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bleeding, by administrating SSRIs to patients receiving interferon-α therapy.[3,4]

Ribavirin was used in combination with interferon in the treatment of patients with CHC in the study by Martin et al.[1] We have reported that ribavirin in combination with interferon, and possibly alone, may reduce the need for clotting factors in hemophilic patients with CHC.[5] In our hospital, we observed a marked reduction in the use of clotting factors in 5 of the 8 hemophilic patients who were being treated for CHC with ribavirin plus interferon. In contrast, no reduction in the use of clotting factors was observed in 47 hemophilic patients previously treated for CHC with interferon alone in our hospital. These findings strongly suggested that the reduced use of clotting factors resulted from the addition of ribavirin.

In the same group of patients, we found that the procoagulant activity of factor VII was elevated in all patients after receiving ribavirin in comparison with activity of factor VII before ribavirin administration.[6] In patients with CHC, the international normalized ratio (INR; prothrombin time) decreased continuously during therapy with peginterferon plus ribavirin in patients both with and without coagulation disorder; INR increased to the pretreatment value after therapy (data not shown). It is possible that ribavirin enhanced coagulation factor activity in both types of patients, reducing the risk of bleeding during therapy. These findings suggest that addition of ribavirin to interferon may be the reason why the risk of bleeding was lower than during antiviral therapy for patients with CHC in the report by Martin and colleagues.[1]

Weinrieb et al.[2] reported that critical upper gastrointestinal bleeding occurred in a patient with CHC being treated with interferon plus ribavirin and an SSRI. However, this patient was suffering end-stage liver disease and was also being given aspirin. We believe that this patient was at risk for bleeding even without an SSRI administration.

Interferon plus ribavirin is now standard therapy for patients with CHC because the efficacy of this combination is higher than that of interferon monotherapy.[9] Morasco et al.[10] reported that use of prophylactic SSRIs to prevent interferon-α induced depression in patients with CHC was not beneficial. Once a patient develops depressive symptoms, however, SSRIs can be successfully used to treat depression in patients with CHC who are receiving interferon-α therapy and ribavirin treatment. From Martin and colleagues' results[1] and ours, the risk of bleeding due to SSRIs looks likely to be relatively low when ribavirin is administered in addition to interferon for patients with CHC. When treating patients with CHC, physicians have to carefully observe whether bleeding episodes will increase after the reduction or stop of ribavirin due to ribavirin-induced anemia. In addition, when we treat other diseases, including chronic hepatitis B, leukemia, and renal cell carcinoma, with interferon alone, we may have to watch for bleeding during SSRI administration. Further randomized controlled studies of bleeding risk in patients given or not given SSRIs during therapy with interferon plus ribavirin are warranted.

Dr. Martin was shown this letter and declined to comment.

The authors report no financial affiliation or other relationship relevant to the subject of this letter.

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Effect of Ribavirin, in Combination With Interferon in Patients With Hepatitis C, on the Bleeding Risk Associated With Selective Serotonin Reuptake Inhibitors

Sir: We read with great interest the recent article by Martin et al.[1] reporting that the bleeding risk associated with use of selective serotonin reuptake inhibitors (SSRIs) during antiviral therapy for chronic hepatitis C (CHC) is lower than that previously reported.[2] Several reports had revealed the increasing risk of bleeding, including retinal hemorrhages or gastrointestinal

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Takashi Honda, M.D., Ph.D.
Yoshiaki Katano, M.D., Ph.D.
Hidenori Toyoda, M.D., Ph.D.
Kazuhiko Hayashi, M.D., Ph.D.
Masatoshi Ishigami, M.D., Ph.D.
Isao Nakano, M.D., Ph.D.
Hidemi Goto, M.D., Ph.D.
Department of Gastroenterology
Koji Yamamoto, M.D., Ph.D.
Junki Takamatsu, M.D., Ph.D.
Department of Transfusion Medicine
Nagoya University Graduate School of Medicine
Nagoya, Japan

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Review Article

Intravenous Immunoglobulin Therapy for Acquired Coagulation Inhibitors: A Critical Review

Koji Yamamoto,[a] Junki Takamatsu,[a] Hidehiko Saito[b]

[a]*Department of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan;*
[b]*Nagoya Central Hospital, Nagoya, Japan*

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Abstract

Intravenous immunoglobulin (IVIG) therapy has been used for autoimmune diseases and disorders involving autoantibodies, including coagulation inhibitors. In this review, we have evaluated the efficacy and safety of IVIG therapy for acquired coagulation inhibitors, including factor VIII inhibitor, and for acquired von Willebrand syndrome on the basis of 44 reports published between 1965 and 2005. Among 35 patients with factor VIII inhibitor, we estimated the efficacy of IVIG therapy alone (which includes complete remissions and partial responses with a clinical benefit) to be 30% (11 cases), whereas the response to combination therapy with IVIG plus immunosuppressive agents (eg, corticosteroid, cyclophosphamide) seemed to be better (approximately 70%, 33/45 cases) than with IVIG therapy alone. In acquired von Willebrand syndrome, the efficacy of IVIG therapy was estimated to be 30%. The response to IVIG therapy appears to occur rapidly, and coagulation inhibitors seem to be neutralized immediately. Moreover, severe complications or side effects rarely occur during IVIG treatment. IVIG therapy thus may be considered one choice for treating acquired coagulation inhibitors, although its efficacy improves when used in combination with immunosuppressive agents.
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Key words: Intravenous immunoglobulin therapy; Acquired coagulation inhibitors; Autoimmune disease; Factor VIII inhibitor; von Willebrand syndrome

1. Introduction

Intravenous immunoglobulin (IVIG), a highly purified immunoglobulin G (IgG) fraction derived from pooled human plasma, is currently one of the most widely used plasma components in the world [1,2]. It was originally introduced as replacement therapy for patients with primary immunodeficiency disorders. In 1981, Imbach et al reported a serendipitous observation that a high-dose infusion of IVIG (2 g/kg of body weight infused over 5 days) was able to transiently increase the platelet count in children with idiopathic thrombocytopenic purpura (ITP) [3]. With the encouragement of this and other reports on ITP [4], the clinical applications of IVIG have increased markedly over the past 25 years to include many autoimmune diseases. IVIG has been shown to be efficacious in clinical trials for graft-versus-host disease [5], myasthenia gravis [6], Guillain-Barré syndrome [7], Kawasaki disease [8], and chronic inflammatory demyelinating polyneuropathy [9]. It has also been used to treat immune neutropenia and coagulation inhibitors [10-12], but its efficacy and safety have not been firmly established.

Coagulation inhibitors, antibodies against individual clotting factors, interfere with blood coagulation. The most common coagulation inhibitor is factor VIII inhibitor, an antibody against factor VIII that neutralizes the coagulant activity of factor VIII. Factor VIII inhibitor develops in patients with hemophilia A as an alloantibody after replacement therapy or spontaneously as an autoantibody in nonhemophilic patients [13], including postpartum patients and those with autoimmune disease, malignancy, or diabetes [14]. Once developed in such patients, factor VIII inhibitor poses a serious problem for the management of bleeding episodes, because any infused factor VIII will be rapidly neutralized and will not be available to induce hemostasis [15]. Although IVIG therapy has been used as one of the immunotherapies

Correspondence and reprint requests: Koji Yamamoto, Department of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan; fax: 81-052-744-2610 (e-mail: kojiy@med.nagoya-u.ac.jp).

for eradicating coagulation inhibitors, such an indication is considered off label [2].

The aim of this review is to examine the efficacy and safety of IVIG therapy in patients with acquired inhibitors against factors VIII, IX, or V, and in patients with acquired von Willebrand disease. Cases with lupus anticoagulant were not included in this review. An electronic search of the Medline/PubMed database from 1965 to 2005 was performed to identify relevant articles. This search yielded 108 citations, 72 of which were considered appropriate and reviewed. The bibliography of each review paper was examined to identify articles that may have been missed by our electronic searches.

2. History

In 1983, Nilsson et al reported an interesting observation [11]. A patient with severe hemophilia B and factor IX inhibitor was treated with extracorporeal protein A–Sepharose adsorption to remove the inhibitor, followed by the administration of factor IX concentrate and cyclophosphamide. This procedure produced a 15-fold increase in factor IX inhibitor on one occasion but did not cause any increase of the inhibitor titer on another occasion, when 5 g of IVIG was also given to the patient to restore the reduced IgG level. The investigators suggested that the administration of IVIG appeared to suppress antibody synthesis in hemophilia B patients with factor IX inhibitor.

Three groups of investigators reported the use of IVIG in the management of factor VIII inhibitors in 1984 [12,16,17]. IVIG therapy combined with vincristine produced a transient disappearance of acquired factor VIII inhibitor along with a slow rise of factor VIII activity in a 13-year-old boy with autoimmune disease [16]. IVIG therapy was ineffective in 2 patients with hemophilia A inhibitor [17]. Sultan et al [12] reported that IVIG therapy (0.4 g/kg body weight per day for 5 days) resulted in the rapid, marked, and prolonged suppression of factor VIII inhibitor in 2 patients with acquired factor VIII antibody (autoantibody) but that it had little or no effect in 2 hemophilic patients with factor VIII antibody (alloantibody). They showed by in vitro experiments that IVIG preparations were able to neutralize the anti–factor VIII activity of the patients' plasma and the IgG fraction of the patients' sera. Many articles were subsequently published on the effect of IVIG on acquired factor VIII inhibitors, as is discussed later.

3. Possible Mechanisms of Action

The rapid rise in the platelet count in ITP following IVIG administration is thought to occur through binding to and blocking Fcγ receptors on macrophages, thereby preventing the removal of antibody-coated platelets by the reticuloendothelial system in the spleen and liver [4]. This mechanism, however, does not appear to explain the effect on coagulation inhibitors.

Several hypotheses on the mechanisms of action of IVIG on factor VIII inhibitor have been put forward. Sultan et al and Kazatchkine and Kaveri postulated that anti-idiotypic antibodies present in IVIG preparations neutralize factor VIII autoantibodies [12,18]. $F(ab')_2$ fragments from IVIG preparations inhibited anti–factor VIII activity in $F(ab')_2$ fragments from the patient's plasma. Anti–factor VIII $F(ab')_2$ fragments were specifically retained on an affinity column of Sepharose-bound $F(ab')_2$ from IVIG, indicating that a direct interaction occurred through the antibody-binding sites of both immunoglobulins [19]. Anti-idiotypes against various autoantibodies were shown to be present in pooled normal human polyspecific immunoglobulin. In addition, IgG prepared from elderly donors and multiparous women was reported to contain a higher frequency of neutralizing antibodies against factor VIII autoantibodies [20]. It is puzzling that such an in vitro antibody-neutralizing effect was not always demonstrated, even though in vivo administration of IVIG produced a marked reduction of the inhibitor titer [21,22].

The fall in inhibitor titer following IVIG therapy without simultaneous immunosuppressive treatment appears to be rapid (within several days) in most cases [12,23,24] but is slow (more than 10 days) in others [22,25]. There must be slow effects of IVIG on autoantibody production. In addition to its direct and immediate action on antibodies, IVIG has been proposed to suppress antibody formation by B-cells, a process mediated through the down-regulation of Fcγ receptors [26]. Furthermore, IVIG may induce T-cell suppressor activity [27]. These observations taken together suggest that IVIG exerts its effect on the inhibitor titer through more than one mode of action.

4. Efficacy

4.1. Factor VIII Inhibitor

We extensively reviewed the international literature published from 1965 to 2005. The typical IVIG dosage used for treating factor VIII inhibitor was 0.4 g/kg per day for 5 consecutive days.

The efficacy criteria (ie, the response to IVIG therapy) were as follows [28]: Complete remission (CR) was defined as the disappearance of the inhibitor, partial response (PR) was defined as a decrease in the inhibitor titer by at least 25% of the baseline value, and failure was defined as other than CR and PR.

In Table 1, we present all of the cases in which the efficacy of IVIG treatment alone was evaluated [12,22-25,28-40]. The response to IVIG therapy alone was failure in 11 cases (31.4%) and PR in 21 cases (60.0%), but with a subsequent clinical benefit in only 8 patients. Finally, 3 patients (8.6%) achieved CR. The efficacy of IVIG therapy alone, which includes CR and PR with a clinical benefit, among these 35 patients was estimated to be 31.4% (11 cases). In most cases of CR or PR, the response to IVIG treatment was rapid, and factor VIII inhibitor seemed to be neutralized immediately.

We summarize the responses to combined therapy with IVIG plus immunosuppressive agents in Table 2 [21,25, 28,32,35,38-52]. The response to IVIG plus steroid and/or cyclophosphamide therapy was better than to IVIG treatment alone. CR was achieved in 19 (73%) of 26 patients who were treated with IVIG plus steroid. In addition, 14 (74%) of 19 patients who received IVIG plus steroid and

Table 1.
Evaluable Patients from the Literature with Acquired Factor VIII Inhibitor Who Were Treated with Intravenous Immunoglobulin (IVIG)*

No.	Reference	Sex/Age, y	Associated Disease	IVIG Dosage, g/kg per d	Inhibitor Titer, Bethesda U		Response	Clinical Outcome
					Before	Nadir (d†)		
1	Hudak et al [29]	F/40	Postpartum	0.5 × 5 d	16	<1 (105)	CR	Sustained remission
2	Schwartz et al [25]	M/68	CLL	1 × 2 d	1	0 (14)	CR	Sustained remission
3	Schwartz et al [25]	F/83	Diabetes	1 × 2 d	0.9	0 (61)	CR	Sustained remission
4	Sultan et al [12]	M/62	Idiopathic	0.4 × 5 d	25,000	550 (3)	PR	No clinical benefit‡
5	Sultan et al [12]	F/29	Postpartum	0.4 × 5 d	10,500	1000 (3)	PR	No clinical benefit
6	Zimmermann et al [30]	F/64	Idiopathic	0.5 × 8 d	75	10 (25)	PR	Clinical benefit
7	Zimmermann et al [30]	F/70	Idiopathic	0.5 × 8 d	51	3.8 (9)	PR	Clinical benefit
8	Newland et al [22]	F/71	Diabetes	0.4 × 5 d	50	20 (45)	PR	Clinical benefit
9	Heyman et al [31]	M/64	Idiopathic	0.4 × 5 d	47	28 (17)	PR	No clinical benefit
10	Nishida et al [23]	F/39	Idiopathic	0.4 × 5d	115	17 (3)	PR	No clinical benefit
11	Schwerdtfeger et al [32]	F/31	Postpartum	0.5 × 5 d	420	104 (6)	PR	No clinical benefit
12	Sultan et al [33]	M/78	NA	0.4 × 5 d	42	20 (30)	PR	No clinical benefit
13	Sultan et al [33]	M/72	Carcinoma	0.4 × 5 d	38	10 (5)	PR	Transient benefit
14	Schwartz et al [25]	M/54	Alcoholism	1 × 2 d	1228	208 (7)	PR	No clinical benefit
15	Schwartz et al [25]	F/72	Idiopathic	1 × 2 d	880	570 (48)	PR	No clinical benefit
16	Schwartz et al [25]	F/25	Idiopathic	1 × 2 d	280	1.9 (57)	PR	Clinical benefit
17	Schwartz et al [25]	F/38	Postpartum	1 × 2 d	102	56 (22)	PR	Clinical benefit
18	Schwartz et al [25]	M/77	Carcinoma	0.4 × 5 d	39	24 (3)	PR	No clinical benefit
19	Schwartz et al [25]	M/60	Griseofulvin	0.4 × 5 d	29	18 (19)	PR	No clinical benefit
20	Crenier et al [28]	M/65	Cardiomyopathy	0.4 × 5 d	120	72 (30)	PR	No clinical benefit
21	Crenier et al [28]	M/74	Bronchitis	0.4 × 5 d	24	12 (7)	PR	No clinical benefit
22	Michiels et al [24]	F/31	Postpartum	0.5 × 5 d	12	1 (11)	PR	Clinical benefit
23	Lafferty et al [34]	F/42	SLE	0.4 × 5 d	500	185 (NA)	PR	Clinical benefit
24	Walsh et al [35]	F/72	Cholecystitis	30 g × 1 d	6	NA	PR	Clinical benefit
25	Hiller et al [36]	M/57	Surgery	30 g × 5 d	24	20 (2)	F	Transient benefit
26	Casas et al [37]	M/70	Lymphoma	0.4 × 7 d	8.6	35 (NA)	F	Transient benefit
27	Sultan et al [33]	M/45	Vasculitis	0.4 × 5 d	25	28 (NA)	F	NA
28	Pignone et al [38]	F/66	RA	0.4 × 6 d	13	26 (7)	F	NA
29	Hauser et al [39]	F/29	Postpartum	0.4 × 5 d	10	110 (NA)	F	NA
30	Mateo et al [40]	F/82	CLL	0.4 × 5 d	9.5	10 (30)	F	NA
31	Schwartz et al [25]	M/64	Diabetes	1 × 2 d	452	340 (6)	F	No clinical benefit
32	Schwartz et al [25]	F/83	LA	0.4 × 5 d	102	96 (5)	F	No clinical benefit
33	Schwartz et al [25]	F/48	Idiopathic	1 × 2 d	59	46 (2)	F	No clinical benefit
34	Schwartz et al [25]	M/73	Carcinoma	0.4 × 5 d	42	108 (5)	F	No clinical benefit
35	Schwartz et al [25]	M/62	Idiopathic	1 × 2 d	1.4	1.4 (11)	F	No clinical benefit

*CR indicates complete remission; CLL, chronic lymphocytic leukemia; PR, partial response; NA, not available; SLE, systemic lupus erythematosus; F, treatment failure; RA, rheumatoid arthritis; LA, lupus anticoagulant.

†Number of days after starting IVIG treatment.

‡Subjective evaluation by the doctors in charge.

cyclophosphamide reached CR. Only 2 cases of treatment with IVIG plus cyclophosphamide were reported, and these patients achieved CR [52]. Conversely, 18 (75%) of 24 patients treated with steroid plus cyclophosphamide instead of IVIG achieved CR. This degree of efficacy is consistent with the report by Green et al [45]. In these reports, however, the evaluation of efficacy depended on the patients' symptoms (ie, improvement of bleeding tendency), because the disappearance of inhibitors was not followed up.

Thus, the overall efficacy of IVIG therapy alone is almost 30%, whereas that of a combination therapy with IVIG plus steroid and/or cyclophosphamide is approximately 70%.

Recent reports have described patients with acquired factor VIII inhibitors who rapidly responded to immunosuppressive regimens including rituximab, a monoclonal antibody against CD20+ B-cells [53,54]. These data suggest that immunosuppressive therapy using rituximab could become a powerful tool against coagulation inhibitors.

4.2. Acquired von Willebrand Syndrome

Acquired von Willebrand syndrome is a rare bleeding disorder with laboratory findings similar to those of congenital von Willebrand disease. According to an international registry, acquired von Willebrand syndrome is primarily associated with lymphoproliferative diseases, immunologic and cardiovascular disorders, and solid tumors. The prevalence of acquired von Willebrand syndrome in these underlying disorders is still unknown.

IVIG was also effective in stopping bleeding in acquired von Willebrand syndrome [55]. Several groups reported that acquired von Willebrand syndrome associated with systemic lupus erythematosus [56], monoclonal gammopathy [57-60], malignant lymphoma [61], and prostatomegaly [62], and of undefined origin [63,64] responded well to IVIG therapy. Some patients were successfully treated with the combination of IVIG and desmopressin, but the effect was transient

Table 2.
Responses of Patients with Acquired Factor VIII Inhibitor to Immunosuppressive Agents with or without Intravenous Immunoglobulin (IVIG) Therapy

	IVIG + Pr (26 Cases)			IVIG + Pr + Cy (19 Cases)			Pr + Cy (24 cases)		
Reference	CR	PR	F	CR	PR	F	CR	PR	F
Green et al [41]	1								
Carreras et al [21]	1								
Heyman et al [31]			1†						
OiSullivan et al [42]					1				
Pirner et al [43]					1				
Lionett et al [44]	1								
Pignone et al [38]							1		
Green et al [45]							5		5
Hauser et al [39]							1		
Mateo et al [40]	1								
Schwartz et al [25]	1	1							
Crenier et al [28]	1			1					
Lafferty et al [34]					1				
Sohngen et al [46]							2		
Bossi et al [47]	4		1	8		1	3		
Gandini et al [48]	1								
Dykes et al [49]	4	1	2						
Grunewald et al [50]				2			4		
Mazzucconi et al [51]	3	1							
Delgado et al [52]	1			3	1		2		1
Total	19	3	4	14	4	1	18		6

*Pr indicates prednisolone or dexamethasone; Cy, cyclophosphamide; CR, complete remission; PR, partial response; F, treatment failure.

†IVIG dosage: 0.4 g/kg per d for 2 d.

in most cases. According to data from an international registry, the efficacy of IVIG therapy in acquired von Willebrand syndrome was estimated to be 30% (21/63 patients) [65,66]. Of note, however, is that in most cases the efficacy of IVIG was subjectively evaluated (ie, a good response means to stop bleeding) by the doctors in charge. This efficacy is similar to that for treatment with desmopressin (38/119) or with immunosuppressive agents (23/66), but corticosteroids alone were effective in only 19% of patients (12/63).

4.3. Other Coagulation Inhibitors (Factor V or IX Inhibitor)

Patients with inhibitors against factor V or IX are extremely rare. Only one report described acquired factor IX inhibitor developing in a patient with autoimmune polymyositis [67]. Single-agent therapy with IVIG was effective in suppressing inhibitor synthesis and in stopping bleeding. Another report described acquired factor V inhibitor developing in an 82-year-old female patient following abdominal surgery [68]. Nine-day treatment with IVIG (0.4 g/kg per day) was partially effective in suppressing the inhibitor titer and improving the patient's hemorrhagic diathesis.

5. Safety

Adverse reactions to IVIG therapy are usually mild and self-limited: headache, back pain, low-grade fever, myalgia, and chills. The IVIG preparations currently in clinical use are also assumed to carry virtually no risk of transmitting infectious agents. Rarely, however, serious complications can occur. In recent years, thromboembolic complications have occasionally been reported in patients who received IVIG. Stroke, acute myocardial infarction, and deep vein thrombosis were estimated to occur at an incidence of 3% to 5% [69]. Thromboembolism appeared to develop mainly in patients who had other risk factors, such as an advanced age, being bedridden, and a history of thromboembolism. What triggers thromboembolic complications? During 5 courses of treatment with IVIG (24-54 g/day), the plasma IgG concentration was noted to increase 4-fold, and plasma viscosity increased to beyond the normal range [70]. It appears that increased blood viscosity after high-dose IVIG infusion is responsible for thromboembolism. Slow infusion of IVIG (a daily dose of 0.4 g/kg in not less than 8 hours) has been recommended to prevent thromboembolism [71].

Interestingly, our own review of the literature revealed no thromboembolic complications in 80 patients with acquired factor VIII inhibitor who had received IVIG. It is tempting to speculate that the presence of a coagulation inhibitor may counteract thrombosis formation.

6. Discussion

In general, treatments of acquired coagulation inhibitors are divided into 2 approaches: One is to stop the present bleeding events, and the other is to remove inhibitors by immunomodulative therapy. In cases of acute bleeding in patients with factor VIII inhibitors, conventional management consists of human factor VIII concentrate or desmopressin for low inhibitor levels (<5 Bethesda U) and porcine factor VIII or bypass therapy (eg, recombinant activated

factor VII, activated prothrombin complex concentrates) for high inhibitor levels (>5 Besthesda U). On the other hand, immunosuppressive agents (eg, corticosteroid, cyclophosphamide, azathioprine, rituximab) or IVIG has been used to suppress the generation of coagulation inhibitors. Other approaches are plasmapheresis and immunoadsorption using a protein A–Sepharose column to remove coagulation inhibitors, but the indications for these therapies are limited.

Evaluation of the response to one therapeutic modality in the management of coagulation inhibitors is not always easy, for a number of reasons. First, there are only a few inhibitor patients, and thus it is almost impossible to conduct a randomized clinical trial. There have been only a few such trials on acquired coagulation inhibitors [25,45]. This situation influences the evaluation of efficacy because cases of unsuccessful treatment with IVIG may not have been reported, with only successful cases having been evaluated. Second, most patients present with life-threatening bleeding and are treated with several different therapies simultaneously or sequentially. It is difficult, therefore, to assess the outcome of any single modality. Third, it is known that spontaneous fluctuation or disappearance of the inhibitor may occur [72].

As is shown in Table 1, the efficacy of IVIG therapy alone is not very high (ie, 30%). Moreover, the CR rates for combination therapy with IVIG plus glucocorticoid and/or cyclophosphamide (IVIG plus prednisolone/dexamethasone, 73%; IVIG plus prednisolone/dexamethasone and cyclophosphamide, 74%) did not differ from those of immunosuppressive agents without IVIG (prednisolone/dexamethasone plus cyclophosphamide, 75%) (Table 2). However, the clinical benefits of IVIG include a rapid response and fewer adverse effects, which are frequently observed with the chronic administration of glucocorticoid or other immunosuppressive agents. Regarding the use of cyclophosphamide in particular, it is possible for cytotoxicity to induce myelosuppression and secondary malignancy. Thus, IVIG therapy should be considered for acute massive bleeding in patients with acquired coagulation inhibitors because of its faster action. On the other hand, IVIG therapy costs approximately US $10,000 for a 5-day infusion, which is much more costly than other treatments except rituximab. These considerations taken together suggest that the use of IVIG for the management of acquired coagulation inhibitors might be limited, because whether a given treatment is used depends on the balance between cost and benefit.

7. Conclusion

For patients with acquired coagulation inhibitors against factor VIII, the efficacy of IVIG therapy alone was estimated to be 30% in 35 cases. On the other hand, the response to combination therapy with IVIG plus immunosuppressive agents (eg, corticosteroid, cyclophosphamide) seems to be better (ie, 70% in 45 cases) than IVIG as single-agent therapy. IVIG may be considered as one choice of treatment for acquired coagulation inhibitors, especially when a rapid response is required without myelosuppression, but its use alone would be limited because of its lower efficacy and high cost.

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ORIGINAL ARTICLE *VWD*

L1503R is a member of group I mutation and has dominant-negative effect on secretion of full-length VWF multimers: an analysis of two patients with type 2A von Willebrand disease

T. KASHIWAGI,* T. MATSUSHITA,† Y. ITO,‡ K. HIRASHIMA,† N. SANDA,* Y. FUJIMORI,* T. YAMADA,* K. OKUMURA,* A. TAKAGI,*§ T. MURATE,*,§ A. KATSUMI,† J. TAKAMATSU,¶ K. YAMAMOTO,¶ T. NAOE† and T. KOJIMA*§

*Departments of *Pathophysiological Laboratory Sciences; †Hematology and Oncology; ‡Division for Medical Research Engineering, Nagoya University Graduate School of Medicine; §Department of Medical Technology, Nagoya University School of Health Sciences; and ¶Division of Transfusion Medicine, Nagoya University Hospital, Nagoya, Japan*

Summary. Type 2A von Willebrand disease (VWD) is characterized by decreased platelet-dependent function of von Willebrand factor (VWF); this in turn is associated with an absence of high-molecular-weight multimers. Sequence analysis of the VWF gene from two unrelated type 2A VWD patients showed an identical, novel, heterozygous T → G transversion at nucleotide 4508, resulting in the substitution of L1503R in the VWF A2 domain. This substitution, which was not found in 60 unrelated normal individuals, was introduced into a full-length VWF cDNA and subsequently expressed in 293T cells. Only trace amount of the mutant VWF protein was secreted but most of the same was retained in 293T cells. Co-transfection experiment of both wild-type and mutant plasmids indicated the dominant-negative mechanism of disease development; as more of mutant DNA was transfected, VWF secretion was impaired in the media, whereas more of VWF was stored in the cell lysates. Molecular dynamic simulations of structural changes induced by L1503R indicated that the mean value of all-atom root-mean-squared-deviation was shifted from those with wild type or another mutation L1503Q that has been reported to be a group II mutation, which is susceptible to ADAMTS13 proteolysis. Protein instability of L1503R may be responsible for its intracellular retention and perhaps the larger VWF multimers, containing more mutant VWF subunits, are likely to be mal-processed and retained within the cell.

Keywords: von Willebrand factor, von Willebrand disease, dominant inheritance, mutation, type 2A, group

Introduction

von Willebrand factor (VWF), one of the largest plasma proteins in mammals, is an essential factor in the primary haemostasis. It forms a bridge between platelet membrane glycoprotein Ib and constituents of the extracellular matrix [1,2], thereby playing a critical role in the adhesion of platelets to sites of vascular injury. Binding of VWF to platelets is a regulated process. VWF is secreted by endothelial cells into either the blood or subendothelial matrix, and VWF in both locations can contribute to platelet adhesion [3,4].

The VWF functions are attributed to its domain structure, which is composed of D1-D2-D'-D3-A1-A2-A3-B1-B2-B3-C1-C2 [5]. The mature protein lacks the D1 and D2 propolypeptides and consists of 2050 amino acid residues. VWF undergoes extensive post-translational modifications including dimerization through multiple intramolecular disulfide bonds between the carboxyl-terminal ends of the protein [6] and once transported to the Golgi, multimerization through interdimer disulfide bonds between the amino-terminal ends [7,8]. Multimers are composed of various lengths but can contain more than 40 VWF subunits and reach sizes of >20 000 kDa.

Correspondence: Tadashi Matsushita, MD, Department of Hematology and Oncology, Nagoya University Graduate School of Medicine, 65-Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan.
Tel.: +81 52 744-2145; fax: +81 52 744 2161;
e-mail: tmatsu@med.nagoya-u.ac.jp

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Regulating the multimer size is crucial for the balance of haemostasis and thrombosis, because circulation of ultra-large VWF multimers results in the disorder known as thrombotic thrombocytopenic purpura, whereas a reduction in the size of multimers results in the bleeding disorder called von Willebrand disease (VWD) type 2A [9–11]. Type 2A VWD is represented by qualitative defect of VWF multimer size, although plenty of type 2A cases also present quantitative defect of VWF, as revealed by decreased immunoreactive antigens or decreased intensities of lower-sized multimer bands.

The multimer size of VWF is regulated by plasma protease ADAMTS13, which cleaves VWF between Tyr1605 and Met1606 in the A2 domain [12–14]. The A2 domain consists of residues 1480–1672 of VWF and numerous mutations in the A2 domain, which result in type 2A VWD [15–19]. Two distinct pathogenic mechanisms cause type 2A VWD. Group I mutations are characterized by impaired intracellular transport, storage and secretion of high molecular weight multimers, whereas group II mutations are characterized by increased susceptibility of the VWF protein to proteolysis by the ADAMTS13 protease [18]. In this report, we describe a novel mutation in a group I type 2A VWD at which the mutation L1503Q has been described as group II mutation [20]. The amino acid was substituted to arginine and our study indicates that this amino acid position is also important for protein conformation of VWF molecule.

Materials and methods

Patient and sample

Ethical approval for the study was obtained from the Ethics Review Committee of Nagoya University School of Medicine. After obtaining written informed consent, blood samples from the patient were collected in 1/10 volume of 3.13% sodium citrate. Plasma was separated by centrifugation at ×2000 *g* for 20 min, and genomic DNA was extended from peripheral blood leucocytes according to standard procedures [21]. Ristocetin cofactor activity of VWF (VWF:RcoF) was determined by a Latex agglutination test kit (von Willebrand Reagent, Dade Behring, Marburg, Germany). The sensitivity of this test was 0.06 U mL^{-1} and CV was within 20% of five independent assays. Multimer analysis was performed based on a method by Raines *et al.* [22] that uses SDS-1.5% agarose gel electrophoresis and capillary blotting.

VWF mutation analysis

Exon 14, 18, 27, 28 including its exon/intron boundaries of human VWF gene were amplified by PCR in two steps using GeneAmp PCR System 9700 and analysed by ABI PRISM 310 Genetic Analyzer (Applied Biosystems; ABI, Forster City, CA). The fist amplification was performed with primers allowing the specific amplification of the VWF gene and avoiding that of the pseudogene (Table 1) [23]. One

Table 1. Primers.

Name of primer	Length	Sequence	Position*
For PCR and sequencing			
Vi27-u	22	5′-TGTGGGAATATGGAAGTCATTG-3′	Intron27-93
Vi28-d	23	5′-GTATCTTGGCAGATGCATGTAGC-3′	Intron28+69
281 U	19	5′-CTGTCCGAGGCTGAGTTTG-3′	3871
319 L	19	5′-CCGCTCCATCATGTCCACC-3′	3900
672 U	18	5′-CCCATGCCAACCTCAAGC-3′	4263
706 L	18	5′-TTCTCAGGGGCCTGCTTC-3′	4287
1014 U	18	5′-AGGACAGCATCCACGTCA-3′	4605
1080 L	18	5′-CCCCTTTGGACTGTGCCT-3′	4661
For PCR-restriction fragment length polymorphism			
Exon28-SmaI/U			19
Exon28-SmaI/L	20	5′-CAGTGTTGGTCCTGTTGCCG-3′	4716
For mutagenesis			
AU	20	5′-TGCGTTGACCCTGAAGACTG-3′	3568
AL	18	5′-TTTGTCCGATCCTTCCCG-3′	4507
BU	18	5′-CCGGGAAGGATCGGACAA-3′	4509
BL	18	5′-AGAGGTACCGCAGGGCCA-3′	4751

*Nucleotide 1 is an adenine of the ATG-translation initiation codon. Beginning of the intron; a plus sign and the position in the intron. The end of the intron; a minus sign and the position upstream in the intron.

hundred nanogram of genomic DNA were incubated in 10× *TaKaRa Ex Taq* buffer with 20 pmoles of specific primers, 0.32 nmol dNTP, 0.5 U of *TaKaRa Ex Taq* polymerase in final volumes of 20 mL (Takara, Ohtsu, Japan). After 2 min of denaturation at 94°C, samples were subjected to 30 amplification cycles. Each cycle comprised 30 s denaturation at 94°C, 30 s annealing at 60°C, 2 min extension at 72°C. Reaction was terminated by a final extension for 7 min at 72°C. The second PCR conditions were approximately the same except for annealing temperature (55°C) and extension time (1 min). PCR fragments were purified on QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany) and reaction was with Big Dye Terminator Cycle Sequencing FS Ready Reaction kit (ABI) as described previously [24].

To determine the 4508T → G transition in exon 28 of VWF gene, we used mismatch strategies for PCR–restriction fragment length polymorphism (RFLP) analysis. Briefly, PCR was performed using a partially mismatched sense primer, which introduces *Sma*I site only into mutant allele PCR products, and the antisense primer (Table 1) used for DNA amplification of exon 28 of the VWF gene sequence except for pseudogene. The PCR products were digested with *Sma*I and electrophoresed on a 2% agarose gel.

Plasmid construction

Plasmid pSVHVWF1.1 [25] contain a full length normal human VWF cDNA cloned into the expression vector pSV7D [26] as described [27]. Plasmid pSVHL1503R contains a T → G transition of nucleotide 4508 of pSVHVWF1.1 resulting in a substitution of Leu by Arg at amino acid 1503 of the mature VWF. PCR was performed using the pSVHVWF1.1 vector as template with following primers (Table 1). The two PCR fragments obtained with primers AU-AL and with BU-BL were used as template for an additional PCR with Primer AU and BL. The corresponding PCR product was cloned into pBluescript II KS+ and the DNA sequence of the fragment was confirmed. Finally, and the amprified fragment was inserted into pSVHVWF1.1 that was digested with *Nae*I and *Kpn*I.

Expression of recombinant VWF

Human embryo kidney 293T (HEK293T) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and phenol red (Wako chemicals, Osaka, Japan), supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.25 μg mL^{-1} amphotericin B, and 6%(v/v) foetal bovine serum (FBS) at 37°C in 5% CO_2. The cells cultured in 100-mm dishes until they became 50–70% confluent, and then were transiently transfected with plasmids pSVHVWF1.1 (wild type) and pSVHL1503R (mutant) using a calcium phosphate method as described previously [28]. Twenty-four hours after transfection, cells were washed twice with phosphate-buffered saline, and incubated with 10 mL FBS-free DMEM. After 48 h, cell lysates were dissolved in the reporter lysis buffer (Promega, Madison, WI) and collected in line with the cell culture media, followed by centrifugation at ×2500 *g* for 10 min. In transfection experiments using a single construct, 10 μg of DNA was used. Dose-dependent series of co-transfection experiment comprised a total amount of 9 μg DNA with various wild-type and mutant mixture. A pCI plasmid was used to bring the DNA amount to 9 μg. The transfection efficacy was determined for each experiment by measuring the luciferase activity of co-transfected 9.6 μg of pRL-SV40 vector and Dual Luciferase Reporter Assay System (Promega). Antigen levels of recombinant VWF were measured by an enzyme-linked immunosorbent assay (ELISA) using polyclonal rabbit anti-human VWF antibody A082 and peroxidase-conjugated rabbit anti-human VWF antibody P226 (DAKO, Carpinteria, CA) [29]. For each experiment, relative expression level was determined by ELISA and normalized according to the transfection efficacy determined by measuring the luciferase activity.

Molecular dynamics simulation

Molecular dynamics simulations were carried out using the program MOE (Chemical Computing Group Inc., Montreal, QC, Canada) for computer simulation of biomolecules, with the aid of CHARMM27 program for calculation of the force field. The coordinate of the A2 domain of human VWF has been built based on a homology-modelling from the structures of six template proteins [30] and the downloaded PDB file was introduced into our system.

The simulation consisted of molecular mechanical calculation of the minimal energy and heating of the system from the crystalline state at $T = 0$ K to a temperature of 310 K. Coordinates were saved every 0.5 ps and the system was simulated for 10 ns. Comparative analysis of the configurations of wild type and mutant VWF-A2 domain at identical time points and identical environmental conditions was performed during dynamical

changes. This yielded a real-time visualization for the whole simulation period, from 0 to 10 ns (Fig. 4). Calculation of the root-mean-squared-deviation (RMSD) was performed using SVL language of MOE program.

Results

Patients

Patient A was a 17-year-old woman suffering from frequent epistaxis and hypermenorrhoea, and has been diagnosed as having VWD. Multimer analysis showed relative loss in high- and middle-sized molecular weight multimers, although smaller-sized multimers; the second to fourth multimer band, were also decreased. (Fig. 1). The mother of patient A has been also diagnosed as having VWD, although detailed analysis has not been performed. Patient B is a 49-year-old man with frequent episodes of epistaxis and his father and son have also been also diagnosed as having VWD. Multimer analysis of patient B has not been available. Patient A and B had reduced VWF ristocetin cofactor activity (VWF:RcoF; 0.10 U mL^{-1} and 0.09 U mL^{-1}, respectively), whereas the VWF antigen levels were decreased by 0.19 U mL^{-1} and 0.28 U mL^{-1}, respectively (Table 2). So far no familial relationship has been identified between the two families.

Fig. 1. Plasma multimer analysis of patient A: plasma from patient A was electrophoresed on SDS-agarose gel and visualized as described in Materials and methods. NP, normal plasma.

Table 2. Plasma VWF concentrations of the patients.

	VWF: Ag (U mL^{-1})	VWF: RcoF (U mL^{-1})	FVIII: C (U mL^{-1})
Patient A	0.19	0.10	0.327
Patient B	0.28	0.09	0.414

VWF: Ag, VWF antigen; VWF: RcoF, VWF ristocetin cofactor activity; FVIII: C, factor VIII procoagulant activity.

In 2006, patient B visited the hospital and complained of uncontrollable epistaxis. 1-8 deamino-D-arginine vasopressin (DDAVP) (28 μg $body^{-1}$: 0.4 μg kg^{-1}) was administrated followed by prompt haemostasis. Unfortunately, the plasma VWF levels were not studied either before or after DDAVP infusion.

Molecular analysis

In both patients, DNA sequence analysis of the VWF exon 28 identified a mutation, a novel T → G transition at nucleotide 4508 of the cDNA that caused amino acid substitution at position 1503 of a leucine with an arginine, L1503R (supplementary Fig. S1). The mismatch PCR–RFLP (*Sma*I-RFLP) method was employed to detect the 4508 T → G transition in exon 28 of the VWF gene of patient A and B (Fig. 2a). The undigested and digested pattern (247 + 228 bp) found in the patients confirmed heterozygous state for the mutation. On the other hand, genomic DNAs from 60 normal individuals presented the homozygous undigested pattern with one single band of 247 bp (Fig. 2a).

Expression of mutant VWF

To determine the effect of the mutation (L1503R) on VWF structure and function, expression vectors pSVHL1503R were transiently transfected into HEK293T cells. VWF antigen levels were assayed in the cell lysates and conditioned media using VWF-specific ELISA (Fig. 3). Relative expression level was normalized according to the transfection efficacy determined by luciferase activity of co-transfected pRL-SV40 vector. Wild-type recombinant VWF was secreted efficiently, achieving a concentration of 1.07 ± 0.41 μg mL^{-1} (range of triplicates) in conditioned media compared with 0.27 ± 0.10 μg mL^{-1} in cell lysates (data not shown). In contrast, rVWF (L1503R) was secreted poorly, reaching a concentration of only 7.2% of wild type in the conditioned media, whereas there was 71.5% of wild type in cell lysates (Fig. 3a).

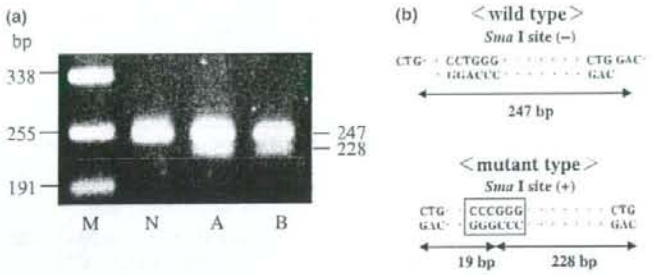

Fig. 2. Mismatch PCR–RFLP analysis: PCR products amplified by two mismatch primers were digested by *Sma*I and analysed by 2% agarose gel electrophoresis as described in the Materials and methods. Lane M, pGEXE/Hinf I digested DNA marker; NP, normal control; A or B is the patient A and B.

Co-expression analysis

Expression analysis of single construct suggested that the mutant protein is not secreted but stored in the cell. Concerning the dominant inheritance of type 2 VWD, it is expected that heterozygous expression of mutant protein may also block the secretion of normal VWF. To mimic the dominant-negative phenotype caused by heterozygosity for the L1503R mutation, plasmid mixtures of wild-type and mutant VWF (L1503R) were co-transfected in HEK293T cells. For a fixed amount of wild-type plasmid (3 μg mL^{-1}), increasing amounts of mutant plasmid caused a dose-dependent inhibition of VWF secretion (Fig. 3b) and a corresponding increase in the level of intracellular VWF (Fig. 3c). Transfection with increasing amounts of only wild-type VWF plasmid did not impair secretion, and intracellular VWF was linearly increased (Fig. 3b, c). These observations indicate that the effects caused by co-transfection with mutant plasmid were specific for the L1503R substitution and are consistent with the proposed

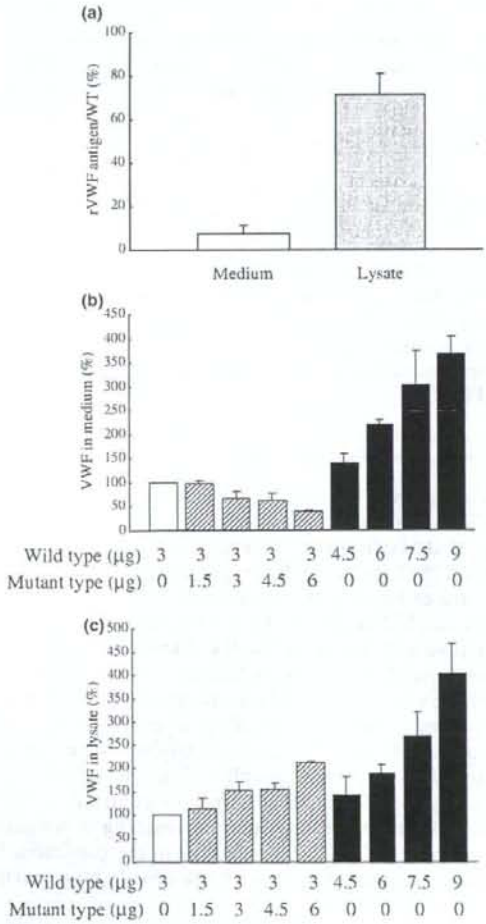

Fig. 3. Transfection experiment of wild type and mutant construct of full-length cDNA of human: VWF wild type and L1503R is expressed in HEK293T cells as described in the Materials and methods. After transfection, cell lysates and media were harvested after 48 h and subjected to ELISA for VWF antigen levels. Expression level was normalized according to the transfection efficacy determined by luciferase activity of co-transfected pRL-SV40 vector. (a), Single transfection analysis; Expression levels of the media (open column) and cell lysates (shaded column) are shown relative to that for expression levels of wild type VWF expressed at the same time. Each column represents the mean ± SD of values obtained in three independent sets of duplicate assays. (b)–(c), Co-transfection of plasmid mixtures of wild type and mutant VWF cDNA; co-transfection was performed by the mixture of varying concentrations (1.5–6 μg mL^{-1}) of mutant plasmids and fixed amount (3 μg) of wild type plasmid (hatched column). As control, varying concentrations (1.5–6 μg mL^{-1}) of wild type plasmid plus 1.5 μg mL^{-1} of same wild type plasmid were transfected (closed column). Relative value of expression is shown relative to that for expression levels of 3 μg of wild type rVWF performed at the same time (open column) as 100%. The VWF antigen levels in the medium (b) and lysate (c) is determined as described above. Each column represents the mean ± SD of values obtained in two independently duplicated transfections.

dominant-negative effect of the L1503R mutation *in vivo*.

Molecular dynamics simulation

Molecular dynamics simulation was used to study the dynamical effects of the mutation introduced into the structural model. Data simulated from wild type human VWF A2 domain were compared with the two mutant forms L1503R and L1503Q. The mutation L1503Q has been classified as group II mutation that causes the increase in the protein's susceptibility to ADAMTS13 proteolysis [20]. Figure 4 shows the results of calculations of RMSD of the structure of the A2 molecule over a period of 1–10 ns. The mean value of all-atom RMSD of the wild type, L1503R and L1503Q was 3.368 ± 0.057, 3.846 ± 0.109, and 3.449 ± 0.131 respectively. The value of L1503R was in the highest level of energy equalization, although those of the wild type and L1503Q were close to each other and were in lower levels (Fig. 4). It is thus suggested that the presence of L1503R mutation strongly alters the mean square deviation and initiates conformational changes.

Discussion

Our patients' plasma showed markedly reduced levels of VWF ristocetin cofactor activity, with 0.2–0.3 U mL^{-1} of VWF antigen levels (Table 2). Plasma multimer analysis of patient A indicated that larger-sized multimer was relatively decreased. Currently, the patient is diagnosed as type 2A VWD, although any pathological mechanism leading to quantitative VWF defect is not excluded.

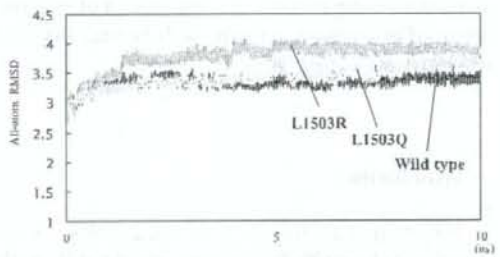

Fig. 4. All-atom root-mean-square deviation (RMSD) from the model of VWF-A2 domain as a function of time: plots of mean square deviations in the configuration of the VWF A2 molecule during the 10 ns of simulation of molecular dynamics. Molecular dynamics simulations were carried out using the program MOE with the aid of CHARMM27 program for calculation of the force field. Detailed methods are described in the Materials and methods. The coordinate of the A2 domain of human VWF has been built based on a homology-modelling from the structures of six template proteins [29] and the downloaded PDB file was introduced into our system. RMSD value of wild type (black), L1503Q (light grey), and L1503R (dark grey) were plotted over time of 10 ns.

By determining the nucleotide sequence of exon 28 of the mutant VWF gene, a single candidate mutation was identified: a T>G substitution at 4508 of the VWF gene, thereby substituting leucine at 1503 to arginine of the VWF A2 domain. This missense mutation was not found in 60 normal individuals. The results of expression analysis of the mutant construct revealed that VWF antigen of mutant L1503R is markedly reduced by <10% in the medium but normal (80%) in the lysates, suggesting that substantial levels of mutant subunit were synthesized but appeared to have been retained within the cell. Thus, the mutation is sufficient to cause reduced secretion of functional VWF and thus it appears to belong to group I of VWD type 2A.

The defect in biosynthesis and secretion of VWF L1503R suggests a clue on the general mechanism by which mutations may be causing VWD that has been inherited in autosomal dominant way. Co-transfection experiment confirmed the dominant-negative mechanism of disease development; as the concentration of transfected mutant DNA is increased, secreted VWF protein is decreased by ~40%. In contrast, VWF antigen was accumulated in the lysates depending on mutant concentrations. Therefore, the mutation L1503R inhibits the secretion of a co-inherited normal allele, thereby causing a dominant form of VWD.

Although detailed VWF level has not been determined, clinical symptom of patient B was improved by DDAVP administration. As DDAVP appears to increase VWF secretion from cell storage, it is suggested that haemostasis had been accomplished by increased secretion of VWF multimers inherited from normal allele.

Structural changes induced by mutations can be studied with molecular dynamics simulations. The all-atom RMSD with respect to the model is shown in Fig. 4 for the simulations of wild type A2 domain, along with that for L1503Q and L1503R. The simulations of three molecules indicated that no dramatic unfolding occurred. However, the mean values of all-atom RMSD of the L1503R were shifted from those with wild type and L1503Q (Fig. 4), suggesting that L1503R is in the higher energy equalization level. This finding suggests that the group I mutation L1503R cause significant deviations, coupled to thermal fluctuations, when compared with group II mutation L1503Q or wild type

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Prevalence of Hepatitis E Virus IgG Antibody in Japanese Patients with Hemophilia

Hidenori Toyoda[a] Takashi Honda[b] Kazuhiko Hayashi[b] Yoshiaki Katano[b] Hidemi Goto[b] Takashi Kumada[a] Kazuaki Takahashi[d] Natsumi Abe[d] Shunji Mishiro[d] Junki Takamatsu[c]

[a]Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Departments of [b]Gastroenterology and [c]Transfusion Medicine, Nagoya University School of Medicine, Nagoya, and [d]Department of Medical Science, Toshiba General Hospital, Tokyo, Japan

Key Words
Hemophilia · Hepatitis E virus · Transfusion transmission

Abstract
Objective: We investigated the prevalence of antibody against hepatitis E virus (HEV) in Japanese patients with hemophilia. ***Methods:*** IgG antibody against HEV was measured in serum of 80 Japanese patients with hemophilia by enzyme-linked immunosorbent assay. The prevalence of HEV antibody was compared with the reported prevalence of HEV antibody in Japanese patients undergoing hemodialysis and in Japanese healthy blood donors. Characteristics of patients and coinfection with other transfusion-transmissible viruses were compared in patients with and without HEV antibody. ***Results:*** Anti-HEV IgG antibody was detected in 13 of 80 patients (16.3%). The prevalence was far higher than that reported in Japanese blood donors (3.7%) and was higher than that in Japanese patients undergoing hemodialysis (9.4%). The patients with HEV antibody were significantly older than those without. HEV antibody was not detected in patients <20 years of age and in patients who had received only virus-inactivated coagulation factors. No association was observed between positivity for anti-HEV antibody and severity of hemophilia or coinfection with other parenterally transmissible viruses. ***Conclusion:*** Our results suggest that the parenteral transmission of HEV may have occurred in Japanese patients with hemophilia via non-virus-inactivated coagulation factors.

Introduction

Infection with hepatitis E virus (HEV), which can cause acute hepatitis E, is an important public health concern in many developing countries, where sanitation is suboptimal; large epidemics of hepatitis E have been reported from Asia, Africa, and Latin America [1]. Although only sporadic cases of acute hepatitis E have been reported in many industrialized countries including the United States, Europe, and Japan [1–5], some healthy individuals in industrialized countries are seropositive for HEV antibodies [6, 7].

A relatively recent report [8] described a patient who was infected with HEV via transfused blood from a vol-

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Hidenori Toyoda, MD, PhD
Department of Gastroenterology, Ogaki Municipal Hospital
4-86 Minaminokawa
Ogaki 503-8502 (Japan)
Tel. +81 584 81 3341, Fax +81 584 75 5715, E-Mail tkumada@he.mirai.ne.jp

untary blood donor, and the potential risk of posttransfusion hepatitis E even in non- or low-endemic countries including Japan was suggested. The parenteral route of HEV transmission, however, remains controversial; some studies have suggested parenteral transmission of HEV, but others have shown no parenteral transmission of this virus [9–20]. Patients with hemophilia are at high risk of infection by transfusion-transmissible viruses due to the frequent use of coagulation factors. High rates of infection by hepatitis C virus (HCV), human immunodeficiency virus (HIV), and GB virus C (GBV-C) have been reported in patients with hemophilia [21–26]. If HEV infection could also have occurred in patients with hemophilia via coagulation factors, the prevalence of seropositivity for HEV antibodies would be high.

We previously investigated the prevalence of IgG antibody against hepatitis A virus (HAV) in Japanese patients with hemophilia [26] and reported a higher prevalence of HAV antibody in Japanese patients with hemophilia in comparison with normal Japanese subjects, suggesting the association between the use of coagulation factor and HAV infection. As for HEV, that is the other hepatitis virus with nonparenteral transmission, we did not investigate its prevalence in hemophilia patients because it had been considered to be rare in Japan. However, it was reported that zoonotic food-borne transmission of HEV to humans sometimes occurs; HEV infection is not so rare in Japan. In the present study, we evaluated the prevalence of antibody against HEV in Japanese patients with hemophilia to investigate the possibility of parenteral transmission of HEV by means of coagulation factors.

Patients and Methods

Patients

Eighty Japanese patients with hemophilia were involved in the study. These patients were selected from among 188 hemophilia patients who were followed up at Nagoya University Hospital and because they had sufficient stored serum samples after 2003. The patient group comprised 80 males, 59 with hemophilia A and 21 with hemophilia B. Fifty-one patients had received both non-virus-inactivated and virus-inactivated coagulation factors, and 29 had received only virus-inactivated coagulation factors. Fifty-four patients had received both domestic and imported coagulation factors that had been manufactured in the United States or in Europe, and 26 had received only domestic coagulation factors. Patients were 39.0 ± 14.4 years of age. No patient had a history of travel abroad. Written informed consent was obtained from all patients before serum samples were obtained. The study was approved by the University Ethics Committee and carried out in compliance with the Helsinki Declaration.

Serologic and Virologic Analyses of HAV, Hepatitis B Virus (HBV), HCV, GBV-C, HIV, and HEV

HAV antibody was measured in serum samples with a commercially available enzyme immunoassay kit (Dainabot, Tokyo, Japan) according to the manufacturer's instructions. HBV surface antigen (HBsAg), HBV surface antibody (HBsAb), and HBV core antibody (HBcAb) were measured with Architect HBsAg QT, Architect HBs, and Architect HBc, respectively (all Abbott Japan, Tokyo). HCV was analyzed by HCV antibody assay (third-generation assay kit; Dainabot), and HCV RNA was analyzed with the Amplicor HCV test, version 2.0 (Roche Diagnostics, Branchburg, N.J., USA). GBV-C RNA was measured by RT-PCR with nested primers deduced from conserved blocks in the 5′-untranslated region by a method described previously [27]. HIV1 infection was confirmed by anti-HIV1 antibody detection achieved with a particle agglutination test (Serodia-HIV; Fuji Rebio, Tokyo, Japan). IgG antibody against HEV was measured in serum by enzyme-linked immunosorbent assay as described by Li et al. [28].

Statistical Analysis

Differences in the proportion of patients with and without HEV antibody were analyzed by χ^2 test. Differences in quantitative variables were analyzed by Mann-Whitney U test. All p values were derived from two-tailed tests, and $p < 0.05$ was accepted as statistically significant.

Results

IgG antibody against HEV was detected in 13 of the 80 patients (16.3%) with hemophilia. The clinical characteristics of patients with and without HEV antibody are shown in table 1. The patients in whom HEV antibody was detected were significantly older than those in whom HEV antibody was not detected (46.9 ± 17.9 vs. 37.4 ± 13.1 years, $p = 0.0346$). No patient <21 years of age had HEV antibody. All patients with HEV antibody had started coagulation factor therapy before 1985. No patient who had received only virus-inactivated coagulation factors was positive for HEV antibody, whereas 13 of 51 patients (25.5%) who had received non-virus-inactivated coagulation factors were positive for HEV antibody. In contrast, HEV antibody was detected in similar percentages of patients who received only domestic and those who had received both domestic and imported coagulation factors (15.4 vs. 16.7%).

The prevalences of HAV, HBV, HCV, HIV, and GBV-C in patients with and without HEV antibody are shown in table 2. No differences were observed in the prevalence rates of these viruses between the two groups.

Table 1. Characteristics of the patients with and without HEV antibody

	HEV antibody positive (n = 13)	HEV antibody negative (n = 67)	p
Age, years (mean ± SD)[a]	46.8 ± 17.1	36.0 ± 13.5	0.0345
Type of hemophilia			
A	9 (15.3)	50 (84.7)	0.9519
B	4 (19.0)	17 (81.0)	
Severity of hemophilia			
Mild	3 (23.1)	10 (76.9)	0.6476
Moderate	0	2 (100)	
Severe	10 (15.4)	55 (84.6)	
Coagulation factors			
Virus-inactivated only	0	29 (100)	
Both non-virus-inactivated and virus-inactivated	13 (25.5)	38 (74.5)	0.0079
Domestic	4 (15.4)	22 (84.6)	
Both domestic and imported	9 (16.7)	45 (83.3)	0.8842

Numbers (and percentages) of patients are shown unless otherwise indicated.

[a] Age at the time of measurement of HEV antibody.

Table 2. Prevalence rates of other transfusion-transmissible viruses in patients with and without HEV antibody

Positive for	HEV antibody positive (n = 13)	HEV antibody negative (n = 67)	p
HAV IgG antibody	6 (46.2)	15 (22.4)	0.1505
HBV surface antigen	1 (7.7)	0	0.3572
HBV surface antibody	8 (61.5)	40 (59.7)	0.9015
HBV core antibody	10 (76.9)	52 (77.6)	0.9566
HCV antibody	13 (100.0)	63 (94.2)	0.8348
HCV RNA	13 (100.0)	63 (94.2)	0.8348
HIV1 antibody	5 (38.5)	27 (40.3)	0.9015
GBV-C RNA[a]	4 (44.4)	12 (30.8)	0.6949

Numbers (and percentages) of patients are shown.

[a] Among 48 patients in whom GBV-C RNA was measured (9 with HEV antibody and 39 without HEV antibody).

Table 3. Reported prevalence rates of HEV antibody in patients with hemophilia and in patients undergoing hemodialysis

Authors (year of publication)	Country	Prevalence (%) of patients with HEV antibody
Patients with hemophilia		
Mannucci et al. [12] (1994)	Italy	0/60 (0)
Barzilai et al. [13] (1995)	Israel	16/188 (8.5)
Klarmann et al. [14] (1995)	Germany	1/37 (2.7)
Zaaijer et al. [15] (1995)	Netherlands	4/296 (1.4)
Buffet et al. [16] (1996)	France	5/63 (7.9)
Our study (2007)	Japan	13/80 (16.3)
Patients undergoing hemodialysis		
Courtney et al. [10] (1994)	Ireland	0/45 (0)
Halfon et al. [11] (1994)	France	16/147 (10.9)
Psichogiou et al. [17] (1996)	Greece	27/420 (6.4)
Fabrizi et al. [18] (1997)	Italy	6/204 (2.9)
Mitsui et al. [20] (2004)	Japan	39/416 (9.4)

Table 4. Prevalence rates of HEV antibody among patients with hemophilia, patients on hemodialysis, and healthy blood donors in Japan

Age, years	Patients with hemophilia (n = 80)	Patients undergoing hemodialysis (n = 416) [20]	Healthy blood donors (n = 5,343) [29]
≤19	0/6		7/812 (0.9)
20–29	1/18 (5.6)	1/33 (3.0)[a]	19/1,043 (1.8)
30–39	5/24 (20.8)		28/1,146 (2.4)
40–49	2/17 (11.8)	3/40 (7.5)	53/966 (5.5)
50–59	3/10 (30.0)	10/109 (9.2)	54/744 (7.3)
≥60	2/5 (40.0)	25/234 (10.7)	39/632 (6.2)

Numbers (and percentages) of patients are shown.

The age range was 16–84 years in patients with hemophilia, 23–91 years in patients undergoing hemodialysis, and 16–69 years in healthy blood donors.

[a] Patients with an age range of 23–39 years.

Discussion

Whether HEV is transmitted parenterally remains controversial (table 3); the existence of transfusion transmission of HEV is still unclear. A high prevalence of anti-HEV antibody was reportedly observed among hemodialysis patients, the majority of whom had a history of blood transfusions [11, 20]. However, other investigators found only a few HEV antibody-positive patients in larger groups of hemodialysis patients [10, 17, 18]. In industrialized countries, the prevalence of positivity for HEV antibody in patients with hemophilia seems to differ between countries [12–16], whereas a higher prevalence in patients with hemophilia than in volunteer blood donors has been reported in nonindustrialized countries [19].

This is the first report that investigated the prevalence of HEV antibody in hemophilia patients in Japan. The prevalence of HEV antibody in our study patients (16.3%) was higher than that previously reported in Japanese blood donors (3.7%) [29] and in Japanese patients undergoing hemodialysis (9.4%) [20] (table 4). The gradual increase in the prevalence of HEV antibody between healthy blood donors, patients undergoing hemodialysis, and patients with hemophilia suggests a possible role of parenteral transmission of HEV. Because coagulation factors that are currently used in Japan are very unlikely to contain IgG antibodies [30, 31], the HEV antibodies that were detected in our patients cannot have been passively acquired from recently used coagulation factors.

The absence of HEV antibody in patients <20 years of age and in patients who had received only virus-inactivated coagulation factors suggests that HEV infection might have occurred by means of non-virus-inactivated coagulation factors that had been used before the mid-1980s. In contrast, we did not find a difference in the prevalence of HEV antibody between patients who received only domestic coagulation factors and those who had a history of using imported coagulation factors, unlike the difference in the prevalence of HIV or HCV genotype 1a infections between Japanese hemophilia patients with and without the use of imported coagulation factors [23]. Recent studies have indicated that hepatitis E is a zoonosis [2, 4, 32–39], and it has been shown that zoonotic food-borne transmission of HEV to humans may play an important role in the occurrence of HEV infection in Japan [38–41]. In addition, silent viremia due to HEV, i.e., the presence of HEV in the bloodstream but without acute hepatitis, has been reported [19, 42, 43]. HEV infection, therefore, may not be rare in Japan [44, 45]. Contamination of domestic, non-virus-inactivated coagulation factors by HEV is, therefore, not unlikely, and the use of domestic coagulation factors could have caused HEV infection in Japanese patients with hemophilia. In addition, the use of coagulation factors that had been manufactured from plasma of individuals from an area where HEV infection is endemic could have caused HEV infections in our patients.

We found no association of HEV antibody with infection by transfusion-transmissible viruses (HBV, HCV, HIV, and GBV-C) or with the presence of HAV antibody. It was difficult to elucidate the characteristics of patients with HEV antibody on the basis of the coinfecting viruses because of the high prevalence of infection by these viruses.

The marked differences between countries in the prevalence of HEV antibody in patients with hemophilia, which sparks controversy over the possibility of parenteral transmission of HEV, might be due to differences in the origins and methods of manufacturing coagulation factors, especially during the period in which non-virus-inactivated coagulation factors were used. Unfortunately, the prevalence of HEV antibodies in volunteer blood donors in the various countries has not been reported, and the origins of the plasma used for manufacturing coagulation factors in these countries are unknown. It is well known that the prevalence of HEV varies widely throughout the world. Plasma of individuals from high-prevalence areas might have had more chance of contamination during the period when non-virus-inactivated coagulation factors were used.

In summary, the high prevalence of HEV antibody in our patients with hemophilia suggests the possibility of a parenteral route of HEV transmission. However, the coagulation factors used in our patients are not now available for examination, so we cannot prove that the coagulation factors used in Japan were contaminated by HEV. Further studies are needed to clarify whether HEV has been transmitted by means of coagulation factors. Tracing the origin of the plasma used for coagulation factors would be helpful in understanding the association between the prevalence of HEV antibody and the use of coagulation factors in patients with hemophilia.

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