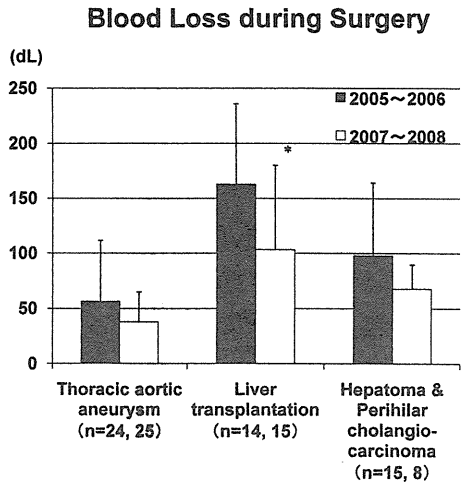

Fig. 2 Volume of blood loss during surgery in cases with massive hemorrhage (> 4,000m*l*) or massive transfusion (> RCC 20U). We performed a comparative analysis (2005 ～ 2006 vs. 2007 ～ 2008) in patients with thoracic aortic aneurysm (n = 24 in 2005 ～ 2006, n = 25 in 2007 ～ 2008), liver transplantation (n = 14 in 2005 ～ 2006, n = 15 in 2007 ～ 2008), and hepatoma/perihilar cholangiocarcinoma (n = 15 in 2005 ～ 2006, n = 8 in 2007 ～ 2008). Closed bars: cases in 2005 ～ 2006; Open bars: cases in 2007 ～ 2008. The data are presented as the mean ± SD. * $p < 0.05$ (by unpaired *t*-test).

に測定した症例（全83例中28例）の約90％で150 mg/d*l* 以下，約60％で100mg/d*l* 以下を示しており，極度の低フィブリノゲン血症が止血不全の要因であると考えられた．

次に，胸部大動脈瘤手術症例において術中の出血量増加時における止血凝固能の変化を評価するため，人工心肺離脱1時間前から1時間ごとに血小板数の測定および血液凝固検査を行った．術中に4,000m*l* を超える出血をきたした場合，ほとんどの症例で血小板数は50,000/μ*l* 以下に減少(13例中12例)，フィブリノゲン値は150mg/d*l* 以下に低下（13例中11例）しており，PTの最低値は平均で23.6％と著明な出血傾向を認めた．止血のために血小板製剤，新鮮凍結血漿の輸血が行われた，代表的な1症例の検査値の推移を示す(Fig. 1A)．大量の血小板製剤，新鮮凍結血漿の輸血にもかかわらず血小板数やフィブリノゲン値はすみやかに改善せず，良好な止血が得られずに長時間を止血に要した．一方，出血量の増加時にフィブリノゲン値の低下（150mg/d*l* 以下）を認めた際，クリオプレシピテート3パック(新鮮凍結血漿15単位分)もしくはフィブリノゲン濃縮製剤3gの投与を行った症例の検査値の推移を示す（Fig. 1B, C)．この治療によってフィブリノゲン値は60～100 mg/d*l* ほど上昇し，それとともに止血は良好となった．Fig. 1Cに示すように，フィブリノゲン値が一気に止血レベルまで上昇したことによりほぼ止血は達成され，さらなる出血量の増加を防ぐことができた．

このような新たな止血治療が，術中の出血量および輸血量に及ぼす影響について解析した．術中大量出血をきたしやすい代表的な手術である胸部大動脈瘤手術，肝臓移植術，肝臓癌・肝門部癌摘出術において，4,000 m*l* 以上の術中大量出血を認めたか，あるいは赤血球製剤20単位以上の大量輸血を行った症例を取り上げ，術中の止血治療としては新鮮凍結血漿の投与が一般的であった2005～2006年と，ほとんどのケースでクリオプレシピテートおよびフィブリノゲン濃縮製剤によるフィブリノゲン補充を行った2007年～2008年とで術中の平均出血量・輸血量を比較検討した．

まず術中出血量について見ると(Fig. 2)，2007～2008年の胸部大動脈瘤手術25例(うちクリオプレシピテートもしくはフィブリノゲン濃縮製剤投与症例は21例)の1例平均出血量は3,764m*l* で，2005～2006年（24例)の1例平均5,647m*l* と比べて約33％減少していた．同様に2007～2008年の肝臓移植術15例（うちフィブリノゲン濃縮製剤投与症例は14例)の平均出血量は10,330 m*l* で，2005～2006年（14例）の平均16,280m*l* と比べて37％の減少であり，肝臓癌・肝門部癌切除術では2007～2008年の8例（うちフィブリノゲン濃縮製剤投与症例は6例)の平均出血量が6,796m*l* で，2005～2006年（15例）の平均9,782m*l* と比べて31％の減少と，3つの手術とも顕著な減少を認めている．

上記の大量出血・大量輸血症例における術中輸血量について解析してみると（Fig. 3)，2007～2008年の胸部大動脈瘤手術25例での1例平均は赤血球製剤(RCC) 32.6単位，新鮮凍結血漿(FFP)46.1単位，血小板製剤(PC)37.2単位であり，1例当たりの血液製剤平均使用量は2005～2006年（24例）の平均（RCC 39.7単位，FFP 62.5単位，PC 47.7単位）と比べて23％の減少であった．肝臓移植術では2007～2008年（15例）の1例平均輸血量がRCC 18.8単位，FFP 16.1単位，PC 10.3単位であり，2005～2006年（14例）の平均（RCC 53.6単位，FFP 47.1単位，PC 34.6単位)と比べて1例当たり67％減と著明に減少していた．肝臓癌・肝門部癌摘出術においては2007～2008年（8例）の1例平均輸血量がRCC 16.5単位，FFP 25.3単位，PC 3.8単位であり，2005～2006年（15例）の平均（RCC 34.4単位，FFP 29.7単位，PC 15.3単位)と比べて1例当たり43％の減少であった．

2007年は術中のフィブリノゲン補充治療としてクリオプレシピテートもしくはフィブリノゲン濃縮製剤の投与を行ったが，クリオプレシピテート中に含まれるフィブリノゲン量を測定したところ，新鮮凍結血漿5単位製剤間でのばらつきが大きく（n＝10；平均値658 mg，最低値423mg，最高値749mg)，クリオプレシピ

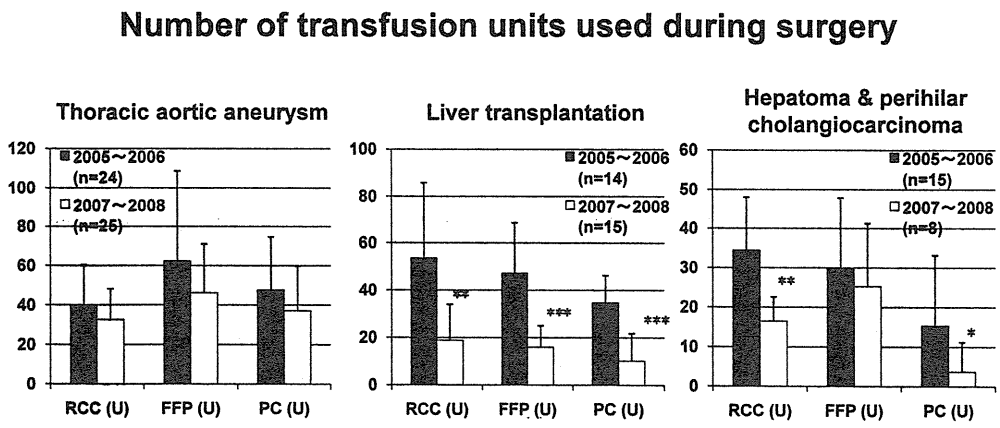

Fig. 3　Transfusion units during surgery in cases with massive hemorrhage (> 4,000m*l*) or massive transfusion (> RCC 20U). We performed a comparative analysis (2005～2006 vs. 2007 ～ 2008) in patients with thoracic aortic aneurysm (n = 24 and 25), liver transplantation (n = 14 and 15), and hepatoma/perihilar cholangiocarcinoma (n = 15 and 8). Closed bars: cases in 2005～2006; Open bars: cases in 2007～2008. The data are presented as the mean ± SD. *$p<0.05$; **$p<0.001$; ***$p<0.0001$ (by unpaired *t*-test).

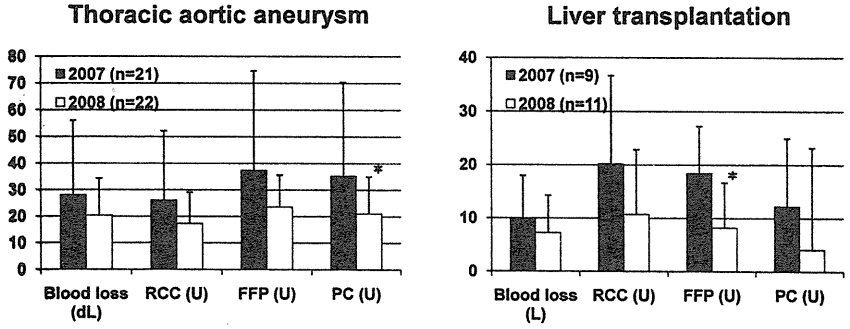

Fig. 4　Volume of blood loss and number of transfusion units during surgery in cases treated with fibrinogen concentrate. We performed a comparative analysis (2007 vs. 2008) in patients with thoracic aortic aneurysm (n = 21 and 22) and liver transplantation (n = 9 and 11). Closed bars: cases in 2007; Open bars: cases in 2008. The data are presented as the mean ± SD. * $p < 0.05$ (by unpaired *t*-test).

テート3パック（新鮮凍結血漿15単位分）の投与ではフィブリノゲン濃縮製剤3gの投与に比べて，血中フィブリノゲン値の上昇度が鈍い傾向を認めた．そこで2008年は術中の低フィブリノゲン血症に対し，原則としてフィブリノゲン濃縮製剤を投与することとした．術中の出血量が1,500～2,000m*l*に達した時点でただちにフィブリノゲン値を測定し，150mg/d*l*を下回っているか，それを下回る勢いで出血が続いていると判断された場合には，フィブリノゲン濃縮製剤3gの投与を行い，不十分なら再投与するという，より積極的なフィブリノゲン補充を行った．胸部外科手術および肝臓移植術の術中にフィブリノゲン濃縮製剤の投与を行った全症例における平均出血量・輸血量について，2007年と2008年の比較検討結果を示す（Fig. 4）．2008年の胸部外科手術22例（うち胸部大動脈瘤17例）での術中平均出血量は2,033m*l*で，2007年(21例，うち胸部大動脈瘤19例)の平均2,804m*l*と比べて27%の減少を認めた．1例当たりの平均輸血量は2008年がRCC 17.3単位，FFP 23.6単位，PC 21.1単位で，2007年(RCC 26.1単位，FFP 37.3単位，PC 35.2単位)と比べて37%の減少であった．一方，2008年の肝臓移植術11例での術中平均出血量は7,252m*l*で，2007年(9例)の平均9,873m*l*と比べて27%の減少であり，1例当たりの平均輸血量も2008年はRCC 10.7単位，FFP 8.2単位，PC 4.1単位で，2007年(RCC 20.2単位，FFP 18.4単位，PC 12.2単位)と比べて55%の著明な減少を認めた．なお，2008年にフィブリノゲン濃縮製剤を術中投与した全54例(肝癌，肝門部癌等の消化器外科症例，血管外科症例を含む）における本製剤の総投与量は253g（1例平均4.7g）であった．

最後に，術中のクリオプレシピテートおよびフィブリノゲン濃縮製剤投与が手術患者の予後に与える影響を検討した．術中の大量出血が原因と考えられた術後

2週間以内の早期死亡症例数は，2007年が1例（胸部解離性大動脈瘤），2008年は2例(胸腹部大動脈瘤，胆嚢癌)で，両製剤の投与を行っていなかった2006年までの4分の1程度に減少した．

考　察

本研究により，術中に大量出血をきたしやすい患者の基礎疾患および術式として，胸部大動脈瘤に対する人工血管置換術，肝臓移植術，肝臓癌・肝門部癌切除術が代表的なものであることが明らかとなった．その原因として，胸部大動脈瘤症例では瘤局所における線溶亢進を主体とした消費性凝固障害(サイレントDIC)および出血量増加による凝固因子の漏出，枯渇が考えられた．また肝硬変を背景とした肝臓癌および肝臓移植術症例では，血小板減少および凝固因子の産生低下と，臓器自体の易出血性が出血傾向をまねいていると推測された．術中の大量出血時に凝固検査が行われている症例は非常に少なく，大量出血時に起こる希釈性凝固障害の評価およびそれに対する適切な止血治療が行われているとは言い難い現状であることが明らかとなった．

術中大量出血をきたした症例では，フィブリノゲン値が150mg/dlを下回ってくるとoozingを主体とする全身性の出血傾向が現れ，外科的処置では止血不可能となる．血小板の凝集にはフィブリノゲンが必須であることからも，術中大量出血時に起こる希釈性凝固障害および止血不全の本態は高度な低フィブリノゲン血症であると考えられた．血液製剤の使用指針では，新鮮凍結血漿の投与基準はフィブリノゲン値100mg/dl以下とされているが，術中出血が持続している場合，検体採取時と検査結果確認時には30分～1時間近い時間差があると考えられ，術野における止血不全が始まっている時点でのフィブリノゲン値は150mg/dl前後であると予想される．したがって，実際の製剤投与時には測定結果よりさらに低フィブリノゲン血症が進行していると考えられるため，止血のためのフィブリノゲン補充治療はフィブリノゲン値100mg/dl以下での開始では遅く，150mg/dlを目安に行うのが適切であると言えよう．出血が持続している状況で血中フィブリノゲン値を一気に止血可能域（150～200mg/dl以上）に上げるためには，含有するフィブリノゲン濃度がけっして高くない上に溶解から投与完了まで長時間を要する新鮮凍結血漿の投与は不適切かつ不十分であり[4)5)]，フィブリノゲンを高濃度に含有する製剤を短時間で投与することが必要であると考えられた[6)7)]．肝移植症例では血中ADAMTS13が著減してlarge multimer vWFによる血小板血栓形成が臓器障害を招く恐れがあり，新鮮凍結血漿の投与が有益である可能性もあるが，術中の新鮮凍結血漿投与という短期的なADAMTS13補充が肝移植患者の術後予後に影響するかどうかについては，今後の検討を待つべきであろう．

クリオプレシピテートとフィブリノゲン濃縮製剤を比較すると，ウィルス不活化処理の有無，備蓄量の確保，フィブリノゲン含有量などから総合的に考えて，フィブリノゲン濃縮製剤の使用が推奨される．海外では術中の低フィブリノゲン血症に対し，米国ではクリオプレシピテートが[8)]，また欧州ではフィブリノゲン濃縮製剤の投与が推奨されており[9)10)]，いずれも供給体制が確立している．我が国でも一刻も早く，術中大量出血時の低フィブリノゲン血症に対してフィブリノゲン濃縮製剤が使用できるよう，厚生労働省および日本赤十字社へ働きかけが必要であると考える．

本研究により，術中の出血量増加時に適宜フィブリノゲン値を測定し，低フィブリノゲン血症に対してはクリオプレシピテートもしくはフィブリノゲン濃縮製剤を投与して積極的にフィブリノゲン補充を行うことが，止血のための輸血治療として非常に有効であることが明らかとなった．両製剤の使用は術中出血量・輸血量を大幅に減少させて手術患者の予後の改善に大きく寄与するとともに，血液製剤使用量の大幅な削減にも貢献しうると考えられる．

結　語

最後に，以下の治療指針を提言する．

「術中大量出血を防ぐ，止血のための輸血指針」

1. 術中大量出血をきたしやすい手術(胸部大動脈瘤に対する人工血管置換術，肝臓移植術，肝臓癌・肝門部癌切除術，産科緊急手術など）では，大量出血の可能性を常に念頭に置き，適宜，止血・凝固検査（血小板数，PT，APTT，フィブリノゲン値の測定)を行う．

2. 術中に循環血液量の50％を超える出血（1,500～2,000ml程度)を認めるか，それに迫る急激な出血が起こった場合，あるいはウージングのような限局しない出血傾向を認めた場合には，すみやかに血算・凝固検査を行う．(検査部は24時間の凝固検査体制を整えるとともに，手術室からの凝固検査用検体に対して，搬送および検査自体を最優先で迅速に対応する．)

3. 低フィブリノゲン血症（＜100～150mg/dl）を認めるか（製剤投与の頃には）それに近づくと判断した場合には，

①フィブリノゲン濃縮製剤（3g程度）

(それが入手できない場合には)

②クリオプレシピテート（3～4パック：新鮮凍結血漿15～20単位分，ABO同型かAB型)

の投与によって一気にフィブリノゲン値の上昇(＞150～200mg/dl）を図る．

（投与後にも適宜凝固検査を行い，不十分なら再投与する．）

4. 出血の速度が緩やかな場合には，新鮮凍結血漿での対応も可．

5. 血小板減少（<50,000/μl）に対しても，原則として低フィブリノゲン血症を改善させた上で適宜血小板輸血を行う．

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CLINICAL USE OF CRYOPRECIPITATE OR FIBRINOGEN CONCENTRATE TO PREVENT MASSIVE HEMORRHAGE DURING SURGERY

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Abstract:

Background: Massive hemorrhage during surgery often results from diluted coagulopathy due to loss of coagulation factors (e.g., fibrinogen), especially in cases of thoracic aortic aneurysm, liver transplantation, and hepatoma/perihilar cholangiocarcinoma. The most important issue in preventing massive hemorrhage during surgery is transfusion therapy for hemostasis. This study analyzed the hemostatic efficacy of cryoprecipitate or fibrinogen concentrate during surgery when massive bleeding occurred.

Patients and Methods: When massive hemorrhage occurred in cases of thoracic aortic aneurysm, liver transplantation, and hepatoma/perihilar cholangiocarcinoma, we measured the fibrinogen level in plasma, and administered cryoprecipitate or fibrinogen concentrate to the patient when the fibrinogen level was below 150 mg/dl (in 2007～2008). The hemostatic efficacy of this treatment was evaluated by counting the volume of blood loss and number of transfusion units in comparison with cases of treatment with fresh frozen plasma (in 2005～2006).

Results: We observed a rapid increase in plasma fibrinogen level and subsequent improvement in hemostasis after cryoprecipitate or fibrinogen concentrate was administered. The average blood loss decreased by 30% and the average number of transfusion units was reduced about 30% to 60% when those agents were given to patients with severe hypofibrinogenemia during surgery. The number of cases of early death due to massive hemorrhage during surgery decreased by 75% in 2007～2008 when fibrinogen concentrate was used.

Conclusion: In patients showing hypofibrinogenemia (i.e. <150 mg/dl) during surgery, administration of fibrinogen concentrate should be effective in establishing hemostatsis, and therefore in reducing blood loss and transfusion volume. This treatment should help to improve the prognosis of patients in surgery, and also to decrease the use of blood products.

Keywords:

massive hemorrhage, diluted coagulopathy, hypofibrinogenemia, thoracic aortic aneurysm, liver transplantation

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ORIGINAL ARTICLE *Inhibitors*

An analysis of factors affecting the incidence of inhibitor formation in patients with congenital haemophilia in Japan

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Summary. Studies conducted in European and North American countries have demonstrated that various factors including races affect the frequency of inhibitor formation in haemophilia patients. The present study was undertaken to analyse factors affecting the incidence of inhibitor formation in Japanese haemophilia A and B patients. Analytical data were retrospectively collected from haemophilia A and B patients born after 1988, the year when monoclonal antibody-purified factor VIII products were first marketed in Japan. Various data were collected from 184 patients (153 cases of haemophilia A; 31 cases of haemophilia B). The sample size of haemophilia B cases was too small to reveal any significant differences between the inhibitor formation group and the inhibitor-free group in any of background variables. For patients with haemophilia A, on the other hand, univariate analysis identified the severity of haemophilia and a positive family history of inhibitor development as risk factors for the formation of inhibitors. In analyses of the clotting factor products used, the incidence of inhibitor formation did not differ significantly between the group treated with plasma-derived products (29.7%) and the group treated with recombinant products (25.0%). When background variables were compared, age was higher in the group treated with plasma-derived products but none of the other background variables differed between the two groups. These results suggest that in Japanese haemophilia patients, the type of clotting factor preparations used for therapy has not influenced the incidence of inhibitor formation.

Keywords: coagulation products, haemophilia, incidence, inhibitor, risk factor

Introduction

Haemostatic treatment for patients with haemophilia has advanced considerably in the past two decades. Safe clotting factor concentrates with high haemostatic activity have become available, and increasing clinical evidence has been accumulated to confirm that regular prophylactic infusions of these products help to prevent the onset and progression of haemophilic arthropathy arising from repeated intra-articular bleeding. It is also evident, however, that the infusion of clotting factor products induce the formation of allo-antibodies (inhibitors), which inactivate factor VIII (or factor IX) in 20–30% of patients with severe haemophilia A and 3–5% of patients with severe haemophilia B. Conventional treatment protocols involving infusion of deficient clotting factors in these patients with inhibitors are poorly effective, and haemostatic control is often difficult in these circumstances, especially in patients with high-responding inhibitors. Moreover, quality of life (QOL) indices are reduced markedly in these patients [1].

Studies conducted in European and North American countries have demonstrated that various genetic factors affect the frequency of inhibitor formation. The most detailed analyses have examined the type and location of

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factor VIII gene mutations, and have revealed that the incidence of inhibitor formation was highest in haemophilia A patients with large deletions, nonsense mutations or inversions [2–9]. A higher incidence of inhibitor formation is known for Black patients [10], and a recent report suggested that differences in factor VIII haplotype contributed to this feature [11]. It is also known that within the same family some patients with haemophilia develop inhibitors whilst others do not, possibly reflecting the polymorphism of genes encoding immunomodulating cytokines [12–14] or indicating the influence of acquired factors [15–20]. With regard to the type of product used for therapy, close attention has been paid to the relationship between the incidence of inhibitor formation and the use of recombinant products or plasma-derived products [21–28].

The present study was undertaken to assess the incidence of inhibitors in patients born after 1988, the year when monoclonal antibody (mAb)-purified factor VIII products were first marketed in Japan. The analyses was restricted to patients that had been treated with clotting factor products for at least 2-years, with the goal of identifying background variables and focusing on whether or not the type of clotting factor products affected the incidence of inhibitor formation.

Methods

Selection of treatment centres included in the survey

Medical centres experienced in the treatment of haemophilia and with comprehensive records enabling a detailed investigation of the history of treatment in individual patients were invited to participate in this study.

Inclusion criteria

Patients with congenital haemophilia A or B satisfying the all following criteria were eligible to study:

1. Born between 1 January 1988 and 31 December 2006.
2. Data on the history of treatment with factor VIII (IX) products available for at least 2 years after the first infusion of clotting factor products.
3. Data on inhibitor assays available for at least 2 years after the first infusion.
4. Informed consent available in writing from the guardian (and also the patient aged 16 years and older).

Investigations

1. Background variables: name initials, gender, birth date, time (year and month) of diagnosis, factor VIII (IX) activity and blood group.
2. Complications: presence/absence of haemophilic arthropathy, hepatitis B, hepatitis C, HIV infection, haemorrhagic disease other than haemophilia and any other severe disease.
3. Neonatal history: manner of delivery and feeding method.
4. Family history: presence/absence of family history of haemophilia with or without inhibitor.
5. Treatment methods: age at the time of first infusion, and administration method of clotting factor products (on demand or regular prophylaxis).
6. Clotting factor products: the name of the clotting factor products and the number of days used during the 2-year period after the first infusion or before detection of inhibitor in patients showing inhibitor formation.
7. Severe bleeding episodes and invasive surgery: presence/absence and time of episode/surgery and the site of bleeding during the 2-year period after the first infusion.
8. Presence/absence of inhibitors: in patients showing inhibitor formation, the inhibitor level on detection, the date of measurement, peak inhibitor level and latest inhibitor level [presence/absence of immune tolerance induction (ITI) therapy if inhibitor had disappeared].

Survey period

The survey was conducted between 1 January 2008 and 31 December 2009.

Statistical analysis

The *t*-test and analysis of variance were used on numerical variables and chi-squared test was employed for nominal variables. In addition, multivariate analysis was conducted by means of logistic regression.

Ethical considerations

The study was conducted in accordance with the Japanese Ethical Guidelines on Epidemiological Studies after approval by the Nara Medical University Ethics Committee. In addition where necessary, the approval of the ethics committee of individual participating medical centres was also obtained.

Results

Analytical data were collected from 184 patients (153 cases of haemophilia A; 31 cases of haemophilia B). Of the 153 patients with haemophilia A, 41 (26.8%) developed inhibitors. In 29 of these 41 patients (70.7%), including four patients who had not received ITI, inhibitors had disappeared by the time of last evaluation. On the other hand inhibitors were persistent

in 12 patients including 10 patients who had received ITI.

In univariate analyses, the background variables found to differ significantly between the inhibitor formation group and the inhibitor-free group were: the severity of haemophilia, family history of haemophilia patients with inhibitor (Table 1), age at the time of the first infusion of clotting factor products (Fig. 1) and factor VIII activity (Fig. 2). No other background variable differed significantly between the two groups (Table 1), including blood group and presence/absence of hepatitis A, hepatitis B and HIV infection. Logistic regression analyses of these variables demonstrated that only the family history of haemophilia patients with inhibitor had a significant influence on inhibitor formation ($P = 0.002$).

One hundred and fifty patients, excluding three haemophilia A patients who had received treatment with factor IX products, were classified into three groups according to the type of clotting factor product used during the 2-year period after the first infusion, or before the detection of inhibitor: (i) patients treated with plasma-derived products alone ($n = 37$), (ii) patients treated with recombinant products alone ($n = 104$), and (iii) patients treated with both plasma-derived products and recombinant products ($n = 9$). The incidence of inhibitor formation did not differ significantly among these three groups (29.7%, 25.0% and 22.2% respectively). Of the 46 patients who received plasma-derived products, nine received only factor VIII products containing von Willebrand factor. Among these nine patients, inhibitor formation was seen in four patients, but the incidence for this group did not differ significantly from that for the other groups. We also compared the peak inhibitor level, the status of inhibitor formation at the time of last observation and the response rate to ITI between the group treated with plasma-derived products alone and the group treated with recombinant products alone. These comparisons revealed no inter-group differences (Table 2). Furthermore, other background variables were compared in these two groups i.e. age, severity of haemophilia, family history of inhibitor, treatment method (on demand or regular prophylaxis) during the 2-year period after the first infusion, the presence/absence of intracranial haemorrhage, other severe bleeding episodes and invasive surgery. The average age of the patients at the end of the survey (31 December 2009) was significantly higher in the group treated with plasma-derived products alone than in the group treated with recombinant products alone, but no other background variable differed significantly between the two groups (Table 3).

Among the 31 patients with haemophilia B, six (19.4%) developed inhibitors. As in the haemophilia A patients, background variables in those haemophilia B patients who developed inhibitors were compared with those that remained inhibitor-free. Univariate analyses indicated that there were no significant inter-group differences in any of the background variables, possibly attributable to some extent to the small sample size.

Table 1. Background variables compared between inhibitor formation cases and inhibitor-free cases with haemophilia A.

Background variable (number of patients)	Inhibitor-free (%)	Inhibitor positive (%)	*P*-value
Severity ($n = 153$)			
Mild	17/112 (15.2)	0/41 (0)	0.0243
Moderate	13/112 (11.6)	4/41 (9.8)	
Severe	82/112 (73.2)	37/41 (90.2)	
Haemophilic arthropathy ($n = 153$)			
Present	25/112 (22.3)	14/41 (34.1)	0.2044
Family history of haemophilia ($n = 148$)			
Positive	46/108 (42.6)	21/40 (52.5)	0.3736
Family history of inhibitor formation ($n = 122$)			
Positive	3/91 (3.3)	9/31 (29.0)	0.0001
Treatment after first infusion ($n = 151$)*			
On demand	78/111 (70.3)	30/40 (75.0)	0.7255
Regular prophylaxis	33/111 (29.7)	10/40 (25.0)	
History of intracranial haemorrhage ($n = 149$)*			
Positive	11/108 (10.2)	8/41 (19.5)	0.2160
History of other severe bleeding ($n = 147$)*			
Positive	8/106 (7.5)	1/41 (2.4)	0.4384
History of invasive surgery ($n = 148$)*			
Positive	9/107 (8.4)	1/41 (2.4)	0.3577
Manner of child delivery ($n = 109$)			
Transvaginal	67/79 (84.8)	27/30 (90.0)	0.7198
Caesarean section	12/79 (15.2)	3/30 (10.0)	
Feeding method ($n = 94$)			
Breast-feeding	26/66 (39.4)	15/28 (53.6)	0.4470
Bottle-feeding	15/66 (22.7)	5/28 (17.9)	
Mixed	25/66 (37.9)	8/28 (28.6)	

*Before detection of inhibitor in inhibitor formation cases and for a 2-year period after the first infusion in inhibitor-free cases.

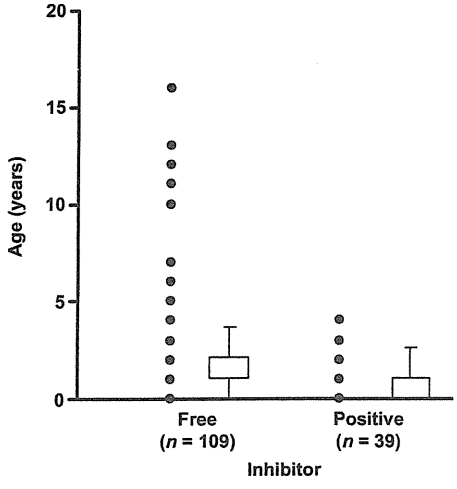

Fig. 1. Age at the time of first administration of clotting factor product (haemophilia A). Age at the time of the first infusion of clotting factor VIII products was significantly earlier in the inhibitor formation group than in the inhibitor-free group ($P < 0.001$).

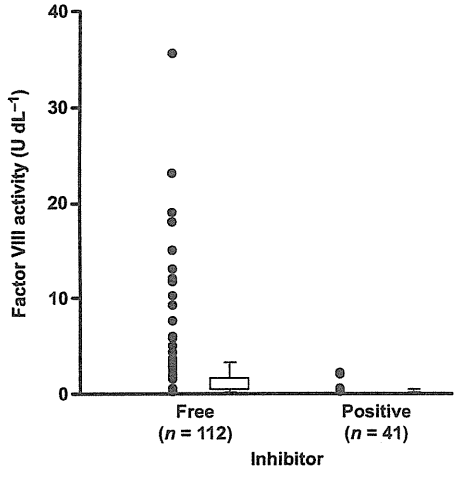

Fig. 2. Factor VIII activity. Factor VIII activity was significantly higher in the inhibitor-free group than in the inhibitor formation group ($P < 0.001$).

Discussion

Of the possible factors affecting the incidence of inhibitor formation in haemophilia patients, the type of factor VIII and IX products used for therapy has been attracting the closest attention. In particular, a retrospective study in France suggested that the incidence of inhibitor formation was lower in haemophilia A patients treated with single plasma-derived products than in patients treated with recombinant products [23]. Subsequently, however, the CANAL study (Concerted Action on Neutralizing Antibodies in severe haemophilia A) indicated that the incidence of inhibitor formation did not differ between patients treated with plasma-derived products (including products containing von Willebrand factor) and patients treated with recombinant products [26], and this result was consistent with a report published at about the same time in the United Kingdom [28]. All of these findings were derived from studies conducted in European and North American countries, primarily involving caucasians. It is known, however, that the formation of inhibitors following treatment with clotting factor products varies between different ethnic populations [10]. The present investigation was conducted, therefore, in Japanese haemophilia patients. The study was retrospective and

Table 2. Status of inhibitor formation in relation to the type of clotting factor products used during the 2-year period after the first infusion in inhibitor-free cases, or before detection of inhibitor in inhibitor formation cases.

Status of inhibitor formation	Plasma-derived products alone $n = 37$ (%)	Recombinant products alone $n = 104$ (%)	*P*-value
Number of inhibitor formation cases	11 (29.7)	26 (25.0)	0.7308
Peak inhibitor level			
≤5 BU mL^{-1}	2 (18.2)	7 (26.9)	0.8829
>5 BU mL^{-1}	9 (81.8)	19 (73.1)	
Status at the time of survey			
Present	4 (36.4)	7 (26.9)	0.8565
Disappeared	7 (63.6)	19 (73.1)	
Outcome of ITI			
Successful	6 (66.7)	17 (73.9)	0.9194
Ongoing	2 (22.2)	4 (23.5)	
Failure	1 (11.1)	2 (8.6)	
Cases not having received ITI among inhibitor disappearing cases	1 (14.3)	2 (10.5)	–

Table 3. Background variables in relation to the type of clotting factor products used during the 2-year period after the first infusion in inhibitor-free cases, or before detection of inhibitor in inhibitor formation cases.

Background variable	Plasma-derived products alone $n = 37$ (%)	Recombinant products alone $n = 104$ (%)	*P*-value
Age (years)*	14.0 ± 5.6	9.7 ± 4.3	0.0491
Severity			
Mild	4 (10.8)	12 (11.5)	0.3342
Moderate	2 (5.4)	15 (14.4)	
Severe	31 (83.8)	77 (74.0)	
Family history of haemophilia†			
Positive	20 (55.6)	42 (42.0)	0.2281
Negative	16 (44.4)	58 (58.0)	
Family history of inhibitor formation†			
Positive	1 (4.2)	8 (9.1)	0.7166
Negative	23 (95.8)	80 (90.9)	
Treatment during the 2-year period after first infusion†			
On demand	29 (78.4)	71 (68.9)	0.3795
Regular prophylaxis	8 (21.6)	32 (31.1)	
Intracranial haemorrhage†			
Present	7 (19.4)	11 (10.9)	0.3091
Absent	29 (80.6)	90 (89.1)	
Other severe bleeding†			
Present	1 (2.9)	6 (6.0)	0.7804
Absent	34 (97.1)	94 (94.0)	
Invasive surgery†			
Present	1 (2.8)	8 (8.0)	0.4903
Absent	35 (97.2)	92 (92.0)	

*Age as of 31 December 2009.
†Excluding cases where data are unavailable.

was designed to include only those patients in whom inhibitor status could be checked for at least 2 years after the first infusion of clotting factor product. In spite of this limitation, the results on our relatively large number of patients born after 1988 (the year when mAb-purified factor VIII products were first marketed in Japan) were consistent with those of the CANAL studies. No differences in the incidence of inhibitor formation were revealed between the Japanese patients treated with plasma-derived products alone and those treated with recombinant products alone. Genetic analyses of haemophilia patients are available at some specialized facilities in Japan, but data of this nature was not comprehensively available for the present study and was not included. The incidence of inhibitor formation appeared to be very high, however, in patients from pedigrees where different family members were inhibitor positive compared to pedigrees where there was no family history of an inhibitor. This finding might have reflected an influence of gene mutation.

In the CANAL study, intensive treatment with clotting factor products at an early age appeared to be closely associated with the incidence of inhibitor formation [20], and we attempted to investigate the influence of high-dose therapy on inhibitor formation by analysing the incidence of inhibitors in patients treated for intracranial haemorrhage, other severe bleeding episodes and invasive surgery within the 2-year period prior to the detection of inhibitor. In these analyses, previous treatment for intracranial haemorrhage had been recorded approximately twice as often in the inhibitor group than in the inhibitor-free cases, although the differences were not statistically significant. In addition, the incidence of inhibitor formation did not differ between the on-demand-treated group and the group receiving regular prophylactic administration of clotting factors.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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ORIGINAL ARTICLE

Life-threatening hemorrhage and prolonged wound healing are remarkable phenotypes manifested by complete plasminogen activator inhibitor-1 deficiency in humans

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Summary. *Background:* Plasminogen activator inhibitor-1 (PAI-1) is the primary physiological regulator of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) activity. A number of studies have shown that elevated levels of PAI-1 are related to pathological states such as an increased risk of arterial thrombotic events and a poor prognosis for cancer patients; however, there are few reports about PAI-1 deficiency in humans because the disorder is very rare. *Objective:* To understand the *in vivo* impact of a complete PAI-1 deficiency, *Serpine1*$^{-/-}$ mice were generated; a number of *in vivo* studies have been conducted to elucidate the function of PAI-1 using *Serpine1*$^{-/-}$ mice. The phenotypes demonstrated in *Serpine1*$^{-/-}$ mice, however, were quite different from those in humans. Therefore, it is necessary to find out and analyze *SERPINE1* deficiency in humans. *Patient and methods:* The patient is a 47-year-old woman who has had multiple episodes of major bleeding. Although most of the patient's blood coagulation factors were functionally normal, her PAI-1 antigen levels were undetectable. Therefore, DNA sequencing of the *SERPINE1* gene were analyzed. *Results:* The proband had a homozygous 1-bp duplication (C) at exon 3 (c.356dupC; p.Ile120AspfsX42). Both wild-type PAI-1 (42.7 kDa) and mutated (Mut) PAI-1 (14.7 kDa) were expressed in COS-1 cells, although the level of Mut PAI-1 expressed in the cell lysates was much lower. Wild-type PAI-1 was observed in the culture supernatant, whereas no Mut PAI-1 was detected in the supernatant. *Conclusions:* Considering the results of the present study, the translation of mouse studies to humans must be performed with great care.

Keywords: bleeding tendency, gene deficiencies, hypermenorrhea, impaired wound healing, plasminogen activator inhibitor-1, Serpine1.

Introduction

The conversion of plasminogen to its active form plasmin is mediated by the proteolytic action of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA); plasmin, in turn, brings about the proteolytic degradation of fibrin clots [1]. Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor (SERPIN) superfamily and is the primary physiological regulator of uPA and tPA activity [2]. A number of studies have shown that elevated levels of PAI-1 are related to pathologies such as an increased risk of arterial thrombotic events [3] and a poor prognosis for cancer patients [4]. There are few reports on PAI-1 deficiencies in humans as the disease is rare, and there is only one report on total PAI-1 deficiency which is genetically identified in humans [5].

To understand the *in vivo* impact of a complete PAI-1 deficiency on various physiologic and pathophysiologic states, *Serpine1*$^{-/-}$ mice were generated [6]. After initial characterization of the deficient mice, it was found that the mice were not only viable, but able to reproduce. Moreover, they did not show any tendency to bleed. These results were very different from those observed in the human case of total PAI-1 deficiency [5], where severe bleeding was observed. Although

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a number of *in vivo* studies have been conducted to elucidate the function of PAI-1 using *Serpine1*$^{-/-}$ mice, such a difference in their fundamental phenotypes makes it difficult to simply apply those results to humans. Here, we report a new case of a genetically characterized, PAI-1-deficient Japanese subject, and discuss the differences observed in PAI-1 deficiencies, as well as differences in the underlying mechanisms, in humans and mice.

Case report

The family background of the proband is shown in Fig. 1. The patient is a 47-year-old woman who has had multiple episodes of major bleeding; omphalorrhagia at birth, postoperative bleeding (after an operation for a ventricular septum defect patch) at the age of 5, and prolonged bleeding from the gingiva after tooth extractions at the age of 10. At age 15, she had her first menstruation and experienced a massive, life-threatening hemorrhage (she lost more than 6 L of blood). All bleeding episodes were characterized by delayed, prolonged bleeding; an initial bleed would stop, only to reinitiate 3–4 days later. This pattern was inconsistent with the early phase bleeding observed with afibrinogenemia, von Willebrand disease (VWD), hemophilia and factor (F)XIII deficiency [7–9]. The patient's surgical incisions were closed after suturing, but completely reopened at the time of suture removal 7–10 days later, based on the fact we thought that the problem was wound healing rather than a coagulation disorder. Blood transfusions were required for all these episodes. After the first menstruation, an oestrogen/progesterone therapy was instituted to simulate pregnancy and prevent menses; however, as bleeding at least twice a year is necessary for healthy uterine and ovarian growth, she was hospitalized during the spring and summer vacations to induce menses. During these hospitalizations, the patient required up to 10 L of blood to compensate for her blood loss. Intensive screening was performed to detect any malfunction of blood coagulation factors, but none were detected; the prothrombin time (PT), activated partial thromboplastin time (APTT), platelet count, platelet function, and plasma levels of fibrinogen, VWF, α2 antiplasmin, and coagulation factors VIII, IX, and XIII were functionally normal determined by clot-time-based assays.

Two decades later, we found that her FXIIa was slightly decreased although her APTT was normal. We investigated the relationship between FXIIa and PAI-1 inactivation [10] and measured the euglobulin clot lysis time (ECLT), which is known to reflect PAI-1 activity and is modified by FXIIa, and found that the ECLT was remarkably shortened. Furthermore, supplementation with either calcium ion or kaolin, which are known to neutralize PAI-1 and shorten ECLT [10,11], did not drastically shorten ECLT; in fact the addition of calcium ion prolonged it. These results suggested a PAI-1 malfunction and prompted us to analyze her PAI-1 levels and the genetic structure of *SERPINE1*.

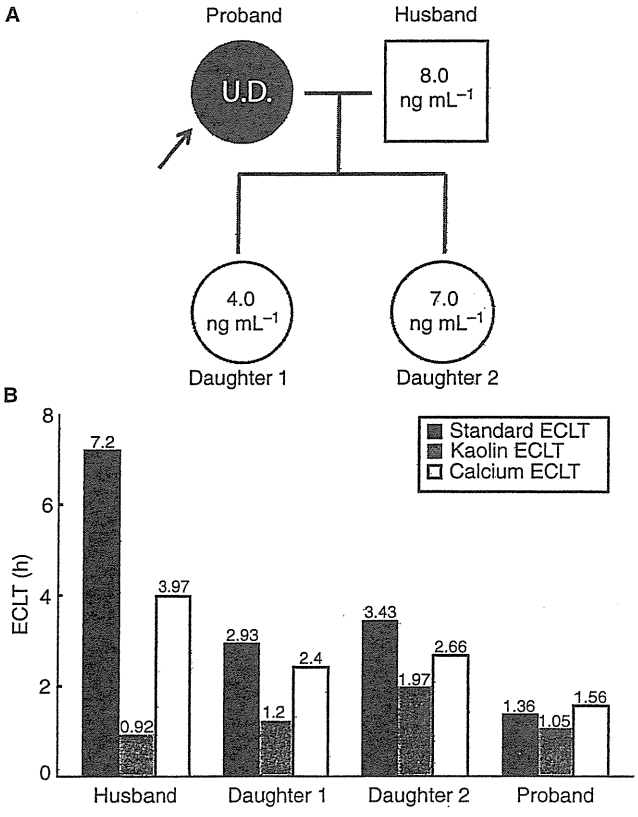

Fig. 1. Lineage of the proband and levels of plasminogen activator inhibitor-1 (PAI-1) antigen (A) and euglobulin clot lysis time (ECLT) (B). PAI-1 levels in the proband were undetectable (UD).

Materials and methods

Sample preparations

The ethical committee of the university hospital gave consent for the use of all human tissues in the present study, and informed consent was obtained from all human subjects. Peripheral blood from the patient and her family members was withdrawn by venipuncture. After the separation of plasma from the whole blood, genomic DNA was extracted from leukocytes with the QIAamp DNA Blood Mini kit (Qiagen, Tokyo, Japan), and total RNA was extracted from platelets using TRIzol (Invitrogen, Tokyo, Japan) according to the manufactures' instructions.

Cutaneous biopsy samples were taken from the ventral regions of three patients, including the proband, who had undergone trans-abdominal operations. These tissues were utilized for total RNA extraction.

Plasma assays

Plasma levels of total PAI-1 antigen were determined by Latex Photometric Immunoassay (Mitsubishi Chemical Medience, Tokyo, Japan). The ECLT assay was conducted using microtiter plates either in the presence or in the absence of calcium ion and kaolin, as previously described [12].

PCR and RT-PCR followed by DNA sequencing

PCR and RT-PCR were carried out with Prime Script II reverse transcriptase and Prime STAR HS DNA polymerase (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The primer sets for *SERPINE1* are summarized in Table 1. PCR and RT-PCR were performed using fibronectin (*FN1*) and all variants of fibronectin, vitronectin (*VTN*), and integrin α1, α2, α3, α4, α5, α6, β1, and β3 (*ITGA1*, *ITGA2*, *ITGA3*, *ITGA4*, *ITGA5*, *ITGA6*, *ITGB1* and *ITGB3*, respectively). Information on primers for these genes is available upon request. All PCR and RT-PCR products were sequenced using ABI Prism3730 (Life Technologies Japan, Tokyo, Japan).

Expression and characterization of mutant PAI-1

A human wild-type (WT) *SERPINE1* cDNA was synthesized by RT-PCR using total RNA extracted from normal human hepatocytes (Lonza, Walkersville, MD, USA) with primers hSERPINE1.EF and hSEPRINE1.ER (Table 1). The 1230-bp PCR amplicon and the pcDNA3.1 plasmid (Invitrogen, Tokyo, Japan) were digested separately with restriction enzymes *Hin*dIII (NEB Japan, Tokyo, Japan) and *Eco*RI (NEB Japan). This was followed by ligation of the digested fragment into the pcDNA3.1 vector, which generated the human WT *SERPINE1* expression vector pcDNA3.1-hSERPINE1-Wt. The *SERPINE1* mutation was introduced using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, La Jolla, CA, USA) according to the manufacturer's instructions with hSERPINE1.mutF and hSEPRINE1.mutR (Table 1) primers, which generated the human mutated *SERPINE1* expression vector pcDNA3.1-hSERPINE1-Mut.

These plasmids were transfected into COS-1 cells using Lipofectoamine2000 (Invitrogen, Japan) according to the manufacturer's instructions. The cell lysates and culture supernatant were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel and transferred to a poly(vinylidene difluoride) (PVDF) membrane (Immobilo Western; Nihon Millipore, Tokyo, Japan) for western blot. The membrane was exposed to a rabbit anti-PAI-1 polyclonal antibody (Cat. no. SC-8979; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a goat anti-rabbit IgG-HRP conjugate (Cell Signaling Technology Japan, Japan), and visualized with a SuperSignal West Pico kit (Thermo Fisher Scientific, Kanagawa, Japan) according to the manufacturer's instructions.

Results

Plasma assays

The total plasma levels of PAI-1 antigen in the proband were undetectable (Fig. 1A), whereas it was 8.0 ng mL^{-1} in her husband (Fig. 1A), and 4.0 and 7.0 ng mL^{-1} in her daughters (Fig. 1A). The average ECLT value for the proband was

Table 1 Sequences of primers used for PCR, RT-PCR, and mutagenesis

Name	Type	Sequence	Exon	Size
For PCR				
hSERPINE1.5'F	F	5'-CAAGGCTATTGGGGTTTGCTC	1	453
hSERPINE1.I1R	R	5'-GAGATTCTGGGGAGGCGAGT		
hSERPINE1.I1F	F	5'-TTGCAGGAAACAAGAAGAGCA	2	457
hSERPINE1.I2R	R	5'-TAAGCCAGAGCCAGCCTTTC		
hSERPINE1.I2F	F	5'-AGAGTGGAGCCCCTTGTGG	3	371
hSERPINE1.I3R	R	5'-CTCTTGAGGCCCAGGAAATG		
hSERPINE1.I3F	F	5'-CCAGCCTGGCAATAGAGTGA	4	348
hSERPINE1.I4R	R	5'-AGGAATGAGATGCAGTTGCTG		
hSERPINE1.I4F	F	5'-AGTGCTGGGTTGCCATCAG	5	313
hSERPINE1.I5R	R	5'-AGCCAGGAGAGAGGGGTTG		
hSERPINE1.I5F	F	5'-TAGGGGATGGGGAAAGGTG	6+7	433
hSERPINE1.I7R	R	5'-CAGTTATCCTGAAGGGCGATG		
hSERPINE1.I7F	F	5'-CCAGTGCCAGCATCCCTCT	8	199
hSERPINE1.I8R	R	5'-GTCGGGACTAGTTCCTGAGAGAAA		
hSERPINE1.I8F	F	5'-GCCTGATGAGCTGTCCCACT	9	484
hSERPINE1.E9R	R	5'-AAAGCTCCTGTAAGCCCCGTA		
hSERPINE1.E9F	F	5'-TTATGGGAGAATTGCACACAGATG	9	403
hSERPINE1.3'R	R	5'-TCCACCCACCTCGGCTTC		
For RT-PCR				
hSERPINE1.E2F	F	5'-GAGGGTGTTTCAGCAGGTGG	2+3+4	531
hSERPINE1.E4R	R	5'-CCGTCTGATTTGTGGAAGAGG		
For expression vectors				
hSERPINE1.EF	F	5'-GGGAAGCTTCAGGATGCAGATGTCTC-CAGCCCTC (AAGCTT; *Hin*dIII site)		1230
hSERPINE1.ER	R	5'-GGGAATTCTCAGGGTTCCATCACTTG-GCCCAT(GAATTC; *Eco*RI site)		
hSERPINE1.mutF	F	5'-GATCAGCACCACAGACGCCGATCTT-CGTCCAGCGGG (CC; the mutation)		
hSERPINE1.mutR	R	5'-CGCTGGACGAAGATCGGCGTCTGTG-GTGCTGATCTC (GG; the mutation)		

1.36 h, which was extremely shortened. ECLT values in the presence of calcium ion and kaolin were 1.56 and 1.05 h, respectively. The value for the husband was 7.20 h, and was significantly shortened by either calcium ion or kaolin, whereas those of the daughters were intermediate at 2.93 and 3.43 h, and moderately shortened by the addition of calcium or kaolin (Fig. 1B). Unfortunately, plasma from the proband's parents was not available for these assays.

Genetic analysis

The promoter, entire coding sequence and all exon–intron junctions of *SERPINE1* from genomic DNA were amplified by PCR. All PCR primer sets listed in Table 1 were functional, and the amplicons were successfully obtained from samples from the proband, her husband, her parents and her daughters (data not shown). Sequence analysis with the forward primer (hSERPINE1.I2F) revealed that a homozygous 1-bp duplication (C) at exon 3 (c.356dupC) existed in the proband (Fig. 2B). This mutation results in a frame shift change with Ile-120 as the first affected amino acid, changing it to an Asp and creating a new reading frame ending in a stop at position 42 (counting starts with Asp as amino acid 1) (p.Ile120AspfsX42; Fig. 4B). This mutation was also verified using the reverse primer, hSERPINE1.I3R (data not shown). This mutation was not present in the husband; however, the daughters (Fig. 2B), as well as her parents (data not shown), were heterozygous for the mutation.

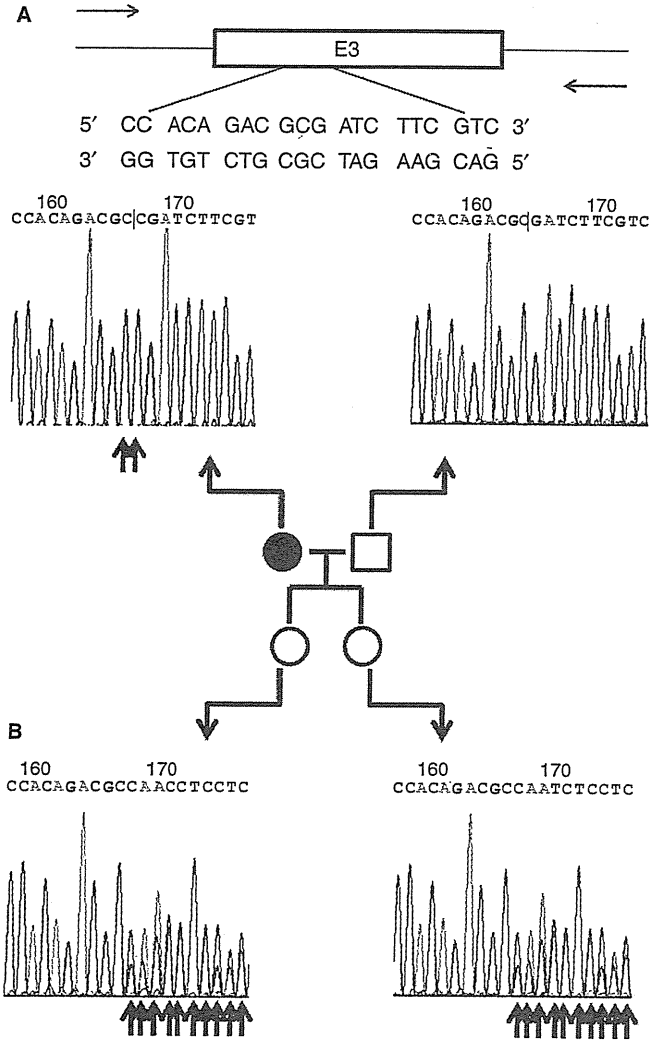

Fig. 2. Identification of the mutation in the genomic DNA. (A) Schema of exon 3 and adjacent introns. Blue and red arrows indicate the forward primer (hSERPINE1.I2F) and reverse primer (hSERPINE1.I3R), respectively. (B) The DNA sequence of the proband and her family. Duplication of 'C' (red arrows) was observed in the proband, but it was not detected in her husband. The daughters were heterozygous (black arrows).

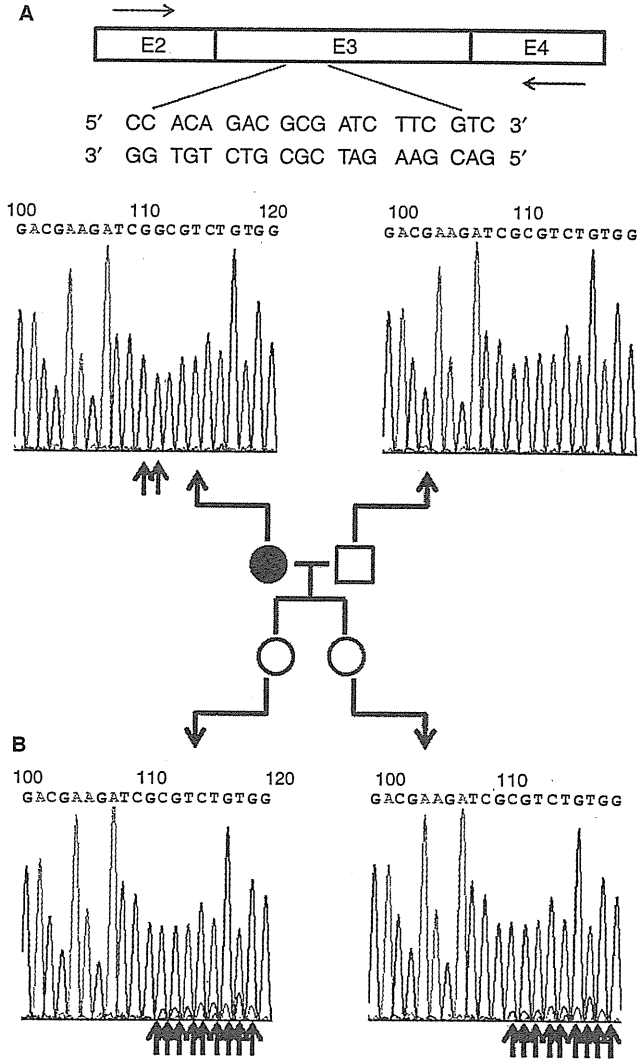

Fig. 3. Identification of the mutation in the cDNA. (A) Schema of exon 3 and adjacent exons. Blue and red arrows indicate the forward primer (hSERPINE1.E2F) and reverse primer (hSERPINE1.E4R), respectively. (B) The DNA sequence of the proband and her family. Duplication of 'G' (red arrows) was observed in the proband, but it was not detected in her husband. Weak overlapping signals were observed for her daughters (black arrows). Note: Background noise was noted with the use of the forward primer when it was used for sequencing. Therefore, the results obtained with the reverse primer are presented herein.

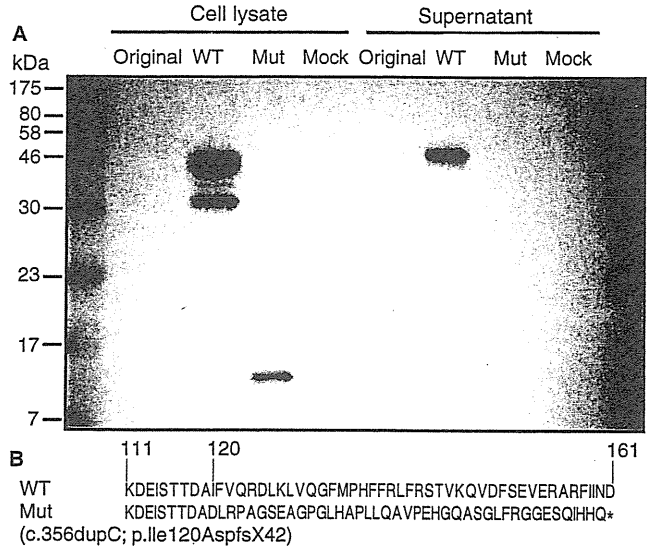

Fig. 4. Characterization of mutant plasminogen activator inhibitor-1 (PAI-1). (A) Recombinant wild-type (WT) and mutated PAI-1 (Mut) expression in COS-1 cells. Non-transfected cells (Original) and cells transfected with pcDNA3.1 (mock) did not produce human PAI-1. WT and Mut PAI-1 were expressed in the cells, although the amounts of Mut were much smaller. However, Mut PAI-1 was not secreted. (B) Deduced amino acid sequence of WT and Mut PAI-1 from 111 to 161.

The *SERPINE1* cDNA encoding exon 3 as well as parts of exons 2 and 4 was synthesized by RT-PCR using hSERPINE1.E2F and hSERPINE1.E4R. The amplicon was successfully obtained from the proband, the husband and the daughters, although the intensity of the band from the proband was much fainter than those of the husband and daughters (data not shown). Sequence analysis with the reverse primer (hSERPINE1.E4R) revealed an identical homozygous 1-bp duplication (C) at exon 3 in the proband (Fig. 3B). As mentioned before, the husband did not have this mutation (Fig. 3B), but sequence data from the daughters clearly indicated that there was transcription from a mutated allele; however, the amounts of mRNA were very small (Fig. 3B). These results imply that the mutated mRNA was very unstable *in vivo*. These findings were also confirmed using the forward primer, hSERPINE1.E2F (data not shown).

Recombinant wild-type and mutated PAI-1

In order to characterize the function of the mutated gene, both wild-type and mutated *SERPINE1* cDNA were inserted into the mammalian expression plasmid pcDNA3.1, and transfected into COS1 cells. Both wild-type PAI-1 (42.7 kDa) and mutated (Mut) PAI-1 (14.7 kDa) were expressed in the cells, although the level of Mut PAI-1 expressed in the cell lysates was much lower (Fig. 4). Wild-type PAI-1 was observed in the culture supernatant of wild-type *SERPINE1*-transfected COS-1 cells, whereas no Mut PAI-1 was detected in supernatant from Mut *SERPINE1*-transfected COS-1 cells (Fig. 4).

Sequence analysis of other genes related to wound healing

Although the recovery time from both spontaneous wounds and surgical incisions was extremely prolonged in the proband, there were no mutations in the genes related to wound healing such as *FN1* and *VTN* as well as related integrins (data not shown). These results indicate that the lack of PAI-1 might be associated with the delayed wound healing.

Discussion

Our research has focused on the investigation of congenital bleeding disorders caused by deficiencies in coagulation factors such as fibrinogen [13], FVIII [14], FIX [15,16], FXIII [17,18] and VWF [19]. In our experience, the bleeding experienced by the proband was not similar to that observed in afibrinogenemia [20] and FXIII deficiency [21], although there was some overlap in symptoms. In fact, we found no functional abnormalities in any of the coagulation factors listed above.

In the past, while investigating recurrent miscarriages in women deficient in fibrinogen and FXIII, we have demonstrated the importance of these two factors in wound healing [17,22–24]. As the proband's surgical incision failed to repair, we investigated genes related to wound healing such as *FN1* and *VTN*, as well as their receptors *ITGA1*, *ITGA2*, *ITGA3*, *ITGA4*, *ITGA5*, *ITGA6*, *ITGB1* and *ITGB3*. After intensive screening of these genes, we found that all of them were fully functional.

Our investigations finally revealed that the proband had a PAI-1 deficiency caused by a previously undescribed homozygous frame shift mutation that resulted in the formation of a premature stop codon. The mutated mRNA was very fragile, the mutated protein was barely expressed and the protein was never secreted from the cells. This mutation was inherited from her parents who both possessed the same heterozygous mutation and was transmitted to her daughters, which confirms germline transmission. Interestingly, interviews with the family revealed no evidence of consanguineous marriage within a third degree relationship.

The phenotype of this patient was similar to those in previous reports on human PAI-1 deficiency, but there were also certain unique characteristics in this case; the severe bleeding was coupled with impaired wound healing.

Severe abnormal bleeding after surgery and during menstruation was the most critical and life-threatening symptom in the proband, which was also reported in a previous case of human PAI-1 deficiency [25]. We did not, however, observe it in *Serpine1*$^{-/-}$ mice, where abnormal bleeding was not observed even after partial amputation of the caecum or tail. However, cardiac fibrosis has been documented in old *Serpine1*$^{-/-}$ mice [26–28], which is also found in young mice with low levels of FVII [29] and mice with low levels of tissue factor [30]. It is therefore possible that the *Serpine1*$^{-/-}$ mice might have as yet unidentified bleeding disorders. Moreover, the expression pattern of PAI-1 in humans and mice is quite different. For example, human PAI-1 is expressed *in utero* (http://

www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.414795), whereas in mice it is not (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.250422). This difference in the expression pattern of PAI-1 *in utero* could explain the large differences in genital bleeding.

PAI-1 indirectly inhibits plasmin via inactivation of uPA and tPA, whereas α2 antiplasmin, which is also a member of the SERPIN superfamily, inhibits plasmin directly. The symptoms of plasminogen deficiency in mice [31,32] are similar to those observed in humans [33]; however, α2 antiplasmin-deficient mice (*Serpinf1*$^{-/-}$) did not present with the bleeding tendencies [34] that are typically observed in humans [35]. Moreover, doubly deficient mice (*Serpine1*$^{-/-}$/*Serpinf1*$^{-/-}$) also do not show a tendency to bleed spontaneously [36]. This could possibly be explained by the presence of additional antifibrinolytic mechanisms; alternatively, it could be because antifibrinolytic reactions triggered by PAI-1 and/or α2 antiplasmin in mice are weaker than those in humans. Furthermore, the proband experienced excessive menstrual bleeding; because genital bleeding does not occur in mice, it was difficult to demonstrate this in the *Serpine1*$^{-/-}$ mice. Thus, even although humans and mice might have the same PAI-1 deficiency, the physiological differences should be carefully considered. Moreover, not only internal differences between humans and mice, but external differences should be evaluated for such comparisons. For example, although it is not typically considered, we need to evaluate the living conditions of mice; experimental mice are bred in cages, whereas humans face daily injuries and occasional surgeries. Thus, these different lifestyles might result in significantly different hemostatic parameters.

The impaired wound healing experienced by this proband was not reported in the earlier case of human complete PAI-1 deficiency [5]. Conflicting results have also been reported in wound healing studies using *Serpine1*$^{-/-}$ mice [37]. Moreover, wound healing was shown to be significantly accelerated in *Serpine1*$^{-/-}$ mice with a skin wound healing model [38], and Carmeliet *et al.* [39] reported that *Serpine1*$^{-/-}$ mice showed better vascular wound healing using a neointima formation model. However, even in the *Serpine1*$^{-/-}$ mouse model opposite results were reported; the vascular repair process, including neointima formation and neovascularization, appeared to be impaired after ferric chloride injury [40]. A critical feature of these studies seems to be the difference in the experimental models, and the magnitude of involvement of the thrombus/fibrin and their stability in wounds, both of which are affected by PAI-1 [41]. Differential interaction of PAI-1 with Vitronectin (VN) during different phases of wound healing might add further complications [1], and well-regulated spatial and temporal changes in the expression of these two proteins seems to be required for appropriate wound healing [41]. It is therefore reasonable to consider that the impaired wound healing observed in the proband is also caused by the PAI-1 deficiency.

While a number of studies have been performed to understand PAI-1 functions using *Serpine1*$^{-/-}$ mice, most have not been validated in humans because the deficiency is very rare. A study of complete PAI-1 deficiency in a family of Old Order Amish with the same gene mutation produced variable symptoms [25]. Therefore, it was speculated that the hemostatic PAI-1 function was exacerbated or attenuated by unidentified factors, the so-called hemostasis modifier genes. PAI-1 also plays a role in many pathophysiological processes other than hemostasis, and these pleiotropic functions might be different in humans and mice. Considering the results of this study, the translation of mouse studies to humans must be performed with great care.

Addendum

Contribution: T. Iwaki, A. Tanaka, Y. Miyawaki, and A. Suzuki performed the experiments; T. Iwaki, T. Urano T. Matsushita, and T. Kojima analyzed the results and created the figures; T. Kobayashi, J. Takamatsu, K. Umemura, and T. Terao undertook the clinical care of the proband and her family; T. Iwaki, T. Urano, and N. Kanayama designed the research and wrote the paper.

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Disclosure of Conflict of Interest

The authors state that they have no conflict of interest.

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Strategies for blood transfusion in critical bleeding

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Background

Blood loss due to bleeding is a reversible process as long as the amount of blood loss is compensated by physiological mechanism and/or fluid therapy. However, the amount and rate of bleeding far greater than compensatory mechanisms leads to a vicious cycle which worsens bleeding and impairs major organ dysfunctions (Fig. 1).

Annual survey of the critical incidents related to anaesthesia by the Japanese Society of Anaesthesiologists (JSA) repeatedly demonstrated that massive bleeding is a major cause of intraoperative cardiac arrest [1,2]. The JSA studies demonstrated that critical bleeding in the perioperative period was the leading cause of intraoperative cardiac arrest and perioperative death in the first week postoperatively (Table 1) [3,4]. About a half of them were related to haemorrhagic shock due to trauma, rupture of the large blood vessels, and so on. The rest were related to intraoperative bleeding due to surgical manipulation. Although the possibility of massive and rapid bleeding was anticipated and some preventive and treatment measures were to be taken to deal with such critical bleeding, prognosis was still grave.

The Subcommittee on Surveillance of Anaesthesia-Related Critical Incidents of the JSA analysed the data. Contributing factors include far greater rate and amount of bleeding than anticipated (Fig. 2), delay in decision to start blood transfusion and to order additional blood products, hesitation to use ABO-compatible blood including group O Blood without cross-matching, delayed transportation of the blood products from the blood banks, and lack of man power. In patients with critical bleeding, blood loss was >12 l/60 kg(body weight) in 35·2% of the patients, and the maximal estimated bleeding rate was >240 ml/60 kg(body weight)/min in 44·9% of the patients. Despite critical bleeding and shortage of blood products due to delayed transportation of the blood products, cross-matching test was waived in 13·4% of the patients, and ABO-compatible blood products including group O blood was used only in 1·3% of the patients. It suggests that using uncross-matched blood might be a major concern in the physicians in Japan even in the life-threatening situation.

Although the national guidelines for blood transfusion made by the Japanese Ministry of Health, Labour and Welfare exist, the issues on critical bleeding were not fully discussed and clear guidelines were not described. Massive bleeding is commonly defined as the loss of one blood volume with in 24-h period. There are many cases where the concept of massive bleeding may not lead to appropriate blood transfusion in a timely fashion. The concept of critical bleeding is arbitrarily defined; the physicians who take care of the briskly bleeding patients feel that bleeding is very likely to result in life-threatening situation in a short period of time, i.e. within a few minutes to a few hours. Immediate and appropriate therapy to stabilize the patient and to avoid secondary damages is required. Therefore, the JSA and the Japan Society of Transfusion Medicine and Cell Therapy decided to make guidelines for treatment of critical bleeding.

Outlines of the guidelines for treatment of critical bleeding

The JSA established "Guidelines for Actions Against Intraoperative Critical Hemorrhage" (Guidelines) in collaboration with the Japanese Society of Blood transfusion and Cell Therapy in 2007. Fundamental concept is life-saving measure is much important than theoretical complications in the life-threatening critical bleeding.

There are a few basic strategies as follows:

(1) Physicians and nurses, medical engineers in the operating room and emergency room, staff in the blood transfusion department, and staff in the blood bank work as a team. Intense communication between them is mandatory.
(2) The commander is in charge of the important decisions related to blood transfusion strategies.

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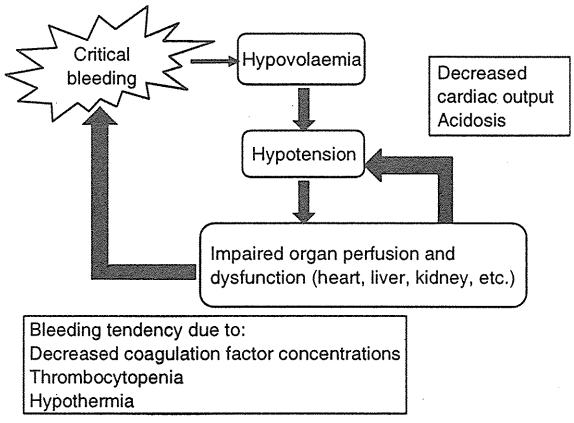

Fig. 1 Vicious cycle due to critical bleeding.

Table 1 Incidence of intraoperative cardiac arrest and postoperative mortality in 7 days

	Number of patients	Mortality after 7 days (%)
Preoperative haemorrhagic shock		
Cardiac arrest	192	88·0
Serious complications other than cardiac arrest	278	44·6
Intraoperative critical bleeding		
Cardiac arrest	103	77·7
Serious complications other than cardiac arrest	438	19·6

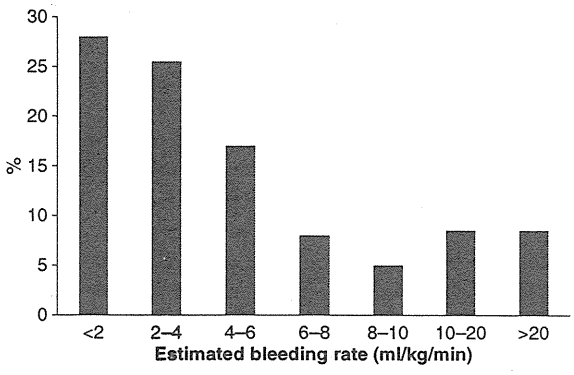

Fig. 2 Estimated bleeding rate in critical bleeding.

(3) Surgeons concentrate on haemostasis rather than on proceeding the planned procedure. Damage control surgery should be considered.
(4) Anaesthesiologists insert a few large bore intravenous lines, draws blood for blood cell counts and coagulation studies, and order blood products according to the amount and speed of haemorrhage, vital signs, laboratory data, and the prospect of haemostasis.
(5) ABO-compatible blood products should be used without hesitation.
(6) Euvolemic status should be maintained to keep adequate perfusion pressures of major organs and to maintain their functions.
(7) Hypothermia should be best avoided to worsen bleeding tendencies.
(8) The institutional structure of blood transfusion system and transport time from the blood bank should be understood by the staff concerning blood transfusion.
(9) The institutional guidelines for critical and massive bleeding should be established according to the Guideline.
(10) Simulation training involving all departments related to blood transfusion should be performed.

Commander and the team

When critical bleeding occurs, one single physician should become the commander who will direct the overall therapy including blood transfusion. The commander declares a state of emergency. Most often, the anaesthesiologist will become the commander in the operating room (OR) because the anaesthesiologist knows the general condition of the patient and is aware of the situation around the OR including storage of blood products in the institution, and transport of blood products from the blood bank. After the life-threatening condition was evaded, the commander declares the end of emergency.

The personnel in the OR, the emergency room (ER), laboratories, department of blood transfusion, and Red Cross blood center work together as a team in the face of critical bleeding (Fig. 3). Close communication amongst all departments is essential. Anaesthesiologists would start new large-bore intravenous lines (16 or 14 gauge cannulae) for fluid resuscitation and blood transfusion, and take blood samples for blood gases, complete blood cell count, coagulation studies, and cross-matching. Arterial and central venous lines may be inserted. The commander would order blood products according to the general condition of the patients, laboratory data, and availability of blood products. Surgeons would concentrate on haemostasis rather than on completing the planned procedure. Damage control surgery should be considered. Medical engineers would prepare for intraoperative autologous blood transfusion and rapid infusion pumps. Nurses would measure the amount of blood loss, and keep contact with the blood bank and check blood product bags.

Postoperative management in the intensive care unit is probably required. Postoperative mechanical ventilation may be required.