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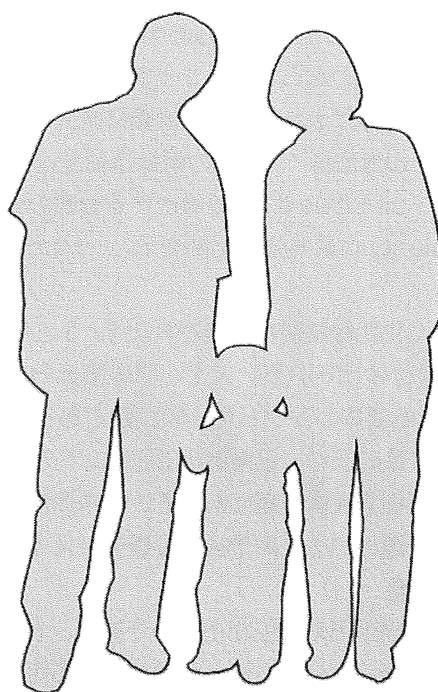
IV.　研究成果の刊行物・別冊

小 児
輸血マニュアル

小児輸血療法研究会

監 修：星　順隆
編 集：長田　広司
堀越　泰雄
小原　明



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4．小児疾患と輸血療法の実際

4）小児心臓外科疾患と輸血

宮田茂樹（国立循環器病センター輸血管理室）

1．先天性心疾患に対する外科手術の特徴

新生児期に発症する先天性心疾患は重症であることが多く、外科的手術が必要となることが多い。新生児期を過ぎた先天性心疾患は、一部の疾患を除き、待機手術となることが多い。また、新生児期に姑息手術を行い、その後修復術を行う場合には、前回手術による癒着等で、出血量が増加する場合も少なからず存在する。

2．人工心肺管理

新生児、乳幼児の人工心肺は、人工心肺充填量と循環血液の比率が成人に比して大きく、血液充填、無血充填を問わず充填液の影響を受けやすいという特殊性を有している。したがって、充填液による影響を最小限にとどめるために、充填液組成の見直しや低容量回路の開発などの努力が続けられている。また、成人に比して基礎代謝量が大きく、心拍出量に対して重要臓器の血流が占める割合が多いため、体重当たりの灌流量を高く設定する必要があり、低充填量でありながら高灌流量が得られる人工心肺システムが重要となる。

3．小児心臓外科における輸血療法

1）赤血球輸血（小児心臓手術患者のヘモグロビン許容限界値）

小児の場合は、余命が長く長期予後が問題となるため、これらに焦点を当てた報告がいくつかなされている。

先天性心疾患の術後患者 243 名において 5 歳時点での神経学的発達に及ぼす影響に対する前向き観察研究がある[1]。人工心肺を用いた症例が 82%で、人工心肺時間は Full-Scale IQ には影響しなかったが、Performance IQ を悪化させる独立危険因子であった。人工心肺中最低ヘマトクリット（Ht）は、家庭環境スコアを調整すると、両心室疾患では、危険因子とならなかったと報告されているが、人工心肺中最低 Ht に関しては単心室疾患も含めた解析が行われていなかった。後ろ向き観察研究の報告もあり、心房中隔欠損症に対しての治療として手術群 26 名とカテーテル治療群 19 名での精神運動発達テストに関する比較検討が行われた。年齢および両親の IQ を調整すると、Full-Scale IQ を始めとする複数のテストで手術群の方が悪い結果となった。人工心肺中最低 Ht が Full-Scale IQ と最も強い相関を示し、Ht が高いほど IQ が高くなる傾向にあったと報告されている[2]。

さらに、この問題に対してランダム化比較試験が実施された[3]。9 ヵ月未満の乳児で、出生時体重 2.3kg 以上の先天性心疾患患者 147 名が対象となっている。人工心肺中最低 Ht を 21.1%±2.4%と低く抑えた群 74 名と 27.7%±3.2%と高くした群 73 名でのランダム化比較試験である。術後 1 歳時点での神経学的発達を調べたスコアは Ht を低く抑えた群で有意に低く、正常者の 2SD 以下の低いスコアの患者が明らかに多かったと報告されている。したがって、先天性心疾患患者において、術中赤血球輸血のトリガー値をあまり低くしすぎると、合併症の増加ならびに精神発達という長期予後に影響を与える可能性がある。

2）血小板ならびに新鮮凍結血漿

出生時、特に未熟児においては、凝固因子の産生が不十分であり、トロンビン産生は遅延する傾向にある。また、先天性心疾患の病態や、その治療により出血傾向が助長される可能性がある。人工心肺使用手術では、その表面への接触やポンプなどによる血流の変化、低体温などにより、血小板の消費、活性化が起こる。また、

希釈性ならびに消費性の凝固障害が手術や人工心肺に伴って発生する。このため、さらに出血傾向が増長される可能性がある。出血に対する血小板輸血のトリガー値として、100,000/μl以上をキープするという記述が海外のガイドラインでは標準的である。また、血小板数が100,000/μl以上であっても、血小板機能低下が認められる場合には、血小板輸血を許容するとの記述も認められる。一方、本邦では、50,000から100,000/μlとするとの記述があるが、これは成人も含めた人工心肺使用手術における記載であり、小児人工心肺使用手術としての明確な基準は示されていない。凝固障害に伴う出血に対しては、新鮮凍結血漿が本邦ではもっぱら用いられるが、新鮮凍結血漿で凝固障害を補正するためには、かなりの量が必要でvolume overloadになる可能性について注意が必要である。このため、海外では、人工心肺手術に伴う凝固障害（特に急性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やフィブリノゲン濃縮製剤が用いられる場合があるが、本邦では、これらの製剤は承認されておらず、今後の大きな課題である。

3）新鮮血（全血）

新鮮血は、輸血に関する全てのコンポーネントを含んでおり、血小板機能などがよく保存されている。したがって、新鮮血をプライミングに使用したり、出血制御困難時に用いることがしばしば考慮される。しかしながら、血小板機能を保存するためには冷蔵することができず、6時間以内に使用する必要があることを考えた場合、核酸増幅法を含めた充分な感染症検査を実施することができないため、その使用は現実的ではない。実際、200症例の先天性心疾患手術において、プライミングを採血後48時間以内の新鮮血で行う群、採血後平均6日後の濃厚赤血球製剤で行う群の2群について、その予後を比較したランダム化比較試験の結果では、出血量、輸血量、患者予後に差はなかったとする報告がある[4]。一方、64症例と少ないものの、生後1ヵ月以内の手術で、新鮮血をプライミング、術中、術後24時間使用した群と、保存濃厚赤血球製剤を用いた群で検討を行ったランダム化比較試験では、ドレーン出血が新鮮血群で少なく、挿管時間、入院期間も短かったとの報告がある[5]。未だ充分にこの問題について検討されているとはいえず、現時点では、新鮮血が、出血制御や患者予後改善に有効であるという明確なエビデンスは存在しない。実際、我々の施設でも10年前までは、院内ドナーから採血した直後の新鮮血を小児心臓手術にしばしば使用していたが、最近10年間は新鮮血を全く使用せずに問題なく手術が施行されている。

まとめ

小児心臓手術については、1）症例によるvariationが大きいため、コントロールされた臨床研究を実施しにくい。2）施設間の比較検討が行いにくい。3）採血による貧血が問題となり、臨床研究の実施に困難を伴う。4）臨床研究のインフォームドコンセントが得られにくい。などの理由から、小児心臓外科の輸血療法に関するエビデンスが少なく、ガイドラインの作成が困難である。今後さらに検討を重ねて、小児心臓外科手術のための最適な輸血療法の確立が重要となる。

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Consecutive national surveys of ABO-incompatible blood transfusion in Japan

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Vox Sanguinis

Background and Objectives Morbidity and mortality from ABO-incompatible transfusion persist as consequences of human error. Even so, insufficient attention has been given to improving transfusion safety within the hospital.

Materials and Methods National surveys of ABO-incompatible blood transfusions were conducted by the Japanese Society of Blood Transfusion, with support from the Ministry of Health, Labor and Welfare. Surveys concluded in 2000 and 2005 analysed ABO-incompatible transfusion data from the previous 5 years (January 1995 to December 1999 and January 2000 to December 2004, respectively). The first survey targeted 777 hospitals and the second, 1355 hospitals. Data were collected through anonymous questionnaires.

Results The first survey achieved a 77·4% response rate (578 of 777 hospitals). The second survey collected data from 251 more hospitals, but with a lower response rate (61·2%, or 829 of 1355 hospitals). The first survey analysed 166 incidents from 578 hospitals, vs. 60 incidents from 829 hospitals in the second survey. The main cause of ABO-incompatible transfusion was identification error between patient and blood product: 55% (91 of 166) in the first survey and 45% (27 of 60) in the second. Patient outcomes included nine preventable deaths from 1995 to 1999, and eight preventable deaths from 2000 to 2004.

Conclusion Misidentification at the bedside persists as the main cause of ABO-incompatible transfusion.

Key words: non-infectious, transfusion complication, transfusion practices (adult), transfusion service operations.

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Introduction

ABO-incompatible transfusion preceded Landsteiner's discovery of human blood groups, but persists more than 100 years later as an important cause of adverse events due to human error [1–3]. Haemovigilance systems in Europe and North America target ABO-incompatible blood transfusion [1,2,4]. In Japan, Red Cross blood centres collect haemovigilance data, but specifically target transfusion-transmitted virus infections and immune phenomena such as allergic reactions, transfusion-related acute lung injury, and transfusion-associated graft-vs.-host disease [5]. Therefore, the actual incidence of ABO-incompatible blood transfusion in our country has been uncertain. In order to investigate and guide methods of prevention, consecutive national surveys were initiated by the Japanese Society of Blood Transfusion (now the Japanese Society of Transfusion Medicine and Cell Therapy) [6,7].

Materials and methods

The Japan Society of Blood Transfusion developed anonymous questionnaires, targeting 777 hospitals from January 1995 to December 1999, and 1355 hospitals from January 2000 to December 2004. Data were analysed and reported in 2000 and in 2005. The first survey solicited cases arising from whole blood (WB), red cell concentrate (RCC) and fresh frozen plasma (FFP) transfusions at 777 hospitals, each having at least 300 beds. The scope of the second survey expanded to include cases arising from platelet concentrate transfusions, and targeted 1355 hospitals, including 777 of the same hospitals targeted in the first survey and 578 additional hospitals with fewer than 300 beds, where at least one transfusion specialist was working. Not only accidents but also incidents (errors without adverse reactions) were solicited. In regard to transfusion oversight, blood transfusion management systems and laboratory testing outside of core hours were investigated in first survey (Tables 1 and 2). To these, the second survey added utilization of electronic equipment for blood transfusion management and product testing (Tables 3 and 4).

Table 1 ABO-incompatible blood transfusion questionnaire form 1 of the first survey (1 January 1995 to 31 December 1999)

I. Did the ABO-incompatible blood transfusion occur in the past 5 years (1 January 1995 to 31 December 1999)?
(The targets are whole blood, red cell concentrates, and fresh frozen plasma; and platelets concentrates should be excluded.)
(1) Yes (Please give details using investigation form 2 on the next page.)
(2) No

II. Questions on system of blood transfusion management

1. Number of hospital beds: Select from the following:
   (1) 300 to less than 400 beds
   (2) 400 to less than 500 beds
   (3) 500 to less than 600 beds
   (4) 600 to less than 700 beds
   (5) 700 to less than 800 beds
   (6) 800 to less than 900 beds
   (7) 900 to less than 1000 beds
   (8) More than 1000 beds
2. Amount of transfused blood components during the last fiscal year: Select from the following:
   (1) 3000 to less than 10 000 units
   (2) 10 000 to less than 20 000 units
   (3) 20 000 to less than 30 000 units
   (4) 30 000 to less than 40 000 units
   (5) 40 000 to less than 50 000 units
   (6) More than 50 000 units
3. Section that manages blood supply:
   (1) Blood transfusion service
   (2) Laboratory
   (3) Pharmacy
   (4) Others
4. Pretransfusion testing out of core hours:
   (1) Duty by laboratory technician
   (2) The doctor takes charge
   (3) Laboratory technician's system of on call
   (4) Others
5. Doctor accredited by the Japan Society of Blood Transfusion:
   (1) Yes
   (2) No
6. Laboratory specialist accredited by the Japan Society of Blood Transfusion:
   (1) Yes
   (2) No
7. Hospital transfusion therapy committee:
   (1) Yes
   (2) No
8. Please describe any special method to prevent of ABO-incompatible blood transfusion in your hospital.

Results

A 74·4% response rate was achieved in the 1995–99 survey, corresponding to 578 of 777 hospitals. A 61·2% response rate was achieved in the 2000–04 survey, corresponding to 829 of 1355 hospitals. From 578 participating hospitals in the first survey came 166 case reports, vs. only 60 case reports from the 829 hospitals participating in the second survey including six cases reported from hospitals with fewer 300 beds (Table 5). These cases include those without adverse reactions. Nevertheless, the number of fatalities reported in each survey was nearly equal: nine in the first survey and eight in the second. In the second survey, the mean number of transfused blood components reported from 540 hospitals during survey period was 14 855 bags, but in first survey the exact number of transfused blood components was not

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Table 2 ABO-incompatible blood transfusion questionnaires form 2 (case report) of the first survey (1 January 1995 to 31 December 1999)

1. Content of case:
(Please describe details and the reason for the discovery of ABO-incompatible blood transfusion.)
2. Persons concerned who made a mistake:
   (1) Doctor
   (2) Nurse
   (3) Laboratory technician
   (4) Others ( )
3. Time period:
   (1) Regular (daylight) hours
   (2) Out of core hours
4. Was it an urgent blood transfusion?
   (1) Yes
   (2) No
5. Site of blood transfusion:
   (1) Ward
   (2) Operation room
   (3) ICU
   (4) Emergency room
   (5) Others
6. Blood product:
   (1) Whole blood
   (2) Red cell concentrates
   (3) Fresh frozen plasma
7. ABO type:
   Blood type of blood preparation
   Patient's blood type
8. Amount of blood transfusion (ml):
9. How long did it take you to become aware of ABO-incompatible blood transfusion from the beginning of transfusion?
10. Did you explain the situation to the patient and family?
   (1) Yes
   (2) No
   (3) Uncertain
11. Was there any symptom of shock?
   (1) Yes
   (2) No
   (3) Unknown
12. Was there any sign of haemolysis?
   (1) Yes
   (2) No
   (3) Unknown
13. Was there any sign of disseminated intravascular coagulation?
   (1) Yes
   (2) No
   (3) Unknown
14. Was there any sign of renal insufficiency?
   (1) Yes
   (2) No
   (3) Unknown
15. What kind of treatment was performed?
16. Outcome:
   (1) Death
   (2) Survival with adverse effects
   (3) Survival without adverse effects
17. Improvement plan concerning ABO-incompatible blood transfusion prevention adopted after the case occurred:
18. Others
(If you think there is anything else pertinent to this case, please describe the details.)

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Table 3 ABO-incompatible blood transfusion questionnaire form 1 of the second survey (1 January 2000 to 31 December 2004)

I. Did the ABO-incompatible blood transfusion occur in the past 5 years (1 January 2000 to 31 December 2004)?
(The targets are whole blood, red cell concentrates, fresh frozen plasma, and platelet concentrates.)
(1) Yes (Please give details using investigation form 2.)
(2) No
II. Questions on system of blood transfusion management
1. How many beds does your hospital have?
( ) beds
2. How many units of total blood transfusion products were administered over 5 years 1 January 2000 to 31 December 2004?
Whole blood ( ) units, ( ) bags
Red cell concentrates ( ) units, ( ) bags
Fresh frozen plasma ( ) units, ( ) bags
Platelets concentrates ( ) units, ( ) bags
3–8. Same as those of the first survey
9. Do you electronically verify patients and blood products before transfusion at bedside?
(1) Yes
(2) No
(3) Only in a part of the ward
10. Is a computer-based ordering system used to request the blood supply?
(1) Yes
(2) No
(3) Its introduction is scheduled
11. Is the ordering computer system used to request the pretransfusion testing?
(1) Yes
(2) No
(3) Its introduction is scheduled
12. Is a computer-based system used for the stock-taking and managing the delivery of the blood products?
(1) Yes
(2) No
(3) Its introduction is scheduled
13. Is an automatic blood transfusion testing machine used?
(1) Yes
(2) No
(3) Its introduction is scheduled

Table 4 ABO-incompatible blood transfusion questionnaire form 2 (case report) of the second survey (1 January 2000 to 31 December 2004)

1–18. Same as those of the first survey
19. Did it occur before introducing the portable digital assistant to blood transfusion confirmation at the bed side?
(1) Yes
(2) No

Table 5 Analysed data

	First survey	Second survey	
Survey period	1 January 1995 to 31 December 1999	1 January 2000 to 31 December 2004	
Target hospital	777	1355	
> 300 beds	777	777[a]	
< 300 beds	0	578	
Response (%)	578 (74·4)	829 (61·2)	
> 300 beds	578 (74·4)	502 (64·2)	
< 300 beds		327 (55·7)	
Reported cases[b]			
	WB + RCC + FFP[c]	RCC + FFP[d]	PC[e]
> 300 beds	166	48	6
< 300 beds	0	4	2
Total	166	52	8

[a]777 hospitals the same as those targeted in the first survey.
[b]Reported cases including those without adverse reactions.
[c]Cases arising from whole blood (WB), red cell concentrate (RCC), and fresh frozen plasma (FFP), including those arising from unknown components.
[d]Cases arising from RCC and FFP, including those arising from unknown components.
[e]Cases arising from platelet concentrate.

Table 6 Number of reports according to the type of blood product

	First survey[a]	Second survey[b]
Whole blood major mismatch	3	0
Whole blood minor mismatch	2	0
Red cell concentrate major mismatch	48	22
Red cell concentrate minor mismatch	38	9
Fresh frozen plasma	71	19
Platelet concentrate	Not reported	8
Unknown	4	2
Total	166	60

[a]1 January 1995 to 31 December 1999.
[b]1 January 2000 to 31 December 2004.

collected. The number of reported cases of ABO-incompatible blood transfusion according to the number of hospital beds is shown in Fig. 1. A decrease in the number of reported cases was recognized in large hospitals, defined as having more than 700 beds. Table 6 shows the numbers of reported cases according to the type of blood product. A decrease of RCC minor mismatch and FFP was more remarkable than that of RCC major mismatch. Outcomes in patients receiving RCC major mismatch included nine deaths in the first survey and eight in the second. The cause of death includes the possibility of underlying disease in nine of 17 cases according to the contents of cases in questionnaire form 2. In six of the remaining eight deaths, unambiguously due to ABO-incompatible transfusion, the patients were of group O blood type. Data from the second survey suggest a risk of ABO-incompatible

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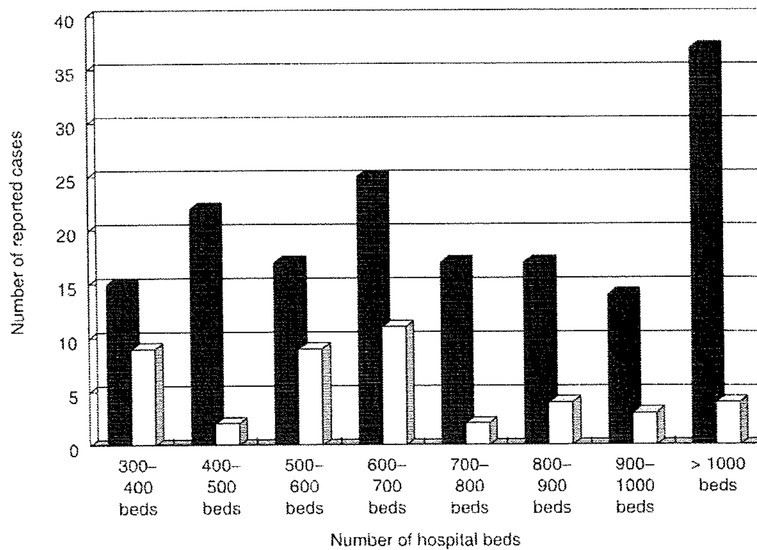

Fig. 1 Number of reported cases of accidental ABO-incompatible blood transfusion of red cell concentrate and fresh frozen plasma according to the number of hospital beds.
■: Number of reported cases of ABO-incompatible blood transfusion of whole blood, red cell concentrates, and fresh frozen plasma in the first survey (1 January 1995 to 31 December 1999).
□: Number of reported cases of ABO-incompatible blood transfusion from red cell concentrates and fresh frozen plasma reported only from hospitals having at least 300 beds in the second survey (1 January 2000 to 31 December 2004).

Table 7 Pretransfusion testing out of core hours

	Number of hospitals		
	First survey[a]		Second survey[b]
	> 300 beds (%)	> 300 beds (%)	< 300 beds (%)
Duty of laboratory specialist	347 (60·35)	476 (75·1)	26 (13·9)
Laboratory specialist on call	163 (28·35)	147 (23·2)	157 (83·9)
The doctor takes charge	43 (7·5)	4 (0·6)	2 (1·1)
Others	22 (3·8)	7 (1·1)	2 (1·1)
Total	575 (100)	634 (100)	187 (100)

[a]1 January 1995 to 31 December 1999.
[b]1 January 2000 to 31 December 2004.

transfusion as 1 : 200 000 and a risk of the death as 1 : 3 000 000. The status of pretransfusion testing out of core hours is shown in Table 7. Electronic correlation of patients and blood products seems to have had limited implementation in 1999, when the first survey was executed, but was reported in 8·8% of facilities in 2004 when the second survey was executed.

Main causes of transfusion error

Identification error between patient and blood product

The main cause of transfusion error was misidentification between patient and blood product: 55% of cases (91 of 166) in the first survey, and 45% (27 of 60) in the second (Table 8). RCC major mismatch comprised 36 cases in the first survey and 14 cases in the second survey. Among the reported cases, no technology-based identification systems were in place.

Phlebotomy error

Phlebotomy errors were reported in 2% of cases (four of 166) in the first survey, and 3% (two of 60) in the second. All phlebotomy errors were emergency situations where the blood typing and cross-matching were performed on the same specimen.

Prescription error

Prescription errors were reported in 11% of cases (19 of 166) in the first survey, and 13% (eight of 60) in the second. In these cases, blood component orders of an incorrect ABO blood group were sent to the laboratory. Fresh frozen plasma

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Table 8 Main causes of transfusion error

	First survey[a]	Second survey[b]
Identification error	91	27
Phlebotomy error	4	2
Prescription error[c]	19	8
Testing error by doctor	21	10
Laboratory error outside of core hours	12	6
Laboratory error during core hours	5	4
Other	14	3
Total	166	60

[a]1 January 1995 to 31 December 1999.
[b]1 January 2000 to 31 December 2004.
[c]Blood components orders of incorrect ABO blood group.

or platelet concentrate orders of an incorrect ABO blood group sent to the laboratory were undetected by laboratory methods due to the omission of the minor cross-match. No reported prescription error was associated with an RCC major mismatch.

Testing error by doctors

Testing errors by doctors were reported in 13% of cases (21 of 166) in the first survey, and 17% (10 of 60) in the second. In hospitals where these errors arose, laboratory services for blood transfusion were not available.

Laboratory error outside of core hours

Laboratory errors outside of core hours were reported in 7% of cases (12 of 166) in the first survey, and 10% (six of 60) in the second. These errors included technical testing errors in 10 cases, issuance of the wrong units in four cases, and use of the wrong patient sample for testing in one case, and, in four cases the details of errors were not reported.

Laboratory error during regular (daylight) hours

Laboratory errors during regular (daylight) hours were reported in 3% of cases (five of 166) in the first survey, and 7% (four of 60) in the second. These errors included technical testing errors in three cases, clerical error in transcription in one case, issuance of the wrong units in two cases, and use of the wrong patient sample in three cases.

Other errors

In the first survey: a wrong blood type was displayed at the bedside in one case; 11 cases had no reports about the main cause; and in two cases, a main cause could not be clearly discerned. In the second survey, two ABO-incompatible bone marrow transplant recipients received the wrong blood, and in one other case, incompatible FFP was taken from an operating room refrigerator.

Discussion

Based on data from the second survey, the risk of ABO-incompatible transfusion and that of death is about half of those reported by Serious Hazards of Transfusion (SHOT) [1]. In Japan, at least 8000 hospitals transfuse blood, perhaps more if the smallest hospitals are counted, but this investigation focused on the hospitals responsible for about 80% of the blood products transfused in Japan. The Japanese Red Cross (JRC) is the only supplier of allogeneic blood components used in Japan. The collection of allogeneic blood by a hospital transfusion service is rare and permitted in emergency cases if the JRC has failed to supply the blood products to hospitals. The total amount of all blood components supplied by the JRC corresponded to the total amount of blood components transfused in Japan. In the fiscal year of 2004, when the second survey was done, the total amount of blood components supplied by the JRC Blood Center was 16 668 784 units, and the total amount of blood components transfused in the 829 hospitals which responded to the second survey was 7 962 317 units, with about 47·8% of blood components supplied by the blood centre.

ABO-incompatible blood transfusion arises from human error [8]. Eighty per cent of ABO-incompatible blood transfusions were reported from the clinical setting of a ward or operating room and 20% were reported from a laboratory. No reported errors were associated with blood banking procedures of the JRC. There were no mislabelling of units or, weak A or B antigens typed as O. This underscores the value of an incident reporting system that collects data from hospitals, and provides analytical feedback to each facility [9–11]. Identification errors between patients and blood products provoke most RCC major mismatch transfusions. Preventive efforts are important because these errors are eminently preventable. Many hospitals had their own transfusion procedural manual, including the final identification between patients and blood products in the clinical area. In many cases, procedural deviations occurred, including half of the hospitals that maintained their own procedures. Following the first survey, a standardized blood transfusion procedure manual emphasizing the final identification between patients and blood products was developed by the Japanese Society of Blood Transfusion, and this procedure has been widely propagated through distributing a poster showing the procedural manual by the Japanese Society of Blood Transfusion and JRC [6]. The second survey collected only about 30% as many identification errors as were reported in the first survey, even with the participation of an additional 251 hospitals. It may be that the dissemination of a standard procedure contributed to a decrease in identification error. This was the main intervention undertaken to reduce the incidence of ABO-incompatible transfusion after the first survey. However, incorrect blood recipient identification at

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the patient's bedside persists as the main cause of ABO-incompatible transfusion. Education programmes may be helpful to the extent that they reach all staff involved in transfusion. This is challenging under the best of circumstances, and more so where staff turnover is high. It thus behooves us to monitor employment trends in the healthcare sector. Technological interventions also have the potential to interdict human error, provided that the technology is not bypassed for reasons of expediency or lack of understanding [12-15]. The introduction of electronic correlation of patients and blood products has progressed in large-scale hospitals. Pretransfusion testing out of core hours is another problem. In 7·5% of hospitals in the first survey, laboratory services for blood transfusion out of core hours were not available, thus forcing clinicians into the role of laboratory professionals. The number of facilities where a doctor performs pretransfusion testing outside of core hours decreased from the first survey, and the number of facilities where laboratory staff perform all testing increased. Even so, laboratory staff who do not routinely perform transfusion-related testing are likely to be more error prone than those who are devoted to the blood bank or transfusion service. These were the main differences between the two surveys.

The second national survey of ABO-incompatible blood transfusion was completed 5 years after first survey. Ideally, investigative data should be collected continuously and reported at least annually, as occurs in other countries with formal haemovigilance systems [1,2]. We aspire to blend the Japanese experience described herein with international best practices described elsewhere, with the ultimate goal of mitigating the needless morbidity and mortality arising from human error.

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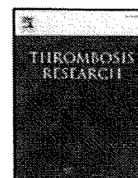

Letter to the Editors-in-Chief

A novel cholesterol absorption inhibitor, ezetimibe, decreases adipose-derived and vascular PAI-1 expression *in vivo*

To the Editor:

Hypercholesterolemia is an independent risk factor for the development of atherosclerosis and cardiovascular/thrombotic diseases. The increased incidence of cardiovascular disease may be associated with elevated levels of coagulation factors and plasminogen activator inhibitor-1 (PAI-1) in plasma, which is the primary inhibitor of plasminogen activation *in vivo* [1]. PAI-1 levels in plasma are elevated in hyperlipidemic and/or obese patients [2], and adipose tissue is one of primary sources of PAI-1 production in this condition [3]. These observations suggest that PAI-1 expression could be substantially influenced by lipid metabolism. For example, PAI-1 expression in cultured adipocytes is strongly upregulated by glucocorticoids, insulin, tumor necrosis factor-α, and LDL, some of which have been found to be frequently elevated in subjects with hypercholesterolemia or metabolic syndrome [4].

Ezetimibe is the first cholesterol absorption inhibitor that inhibits the absorption of biliary and dietary cholesterol from the small intestine [5]. This agent specifically blocks the cholesterol transporter, Niemann-Pick like protein 1, enriched in the brush border membrane of small intestine [6]. The consequences of cholesterol absorption inhibition include decreased cholesterol delivery to the liver, reduced hepatocyte cholesterol stores, increased LDL production, but still increased LDL clearance from the serum, and subsequently, decreased serum LDL-C levels. Although it was reported that ezetimibe was associated with decreased platelet aggregation and LDL tendency to peroxidation [7], the effect of ezetimibe on the coagulation or fibrinolytic pathway has not been investigated.

To answer this question, we have investigated the effect of ezetimibe on the expression of PAI-1 in mice *in vivo*. Twelve to twenty-month-old (middle-aged) male C57BL/6J mice (n = 4) were orally administered with 5 mg/kg/day of ezetimibe for 7 days, and then, sacrificed. This dose of ezetimibe is the lowest dose that maximally inhibits cholesterol absorption in mice [8]. We prepared the age-matched control group (ezetimibe naive, n = 4). All procedures were carried out according to the protocol approved by the Animal Care and Use Committee of Nagoya University. We harvested several tissues including aorta and adipose tissues, which have been regarded to be a major source of PAI-1 [9,10], and then, quantitated PAI-1 mRNA expression by competitive RT-PCR [9,10]. The intensity of the bands resulting from competitive RT-PCR was measured by densitometer and the concentration of target PAI-1 mRNA could be determined by extrapolation using the competitor RNA (containing 1×10^6 molecules) standard curve. The intensity of the bands for the target PAI-1 mRNA (upper bands in each lane) was decreased in both tissues from ezetimibe-treated mice (Fig. 1), indicating the expression of PAI-1 gene was suppressed by this agent. After quantification of the intensity of the bands, we found that ezetimibe decreased the expression of PAI-1 mRNA in aortas and adipose tissues by 60-70% of the control (i.e., ezetimibe naive) mice (aorta: 0.97 ± 0.29 pg PAI-1 mRNA/μg total RNA in ezetimibe-naive mice *vs.* 0.27 ± 0.076 pg in ezetimibe-treated mice; adipose: 1.34 ± 0.33 pg PAI-1 mRNA/μg total RNA in ezetimibe-naive mice *vs.* 0.46 ± 0.12 pg in ezetimibe-treated mice; in Fig. 2). As plasma cholesterol levels were not significantly affected by 7-day-administration with ezetimibe in these mice (e.g., 132 ± 27 mg/dl in ezetimibe-naive mice *vs.* 125 ± 34 mg/dl in ezetimibe-treated mice), ezetimibe could suppress the expression of PAI-1 gene in specific tissues independently of cholesterol lowering action. In addition, active PAI-1 antigen levels in plasma measured by t-PA binding assay, were not significantly changed by ezetimibe in these mice (e.g., 3.28 ± 0.74 ng/ml in ezetimibe-naive mice *vs.* 3.06 ± 0.51 ng/ml in ezetimibe-treated mice), probably due to the observation that PAI-1 expression in the liver was not affected by such a short period of ezetimibe treatment (e.g., 0.073 ± 0.018 pg PAI-1 mRNA/μg total RNA in ezetimibe-naive mice *vs.* 0.067 ± 0.022 pg in ezetimibe-treated mice).

The mechanism of PAI-1 suppression by ezetimibe *in vivo* remains to be elucidated, but it could be speculated as follows. The major effect of ezetimibe therapy is the prevention of absorption of cholesterol from the small intestine, thus reducing the half-life of LDL in the plasma as a result of increased uptake of the lipoprotein. This results in the enrichment of the LDL particles with a fresh LDL population, which is less prone to oxidative stress than the old LDL population. The LDL-associated antioxidants are therefore less consumed by the oxidative stress in the fresh *vs.* the old LDL particles, leaving lipoproteins which

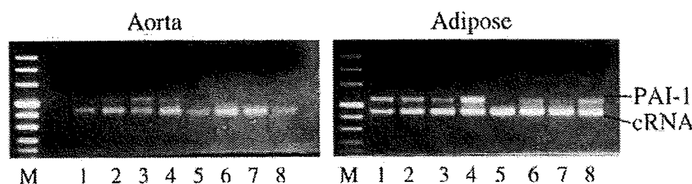

Fig. 1. Twelve to twenty-month-old mice was administered with ezetimibe (5 mg/kg/day) for one week, and the age-matched control group (ezetimibe naive) was prepared (n = 4, respectively). Aortas and adipose tissues were harvested and analyzed for PAI-1 mRNA expression by competitive RT-PCR. The upper bands (540 bp) correspond to RT-PCR products of PAI-1 mRNA and the lower bands (438 bp) correspond to those of competitor RNA (cRNA) containing 1×10^6 molecules. Lanes 1-4: control group; lanes 5-8: ezetimibe-treated group. M: DNA Molecular Weight Marker VIII (Roche).

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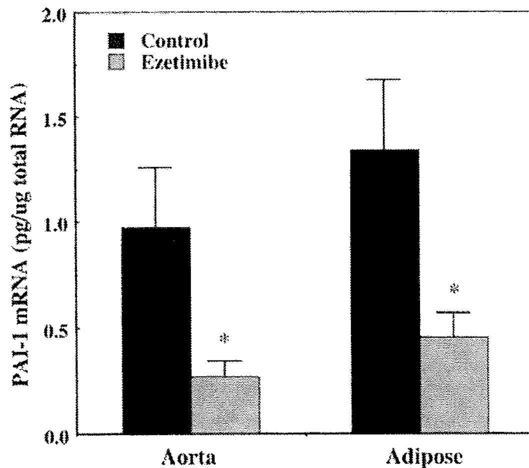

Fig. 2. Quantitative data of PAI-1 mRNA (pg/μg total tissue RNA) expressed in the aorta and adipose tissues from control (ezetimibe naive) and ezetimibe-treated mice (n = 4, respectively). Closed bars: control (ezetimibe naive) group; hatched bars: ezetimibe-treated group. The data are presented as the mean and SD. *$p < 0.01$ (statistically analyzed by one-way ANOVA).

are enriched with antioxidants [11]. As oxidative stress induces PAI-1 expression [12], we speculate that antioxidants generated during ezetimibe therapy could suppress PAI-1 expression in vascular and adipose tissues.

Statins, which have been broadly used for lowering serum cholesterol levels by inhibiting cholesterol biosynthesis in the liver, exert pleiotropic and beneficial effects on coagulation and fibrinolytic system [13], which are regarded to be independent of cholesterol lowering action. Existing research has demonstrated that statins can down-regulate PAI-1 expression *in vivo* [14]. For example, four-week-administration with atorvastatin in rabbits decreased PAI-1 expression up to 15% in adipose tissues [15]. A novel cholesterol absorption inhibitor, ezetimibe, may also exert an anti-thrombotic effect by suppressing PAI-1 expression in vascular and adipose tissues *in vivo*. This effect of ezetimibe should lower the risk of thrombotic complications in patients with hypercholesterolemia or metabolic syndrome.

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LETTER TO THE EDITOR

Stress-induced PAI-1 expression is suppressed by pitavastatin in vivo

Koji Yamamoto · Kyosuke Takeshita · Tetsuhito Kojima · Junki Takamatsu

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Thromboembolism, including myocardial infarction, cerebral infarction, and pulmonary embolism, is frequently induced by a variety of stressors. Indeed, mental, and physical stressors decrease fibrinolytic activity [1] and contribute to the occurrence of thrombotic complications. We have already reported that plasminogen activator inhibitor-1 (PAI-1) expression is dramatically induced by restraint (immobilization) stress, a typical physicopsychological stress [2], with maximal induction in the adipose tissue in vivo, a change contributing to the development of tissue thrombosis [3]. PAI-1 regulates fibrinolysis by inhibiting plasminogen activation and elevated levels of plasma PAI-1 are observed in a variety of thrombotic conditions. In obese humans, increased plasma PAI-1 levels correlated with the amounts of visceral fat, suggesting that adipose tissue is the primary source of PAI-1 in this condition [4]. Statins, 3-hydroxy-methylglutaryl coenzyme A reductase inhibitors, have been widely used for the prevention of cardiovascular diseases primarily with their lowering serum cholesterol levels. Statins also exert pleiotropic and beneficial effects on the coagulation and fibrinolytic systems [5], which are regarded to be independent of cholesterol-lowering action.

The study described below demonstrated that pitavastatin attenuated the upregulation of PAI-1 gene in restraint-stressed mice. Twelve to sixteen-month-old male C57BL/6 J mice were administered orally 10 mg/kg/day of pitavastatin or atorvastatin for 3 weeks before the animals were received restraint stress. The dosage of agents we used is regarded to be much excess in comparison with clinical dose because rodents metabolize statins more rapidly than humans. Restraint stress, plasma collection, RNA extraction and quantitative RT-PCR assay were performed, as described previously [3]. PAI-1 antigen levels in plasma were quantified by a sandwich ELISA, as described previously [6]. All procedures were carried out according to the protocol approved by the Animal Care and Use Committee of Nagoya University. Twenty hours of restraint stress to mice caused a substantial induction of PAI-1 antigen in plasma and of PAI-1 mRNA in the liver and adipose tissues, which have been regarded as major sources of PAI-1 [3]. PAI-1 antigen in plasma was dramatically elevated after a 20 h-restraint stress, but this increase attenuated by 40% in mice pretreated with pitavastatin (Fig. 1, left panel). Free PAI-1 activity measured by t-PA binding assay was also elevated by stress and its increase was attenuated by pretreatment with pitavastatin in parallel with PAI-1 antigen level (not shown). Although t-PA antigen levels measured by ELISA were elevated after restraint stress, the degree of elevation (by 2-fold, not shown) was much smaller than PAI-1 induction (by 7-fold), showing that a prothrombotic state was induced by restraint stress. Pitavastatin also suppressed the induction of PAI-1 mRNA by restraint stress in the liver and adipose tissues about 60% of the control (i.e., pitavastatin naive) mice (Fig. 1, middle and right panels), while atorvastatin did not (not shown). As plasma cholesterol levels were not affected by statins in these mice (not shown), pitavastatin may suppress the upregulation of PAI-1 gene independent of its cholesterol-lowering action in restraint-stressed mice. It has been reported that statins reduce the PAI-1 expression by

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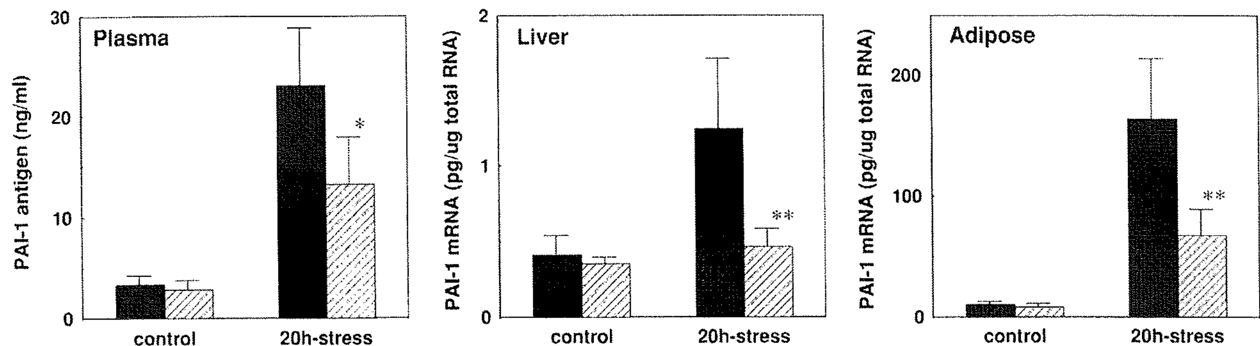

Fig. 1 Twelve to sixteen-month-old mice were administered pitavastatin (10 mg/kg/day) for 3 weeks ($n = 6$, respectively), followed by 20-h-restraint stress. As a control group, non-stressed mice and 20-h-stressed mice without pitavastatin administration were prepared ($n = 6$, respectively). The plasma was collected and measured for PAI-1 antigen (ng/ml) by ELISA assay. Liver and adipose tissue were harvested and analyzed for PAI-1 mRNA (pg/μg total tissue RNA) by competitive RT-PCR. *Closed bars* control (pitavastatin naive) group, *hatched bars* pitavastatin-treated group. The data are presented as the mean and SD. $*P < 0.05$, $**P < 0.02$ (statistically analyzed by one-way ANOVA)

suppressing the formation of geranylgeranylated proteins required for the proper synthesis of PAI-1 [7], and this may be one of the mechanisms by which pitavastatin attenuates the PAI-1 induction in restraint-stressed mice.

Several differences are observed in the pleiotropic effects of statins. Pitavastatin may more strongly suppress the molecular responses against stress insults, which include the induction of cytokine-induced nuclear factor-κB (NF-κB) and the production of oxidative stress markers in the ischemic model, in comparison with atorvastatin [8, 9]. The expression of PAI-1 gene is upregulated by oxidative stress markers (e.g., 4-hydroxynonenal and 8-hydroxy-2′-deoxyguanosine) [10] and NF-κB, both of which could be induced by stress-related inflammatory cytokines (e.g., TNF-α). Taken together, it is speculated that pitavastatin may attenuate the stress-induced PAI-1 expression through the inhibition of TNF-α-induced NF-κB activation and its anti-oxidative effect. Although there have been some reports on the inhibitory effect of atorvastatin on PAI-1 expression in vitro or ex vivo [11], this agent may have less anti-oxidant potential and less ability to block NF-κB activation than pitavastatin [12], resulting in the lack of suppressive effect on the stress-induced PAI-1 expression. Finally, the finding in this study suggests that pitavastatin contributes, in part, to the prevention of thrombotic cardiovascular diseases associated with physicopsychological stress although further studies are required to elucidate its mechanism.

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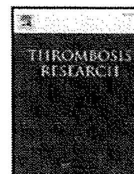

Regular Article

A novel splice site mutation in intron C of *PROS1* leads to markedly reduced mutant mRNA level, absence of thrombin-sensitive region, and impaired secretion and cofactor activity of mutant protein S☆

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ABSTRACT

Protein S (PS) is a member of the vitamin K-dependent protein family containing similar γ-carboxyglutamic acid (Gla) domains, although only PS has a thrombin-sensitive region (TSR), which is located between the Gla domain and the first epidermal growth factor-like domain. In this study, a novel *PROS1* mutation was identified at the last nucleotide in intron C (c.260-1G>A) in a patient suffering from recurrent deep vein thrombosis associated with PS deficiency. To investigate the molecular mechanisms of PS deficiency caused by the novel *PROS1* mutation, we characterized the mutant mRNA, and the secretion and function of the mutant PS molecule associated with the mutation. RT-PCR was used to detect the aberrant mRNA in the patient's platelets, the amount of which was markedly reduced and lacked the region corresponding to exon 4 coding the TSR of the PS molecule. The recombinant mutant PS lacking the TSR (TSR-lack PS) showed a markedly reduced transient expression/secretion level, 37.9% of that of wild-type (WT) PS. Activated protein C (APC) cofactor activity assay showed that TSR-lack PS had no cofactor activity. Moreover, binding assays of monoclonal antibodies recognizing the PS Gla domain and the Gla residues indicated that the bindings of TSR-lack PS to both of these antibodies were clearly weaker than those of WT PS. These findings suggest that the novel mutation leading to the absence of the TSR not only affected the secretion of mutant PS, but was also responsible for impairment of the Gla domain conformation required for the γ-carboxylation to express APC cofactor activity.

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Introduction

Protein S (PS) is one of the most important natural anticoagulants, as demonstrated by the fact that individuals with PS deficiency have an increased risk of venous thrombosis [1]. PS enhances the activated protein C (APC)-dependent proteolytic inactivation of coagulation factor Va and factor VIIIa [2]. PS also exhibits APC-independent anticoagulant functions, probably through direct inhibition of both the prothrombinase and tenase complexes [3,4], and functions as a nonenzymatic cofactor for tissue factor pathway inhibitor in the inhibition of factor Xa [5–7]. Recently, model mice heterozygous for PS deficiency were generated and showed reduced PS plasma levels and APC cofactor activity in assays of plasma coagulation and thrombin generation [8]. It has also been reported that mice homozygous for PS deficiency were not obtained through mating and that the homozygous-recessive embryos died in utero, indicating the physiological importance of PS [8].

PS is a single-chain 635-amino-acid glycoprotein with a γ-carboxyglutamic acid (Gla) domain, a thrombin-sensitive region (TSR), four consecutive epidermal growth factor (EGF)-like domains, and a large domain homologous to the sex-hormone-binding

Abbreviations: PS, protein S; Gla, γ-carboxyglutamic acid; TSR, thrombin-sensitive region; WT, wild-type; APC, activated protein C; EGF, epidermal growth factor; DVT, deep vein thrombosis; ELISA, enzyme-linked immunosorbent assay; moAb, monoclonal antibody.

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globulins. The Gla domain of PS is highly homologous to the Gla domains of other vitamin K-dependent coagulation factors and required to bind to the surface of negatively charged phospholipid membranes [9]. However, in contrast to other vitamin K-dependent factors, only PS has a unique module consisting of 29 residues, namely, the TSR, between the Gla domain and the EGF1 domain. The TSR is cleaved by thrombin at two sites (Arg-49 and Arg-70) and by factor Xa at Arg-60, resulting in functional inactivation of PS [10,11]. Although the physiological functions of the TSR are not yet fully understood, some studies have shown that the TSR is required for the PS Gla domain to bind to phospholipids [12]. These studies have also indicated that the TSR is not involved in direct interactions with phospholipids, but modulates phospholipid binding and the Gla domain conformation in a non-specific manner [13,14].

The PS gene, *PROS1* (GeneID: 5627), spans 101 kb of genomic DNA containing 15 exons and is transcribed into about 3.3 kb of mRNA. *PROS1* is located near the centromere of chromosome 3q11.1-11.2. To date, more than 200 mutations associated with PS deficiency in humans have been reported; however, only a few studies have investigated the molecular basis of the *PROS1* mutations responsible for PS deficiency. In the present study, we describe a novel splice site mutation in intron C of *PROS1* identified in a patient suffering from severe thrombotic complications associated with PS deficiency, and characterize the molecular effects of the mutation focusing on mutant mRNA levels, secretion of the mutant PS molecule, and the functional role of the TSR.

Materials and methods

Patient and blood samples

Patient

The patient is a Japanese man who had experienced episodes of recurrent deep vein thrombosis (DVT) in his legs since 45 years of age. Following pain in his left leg due to DVT at the age of 69, he was diagnosed with PS deficiency and has subsequently undergone continuous warfarin treatment. The PS levels in the patient's plasma showed total PS antigen, 30%; free PS antigen, 19%; and PS activity, <10% under warfarin therapy.

Blood samples

This study was approved by the ethics committee of the Nagoya University School of Medicine. Following the provision of informed consent, venous blood samples from the patient with PS deficiency as well as normal individuals were collected. No blood samples were available from members of the patient's family. Genomic DNA and total platelet RNA containing PS mRNA were isolated as previously described [15].

Analysis of PS DNA and mRNA in platelets

PCR amplification and sequencing of *PROS1* and PS mRNA was done essentially as described previously elsewhere [15,16].

Mutagenesis and construction of expression vectors

The expression vector pcDNA3 (Invitrogen, San Diego, CA, USA) carrying a full-length WT PS cDNA was generously provided by Dr B. Dahlbäck. Mutant PS cDNA lacking exon 4 was generated by recombinant PCR [17]. The mutant PS molecule was designated as TSR-lack PS in this study.

Quantification of PS expression/secretion by ELISA and pulse-chase analysis

Transient expression of recombinant PS molecules in COS-1 cells and measurement of PS antigen concentration in conditioned media by an enzyme-linked immunosorbent assay (ELISA) were performed essentially following a previously described method [18,19]. Pulse-chase analysis of recombinant PS by radioactive labeling, immunoprecipitation, and electrophoresis were carried out as previously described [20].

Determination of APC cofactor activity of recombinant PS

In order to measure APC cofactor activity, we needed a large amount of recombinant PS. Thus, instead of transient transformants, we established stable transformants expressing recombinant PS molecules in HEK 293 cells as described previously [20]. The APC cofactor activity of recombinant PS was determined by a clotting-based assay as previously described [21].

Binding of the recombinant PS to monoclonal antibodies (moAbs)

Briefly, a microtiter plate was coated with two moAbs: moAb MK21 recognizing the PS Gla domain and moAb M3B recognizing the Gla residues (kindly provided by Dr B. Dahlbäck and Dr J. Stenflo, respectively) [22,23]. We also prepared a microtiter plate coated with anti-PS polyclonal antibody (DAKO, Glostrup, Denmark). For the binding assay, we prepared conditioned media containing recombinant PS molecules transiently expressed in COS-1 cells. Various concentrations of the recombinant PS were incubated in the coated plates with various antibodies overnight at 4 °C and bound proteins were detected by peroxidase-conjugated anti-PS polyclonal antibody (DAKO).

Results

Gene abnormalities in the patient

The DNA-PCR products of all 15 exons, including exon-intron boundaries, of *PROS1* in the patient were directly sequenced. A G-to-A substitution at the last nucleotide of intron C was identified in exon 4 of the DNA-PCR products. This novel mutation (c.260-1G>A) was found to be heterozygous in the patient. The same mutation was not found in the DNA samples of 114 healthy subjects (data not shown).

The total platelet RNA in the patient was tested for the presence of mutant PS mRNA. Using the primers shown in the legend to Fig. 1, the region including exon 4 was amplified by RT-PCR. An aberrant, small RT-PCR product was detected in addition to a band associated with the normal-sized product (404 bp) for the patient, but not for the controls (Fig. 1). Furthermore, the abnormal band showed a significantly lower intensity compared with the normal band. Each product was purified by gel electrophoresis and directly sequenced. The normal sized product showed only the WT sequence of *PROS1*; however, the sequence of the aberrant fragment completely lacked exon 4 (c.260_346del: p.Val87_Asn115del).

The expression/secretion of recombinant PS in COS-1 cells

The failure to transcribe exon 4 causes an in-frame deletion of 29 amino acids that constitute the TSR of the PS molecule. To address the effect of the absence of TSR in the mutant PS on its expression/secretion from cells, transient expression studies with COS-1 cells were performed and the culture media were analyzed for recombinant PS content. To accurately quantify these recombinant PS expression/secretion levels, the concentrations of recombinant PS in the culture media were measured by ELISA (Fig. 2A). The quantity of TSR-lack PS in the culture media was markedly reduced to 37.9% of that of WT PS.

Pulse-chase analysis was carried out to compare the secretion profile of TSR-lack PS with that of WT PS (Fig. 2B). The level of radiolabeled WT PS rapidly decreased in the cells with a half-life of 2

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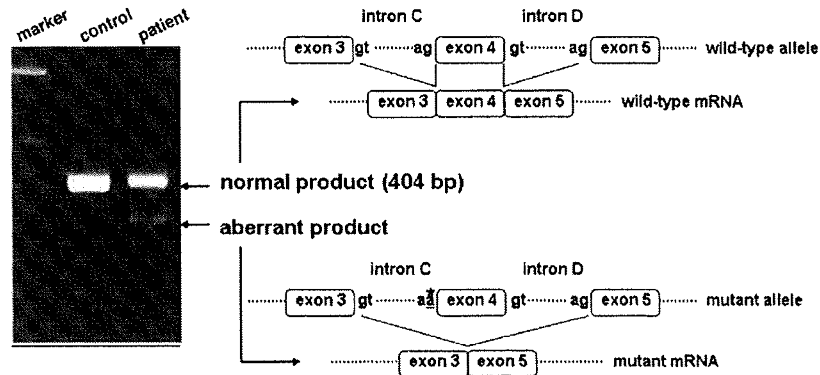

Fig. 1. Analysis of the PS mRNA of the patient with PS deficiency. Left: RT-PCR products amplified with the primer set (5′-AGGCTTCACAAGTCCTGGTTAGGAAGCG-3′ and 5′-CTTTGATTTGAGATTATATCTGTAGCC-3′) were subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide. Right: Schematic diagram indicates the mechanism causing the absence of exon 4 in the aberrant RT-PCR product of the patient induced by the G-to-A transition (*) at the splice site at -1 of exon 4 (c.260-1G>A). Raw sequencing data of the normal RT-PCR product and the aberrant RT-PCR product are not shown.

hours and immediately appeared in the culture media. In contrast, radiolabeled TSR-lack PS slowly disappeared from the cells, with a half-life of approximately 8 hours, and its rate of secretion into the culture media was lower than that of WT PS. Furthermore, the secretion efficiency, measured as the level of PS in the media at 8 hours, was significantly reduced for TSR-lack PS (40% of the initial value) compared with WT PS (70%). Taken together, these findings show not only that the secretion rate of TSR-lack PS is lower than that of WT PS, but also that the secretion efficiency of TSR-lack PS is lower.

APC cofactor activity

The APC cofactor activities of WT PS and TSR-lack PS were examined by a clotting-based assay using the serum-free culture media of the stable transformants. WT PS dose-dependently prolonged the clotting time (10-100 ng/ml), while TSR-lack PS showed no APC cofactor activity (Fig. 3).

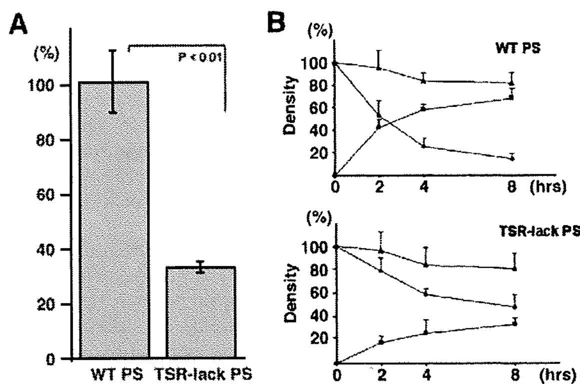

Fig. 2. Transient expression/secretion of WT PS and TSR-lack PS in COS-1 cells. (A) ELISA results of concentration of WT PS and TSR-lack PS. Mean value of WT PS is assigned as 100%. Values represent mean ± SD of 6 transfection experiments for both WT PS and TSR-lack PS. Comparison between TSR-lack PS and WT PS expression levels was performed using unpaired *t*-test. (B) Pulse-chase analysis using transient expression in COS-1 cells. Radiolabeled media and cell lysates were immunoprecipitated and subjected to SDS-PAGE. The radioactivity of the PS bands on the dried gels was measured using an image analyzer. The amount of radioactive PS in the cell lysates at the beginning of the experiment is assigned a value of 100%. Graphs represent radioactivity recovered from cell lysates (♦), media (■), or the total (▲) at each time point. Total radioactivity was calculated as the sum of radioactivities recovered from media and lysates. Values represent mean ± SD of 3 (WT PS) or 4 (TSR-lack PS) independent experiments.

Binding of recombinant PS to moAbs recognizing the PS Gla domain and the Gla residues

Next, to identify the effect of the absence of the TSR in the PS molecule on its Gla domain conformation and γ-carboxylation, we performed moAbs binding assays using the serum-free culture media of transient transformants (Fig. 4). WT PS and TSR-lack PS showed similar binding to the anti-PS polyclonal antibody. In contrast, in the Ca^{2+}-dependent and conformation-dependent moAb MK21 (recognizing the PS Gla domain) binding assay, the binding of TSR-lack PS was weaker than that of WT PS, as described in previous reports [14]. In addition, the binding of TSR-lack PS to the moAb M3B (recognizing the Gla residues) was also clearly weaker than that of WT PS, suggesting that TSR-lack PS has impaired γ-carboxylation.

Discussion

In the present study, the DNA analysis of a patient with PS deficiency revealed a G-to-A transition at the last nucleotide of intron C of *PROS1* (c.260-1G>A). This novel point mutation abolishes the invariant AG dinucleotide in the acceptor splice site of intron C and

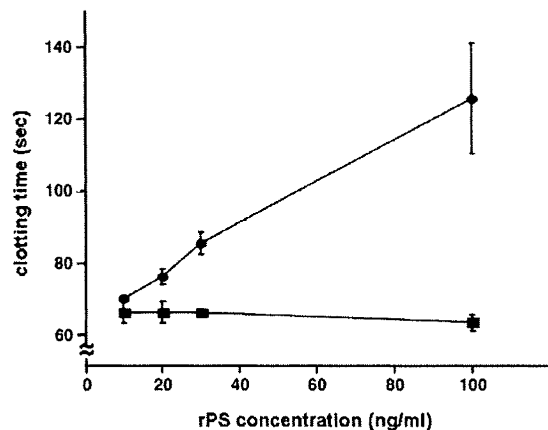

Fig. 3. APC cofactor activity of WT PS and TSR-lack PS. WT PS and TSR-lack PS at a range of concentrations (0-100 ng/mL) in serum-free media of stable transformants were incubated with PS-depleted plasma, factor Va, and APC for 2 min. Clotting was initiated by addition of $CaCl_2$, and clotting time was measured using ST art4. Values represent mean ± SD of 6 independent experiments. (●) indicates WT PS; (■), TSR-lack PS.

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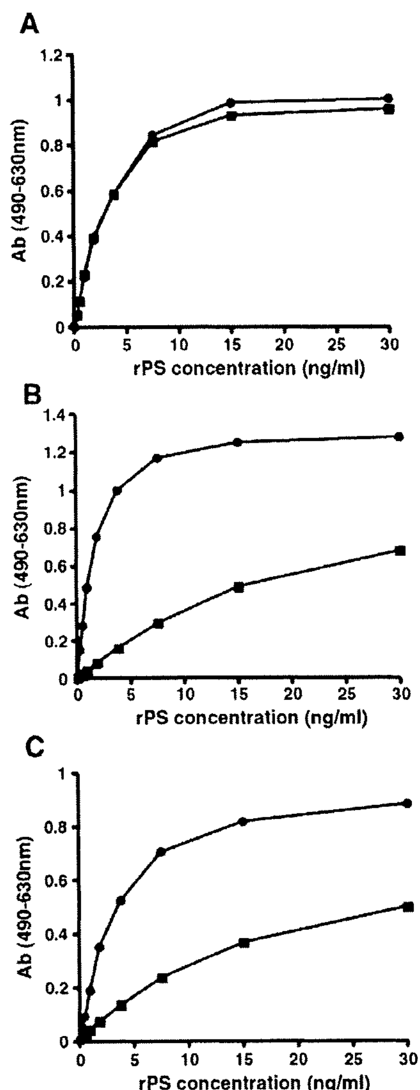

Fig. 4. Characterization of the Gla domain conformation and the γ-carboxylation of recombinant PS. The binding of WT PS (●), and TSR-lack PS (■) to polyclonal anti-PS antibody (A), moAb MK21 recognizing the PS Gla domain that is Ca^{2+}- and conformation-dependent (B), and the moAb M3B recognizing the Gla residues (C) in the culture media were measured by microtiter plate assay. The bound recombinant PS molecules were detected using peroxidase-conjugated anti-PS polyclonal antibody.

may subsequently induce cryptic splicing of the mutated mRNA [24]. Okamoto et al. reported that the region corresponding to exon 4 of *PROS1* containing a nonsense mutation (c.308C>G: p.Ser103X) was absent in mutant mRNA, and that the amount of the abnormal PS transcript was markedly reduced in a patient with a quantitative PS deficiency [25]. In fact, our RT-PCR analysis also revealed the presence of aberrant mRNA lacking a section corresponding to exon 4, the amount of which was markedly reduced in our patient. It was supposed that this splice site mutation affected the splicing of the aberrant pre-mRNA and impaired the mRNA processing efficiency, and possibly also the *in vivo* stability of the aberrant mRNA which could be lower than that of WT mRNA, resulting in the low mutant mRNA level [26]. It is apparent that the reduced mRNA level associated with the failed transcription of an exon caused by the splice site mutation is mainly responsible for the quantitative PS deficiency in our patient.

The failed transcription of exon 4 causes an in-frame deletion of 29 amino acids that constitute the TSR of the PS molecule (c.260_346del: p.Val87_Asn115del). The complete deletion of a domain as important as the TSR is expected to affect protein structure and function. To demonstrate the effect of the absence of the TSR in the PS molecule on its expression/secretion from cells, we carried out transient expression analysis using COS-1 cells. Measurement of the amount of protein secreted into the culture media by ELISA is useful for assessing the overall efficiency of the protein expression/secretion pathway because an ELISA result depends on the efficiency of every step of the expression/secretion pathway, including transcription, mRNA stability, translation, secretion, and protein stability in the culture medium. In addition, we performed pulse-chase analysis to focus on the steps from primary protein synthesis to secretion in the protein expression/secretion pathway. Our ELISA result showed that the amount of TSR-lack PS secreted into the culture medium was markedly reduced to 37.9% of that of WT PS, indicating that some steps in the protein expression/secretion pathway were impaired. Moreover, the data from pulse-chase analysis also showed that the secretion efficiency of TSR-lack PS was significantly lower than that of WT PS. Thus, it is considered that the absence of the TSR in the PS molecule leads to its impaired secretion, resulting in the quantitative PS deficiency similar to the missense mutations associated with quantitative PS deficiency reported previously [27]. The present study indicates that not only the decrease in the level of mutant mRNA, but also the impairment of mutant PS secretion, is responsible for the quantitative PS deficiency in the patient.

In some reports, the TSR of PS is described as being essential for APC cofactor activity, because the TSR is necessary to maintain the correct conformation of the PS Gla domain for its binding to membranes [12,13]. Our data clearly show that TSR-lack PS has no APC cofactor activity, in contrast to WT PS, in the clotting-based assay (Fig. 3). In addition, the binding assay of moAb MK21 recognizing the PS Gla domain showed that TSR-lack PS bound to MK21 with significantly lower affinity than WT PS, indicating that the Gla domain might be incorrectly folded in TSR-lack PS (Fig. 4B). Furie et al reported that the vitamin K-dependent proteins lack biological activity if γ-carboxylation is impaired [28]. We also demonstrated that the binding affinity of TSR-lack PS to the moAb M3B, which recognizes the Gla residues, was much lower than that of WT PS (Fig. 4C), indicating that the γ-carboxylation of TSR-lack PS was at least partially impaired. Therefore, it was speculated that the absence of the TSR affected the conformation of the γ-carboxylase-recognizing region near the Gla domain of the PS molecule, leading to partial impairment of the γ-carboxylation of TSR-lack PS. Our findings suggest that the TSR of PS might have an important role in maintaining the conformation of the Gla domain of PS required for its proper γ-carboxylation, and that the absence of the TSR might result in the loss of APC cofactor activity.

Unfortunately, we were unable to measure the levels of plasma PS before the patient started warfarin therapy, which makes it difficult to discuss the genotype-phenotype relationship in detail. However, our observations suggest that in our patient, the plasma antigen concentration of TSR-lack PS is very low because of the reduced mutant mRNA level and the impaired secretion of TSR-lack PS. Furthermore, even though a small amount of TSR-lack PS was identified in the plasma, it is expected to be inactive as an APC cofactor. Thus, we consider that the patient would have low plasma PS antigen and activity levels even if he had not undergone the warfarin treatment. In this study, we identified a novel splice site mutation in *PROS1* leading to a reduced mutant mRNA level and the absence of the TSR in the mutant PS molecule. We also characterized the mutant TSR-lack PS molecule to investigate the molecular effects of the mutation. These molecular approaches could contribute to a greater understanding of the relationship between the genetic mutation and clinical phenotypes.

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