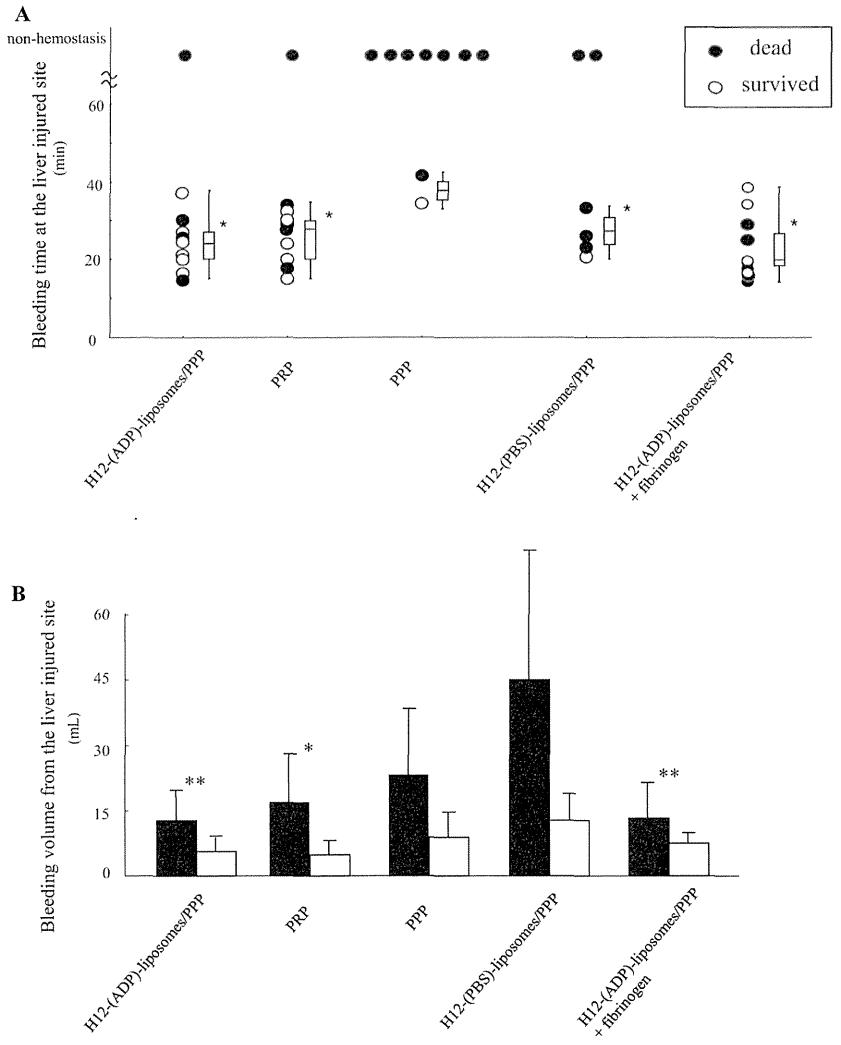

Fig. 6. Liver hemorrhage in rabbits with thrombocytopenia after administration of H12-(ADP)-liposomes/PPP, PRP, PPP, H12-(PBS)-liposome/PPP, or H12-(ADP)-liposomes/PPP with fibrinogen. (A) Bleeding time from liver injury. *p < 0.05 versus PPP. (B) Bleeding volumes from the penetrating liver injury. (■) 0 to 5 minutes; (□) 5 to 10 minutes. *p < 0.05 versus PPP, p < 0.01 versus H12-(PBS)-liposomes/PPP; **p < 0.01 versus PPP, H12-(PBS)-liposomes/PPP.

significantly lower MAP (<40 mmHg) than the other groups (approx. 60 mmHg; Table 2). These two groups also showed severe anemia (Hb < 4 g/dL) due to massive hemorrhage (Table 2). As expected, only rabbits receiving PRP showed a significantly higher PLT count, which might have contributed to the cessation of bleeding (Table 2). Plasma fibrinogen was below the lower limit of detection except for the H12-(ADP)-liposomes/PPP plus fibrinogen group (Table 2), suggesting that supplementation of coagulation factors such as fibrinogen by PPP or PRP administration appeared to be insufficient. However, there were no significant differences in the AT III activities or PTs among the H12-(ADP)-liposomes/PPP, PRP, and H12-(ADP)-liposomes/PPP plus fibrinogen groups, although PPP and H12-(PBS)-liposomes/PPP groups showed marked impairment in these variables (Table 2). The time after which APTT measurement was no longer recorded (75 sec in this case) was exceeded in all groups (data not shown).

Analyses of whole blood coagulation activity

Administration of H12-(ADP)-liposomes/PPP as well as PRP alone significantly shortened the CT in comparison to that observed in the PPP and the H12-(PBS)-liposomes/PPP groups (Table 2). Supplementation of fibrinogen to H12-(ADP)-liposomes/PPP did not significantly affect CT in rabbits (Table 2). Although H12-(ADP)-liposomes/PPP and PRP groups tended to show an increase in the CR compared to that of the PPP or H12-(PBS)-liposomes/PPP groups, supplementation of fibrinogen to H12-(ADP)-liposomes/PPP remarkably augmented CR (Table 2), suggesting that fibrinogen potently affected CR in the Sonoclot analyses. Representative data in each group are shown in Fig. 4.

Electron microscopic examinations

Clot formation adjacent to the injured site in the liver was observed after administration of H12-(ADP)-liposomes/PPP (Fig. 7A). These clots involved both PLTs and fibrin (Fig. 7B). Electron microscopic assessment of the lesion revealed liposomes (approx. 0.2 to 0.4 μm in diameter) around the PLTs or fibrin deposits (Fig. 7B, indicated by arrows), suggesting the presence of H12-(ADP)-liposomes in the lesion.[9] In the uninjured hepatic lobe, a few liposomal particles were found in the sinusoidal space; however, they did not accumulate PLTs or fibrin (Fig. 7C, indicated by left arrow). Kupffer cells as well as splenic macrophages phagocytosed liposomal particles (Fig. 7C, indicated by right arrow; Fig. 7D, indicated by arrows), suggesting degradation of H12-(ADP)-liposomes by reticuloendothelial system; however, no thrombi were found in the spleen.

TABLE 2. Hematologic variables and coagulation factors or activities in rabbits 20 minutes after liver injury*

Variable	H12-(ADP)-liposomes/PPP (n = 10)	PRP (n = 10)	PPP (n = 10)	H12-(PBS)-liposomes/PPP (n = 6)	Fibrinogen + H12-(ADP)-liposomes/PPP (n = 10)
MAP (mmHg)	59 ± 20	59 ± 11	41 ± 12†	39 ± 10†	62 ± 11
Hb concentrations (g/dL)	5.5 ± 2.2	4.8 ± 2.4	3.0 ± 0.9‡	3.8 ± 1.3	5.3 ± 1.5
PLT counts ($\times 10^9$/L)	55 ± 14	70 ± 21§	54 ± 10	48 ± 14	53 ± 10
Fibrinogen concentration (mg/dL)	<55	<55	<55	<55	93 ± 22
AT III activity (%)	39 ± 9	40 ± 10	26 ± 8‡	24 ± 9‡	39 ± 8
PT (sec)	14 ± 3	12 ± 2	>30	>30	14 ± 1
CT (sec)	270 ± 57	306 ± 77	401 ± 149‡	461 ± 196‡	251 ± 112
CR (sec)	2.9 ± 1.6	2.5 ± 1.3	1.8 ± 1.1	1.6 ± 0.3	6.2 ± 3.1‖

* Hematologic variables and coagulation factors were measured in rabbits 20 minutes after liver injury (followed by administration of H12-(ADP)-liposomes/PPP, PRP, PPP, H12-(PBS)-liposomes/PPP, or H12-(ADP)-liposomes/PPP plus fibrinogen). Data are reported as mean ± SD.
† $p < 0.01$.
‡ $p < 0.05$ versus H12-(ADP)-liposomes/PPP or PRP.
§ $p < 0.05$ versus H12-(ADP)-liposomes/PPP, PPP, H12-(PBS)-liposomes/PPP, or H12-(ADP)-liposomes/PPP plus fibrinogen.
‖ $p < 0.05$ versus H12-(ADP)-liposomes/PPP, PRP, PPP, or H12-(PBS)-liposomes/PPP.

Histologic examinations

Neither macro- nor microthrombi were found in the lung, liver (uninjured lobe), kidney, or spleen in the rabbits 24 hours after administration of H12-(ADP)-liposomes or PRP or PPP (Fig. 8). We also carefully examined the tissue specimens from the H12-(ADP)-liposomes/PPP plus fibrinogen group; however, no thrombi were detected in their organs (Fig. 8). Moderate septum thickness in the lung was observed in the PPP group but not other groups (Fig. 8).

DISCUSSION

It is difficult to control exsanguinating hemorrhage from an injured organ in patients with acute thrombocytopenia. Treatment for coagulopathy as well as surgical interventions including damage control surgery is important for their intensive care. Even with damage control intervention, such patients can die due to complications from severe coagulopathy. In the present rabbit model, loss of blood volume reached approximately 45% to 50% of systemic circulation in the PPP group and 35% to 40% of that in the H12-(ADP)-liposomes/PPP or PRP groups (Fig. 6B). Those levels were sufficient to achieve shock criteria Classes IV and III, respectively.[13] Balloon compression alone failed to rescue rabbits with thrombocytopenia from severe hemorrhage, although it likely offered critical damage control. The rabbits' residual PLT counts might have been too low to achieve effective hemostasis because when we deflated the balloon after a 5-minute compression, exsanguinating hemorrhage was observed again in the rabbits with thrombocytopenia (in the absence of agent administration). Therefore, effective treatment against pernicious coagulopathy is required.

PLT transfusion is believed to be quite effective against severe coagulopathy. In line with this, PRP administration after balloon compression rescued half of the rabbits with thrombocytopenia from lethal hemorrhage (Fig. 5). However, PLT concentrates are often not readily available (at least in hospitals, if not prehospital), because of their short shelf life.[14,15] Currently, the US military uses apheresis PLTs prepared on site in its Role 3 hospitals (combat support hospital) in Afghanistan and these are the only Role 3 hospitals currently deployed in active combat.[16]

We previously reported that pretreatment with H12-(ADP)-liposomes effectively prevented noncompressible liver hemorrhage and rescued all rabbits with acute thrombocytopenia. However, in clinical settings, hemostatic treatments are usually performed in response to ongoing exsanguinating hemorrhage in patients. Damage control surgery is also often performed and accompanied by hemostatic treatment. Therefore, we attempted to simulate those conditions in the present model. Infusion of H12-(ADP)-liposomes or PRP showed effective hemostatic potential even after hemorrhage was initiated and it rescued more than half of the rabbits with thrombocytopenia (Fig. 5). However, neither H12-(ADP)-liposomes nor PRP alone without balloon compression rescued any of the rabbits with thrombocytopenia from hemorrhage. Those results suggest that treatments for coagulopathy using PLTs or a PLT substitute as well as damage control intervention are indispensable for the rescue of patients experiencing severe bleeding.

In our previous study, pretreatment with H12-(PBS)-liposomes significantly improved the survival of rabbits with thrombocytopenia from hemorrhage, whereas H12-(PBS)-liposomes were not effective in the present model. In the present study, hemorrhagic injury preceded administration of H12-(PBS)-liposome, whereas in the previous study, hemorrhagic injury followed administration of H12-(PBS)-liposome. Blood loss due to hemorrhage in the

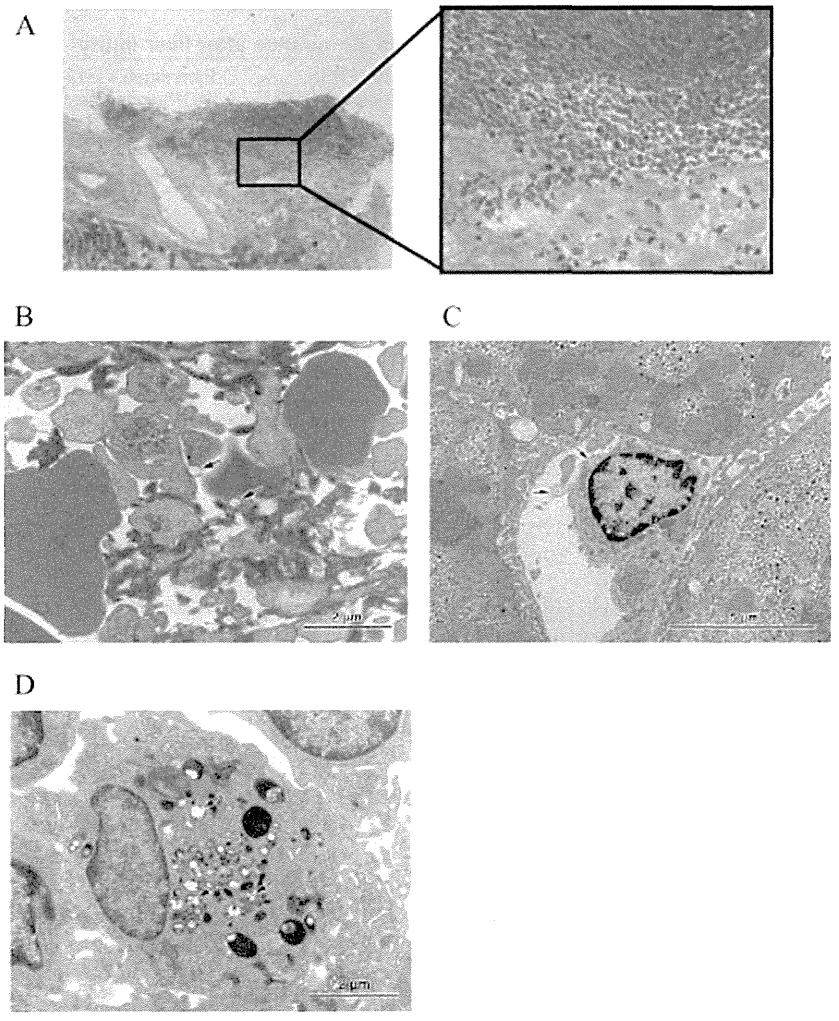

Fig. 7. Microscopic (A) and electron microscopic (B) observations of the penetrating liver injury site in rabbits. Electron microscopic observation of the uninjured hepatic lobe (C) and spleen (D) in rabbits. Specimens were obtained 1 hour after injury from rabbits with thrombocytopenia administered H-12(ADP)-liposomes/PPP. Liposomes are indicated with arrows.

current model was more severe than that of our previous study (bleeding volume from the liver injury site was severalfold larger). These differences between the present and previous studies might have affected the efficacy of H12-(PBS)-liposome. Because ADP plays a key role in both initiating and sustaining integrin αIIbβ3 activation necessary for the development of stable PLT-PLT adhesion contacts,[17] we designed the H12-coated liposomes to contain ADP and release it at the bleeding site.[18] The ADP might be required to induce PLT activation at the bleeding site followed by prompt aggregation of PLTs, resulting in effective hemostasis even in the severe hemorrhagic condition encountered in the present model.

Nevertheless, administration of H12-(ADP)-liposomes was unable to rescue 40% of the rabbits from hemorrhage in this study. In contrast, H12-(ADP)-liposomes achieved 100% survival of rabbits with thrombocytopenia in our previous study.[9] PRP treatment rescued only one-half of the rabbits in this study, whereas PRP pretreatment rescued all subject rabbits in the previous study. Considering this difference in the survival of PLT-transfused rabbits, one can ask why H12-(ADP)-liposome/PPP supported lower survival in the present report than the previous study (60% vs. 100% survival). The reasons are, in fact, not entirely clear, but the marked severity of hemorrhage in the present model might have contributed to the poor survival rates.

To further improve the survival of H12-(ADP)-liposome–treated rabbits, we combined fibrinogen concentrates with H12-(ADP)-liposomes/PPP because there is a consensus that the level of fibrinogen required for effective clot formation to overcome hemorrhaging is at least 100 mg/dL blood.[2,4] However, even though fibrinogen levels were corrected to approximately 100 mg/dL and the CRs markedly improved from 2.1 to 6.2, enhanced survival was not observed. At present, the reason for the failure of fibrinogen supplementation to improve survival from coagulopathy is not known. Theoretically, fibrinogen might contribute to the coagulation step, not to the primary PLT aggregation step. Therefore, further experiments are needed to explore the role of fibrinogen and other coagulation factors in this particular animal model.

Finally, in the present model, we examined the effect of adding fibrinogen concentrate to PPP for administration to the rabbits with thrombocytopenia. However, none of the rabbits survived the experimental conditions (data not shown). Interestingly, blood that accumulated in the peritoneal cavity from the injured liver showed a clot formation. This was not observed in the rabbits receiving PPP alone. In addition, clot formation was not observed at the site of bleeding in the liver in rabbits treated with fibrinogen concentrates added to PPP. Those results suggest that it is important to form the PLT clot in the bleeding site to achieve effective hemostasis. Notably, H12-(ADP)-liposomes effectively formed PLT thrombi in the bleeding site, resulting in effective hemostasis.

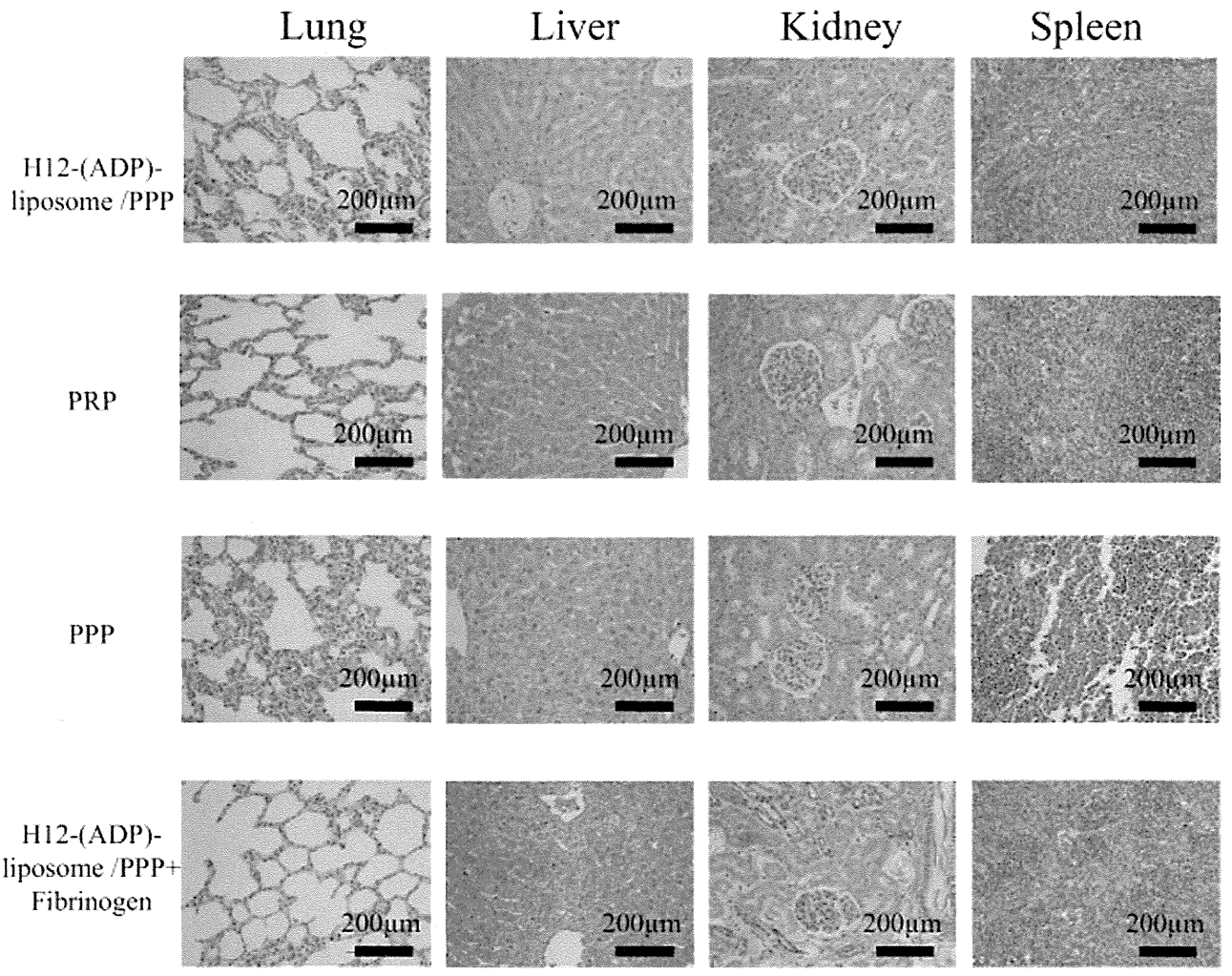

Fig. 8. Histologic findings 24 hours after liver hemorrhage in rabbits with thrombocytopenia. Lung, liver, spleen, and kidney samples were obtained from the rabbits 24 hours after liver injury.

Limitations

Despite achieving hemostasis, several rabbits eventually died even in the H12-(ADP)-liposomes/PPP and the PRP group (Fig. 6A). We noted that the deceased rabbits showed severe anemia. In this study, to exclude the effects of allogeneic RBC transfusion, we utilized autologous blood. However, in clinical settings of acute hemorrhage, allogeneic transfusion of RBCs is the norm. Considering these issues, we will next study the hemostatic efficacy of H12-(ADP)-liposome on exsanguinating hemorrhage in rabbits with acute thrombocytopenia using allogeneic RBCs transfused from other rabbits.

In this study, bleeding was tamponaded immediately after the liver trauma and within 5 minutes, the artificial PLTs were transfused. These brief time periods might well have been required to assure sufficient survival of the experimental model, but certainly do not reflect clinical reality, especially on the battlefield. In conclusion, H12-(ADP)-liposomes might be a safe and effective therapeutic tool during damage control surgery for trauma patients with acute thrombocytopenia with massive bleeding.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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