light chain (residues 1546–2196, A3-C1-C2 domains), activated FV (FVa) [6].

Activated FV functions as an essential molecule of the prothrombinase complex that catalyses the conversion of prothrombin to thrombin by factor Xa in the presence of calcium and a phospholipid membrane. The procoagulant function of FVa is downregulated by the anticoagulant serine protease, an activated protein C (APC) [7] that cleaves to FVa at Arg306, Arg506 and Arg679, resulting in a loss of FVa activity. On the other hand, FV cleaved by APC before thrombin activation, FVac, shows an anticoagulant function as a cofactor in the APC-mediated inactivation of activated FVIII (FVIIIa). Thus, FV plays an important role in the procoagulant pathway as well as in the protein C anticoagulant pathway [8].

Around 75% of FV in blood is in the plasma, with the residual FV in the α-granules of blood platelets. In plasma, FV exists in two isoforms (FV1 and FV2) that have different molecular weights because of partial N-linked glycosylation in the C2 domain [9]. FV1 and FV2 have different characteristics in terms of procoagulant activity, inactivation by APC, and their anticoagulant function in the protein C pathway [10]. Consequently, FV1 has the overall potential to generate more thrombin than FV2.

Factor V deficiency, also known as parahaemophilia, was first described in 1947 by Owren [11]. It is a rare bleeding disorder inherited in an autosomal recessive manner with an incidence of about one in 1 million [1]. Bleeding symptoms in FV-deficient patients are varied; heterozygotes are usually asymptomatic, whereas homozygotes may show a mild, moderate or severe bleeding tendency.

To date, more than 40 identified cases of mutations in the FV gene were described in FV-deficient patients in the homozygous or compound heterozygous state [12]. In this study, we investigated the molecular basis of severe FV deficiency in a Japanese patient, and demonstrated that she was another compound heterozygote for FV gene mutations resulting in the post-transcriptional impairment of FV synthesis and/or secretion.

Materials and methods

Preparation of plasma, genomic DNA and total RNA of platelets

Ethical approval for the study was obtained from the Ethics Committee of the Nagoya University School of Medicine. Following informed consent, blood samples from the patient, family members and volunteers were collected in a 1:10 volume of 3.13% sodium citrate. Plasma was separated by centrifugation at 2000 *g* for 20 min, and aliquots were stored at −70°C until use. The patient had not received substitution therapy for 3 months prior to blood sampling for FV antigen and activity measurements. Genomic DNA was isolated from peripheral blood leucocytes as described previously [13]. Citrated blood samples from the patient and her sibling were centrifuged at 250 *g* for 5 min at 4°C to collect platelet-rich plasma. Subsequently, the total RNA was extracted from platelets by RNA STAT-60 (Tel-Test Inc., Friendswood, TX, USA), and subjected to a reverse transcription (RT) reaction as described below.

FV antigen and activity assays

Factor V procoagulant activity and FV antigen in plasma as well as in culture media containing recombinant FV proteins were measured as described below. FV procoagulant activity was measured by one-stage clotting assay, of which the sensitivity limit and the normal range are 3% and 70–135%, respectively, using human FV-deficient plasma (George King Bio-Medical, Overland, KS, USA) and Simplastin (Biomerieux, Inc., Durham, NC, USA). FV antigen was measured by enzyme-linked immunosorbent assay (ELISA), of which the sensitivity limit and the normal range are 1% and 70–135%, respectively, using an affinity-purified sheep anti-human FV IgG as a coating antibody with a peroxidase-conjugated sheep anti-FV antibody as a second antibody, according to the manufacturer's protocol (Cedarlane Lab. Ltd, Hornby, ON, Canada). In both assays, FV levels were expressed as a percentage of control plasma pooled from 25 healthy individuals.

PCR and DNA sequencing

The polymerase chain reaction (PCR) primers were synthesized to amplify all exons and splicing junctions of the FV gene, based on the reported genomic DNA sequence of human FV (GenBank Z99572). Information of the primer sequences is available from the authors. PCR amplification of the FV gene was performed with rTaq polymerase or exTaq polymerase (Takara Bio Inc., Kusatsu, Japan) in 30–35 cycles under the following conditions: 30 s denaturing at 94°C, 30 s annealing at 47–58°C and 30 s extension at 72°C.

Polymerase chain reaction products were separated by agarose gel electrophoresis, and authentic fragments were collected and purified with a QUAEX II kit (Qiagen K.K., Tokyo, Japan). The samples were then directly sequenced by a Big Dye Terminator

Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) using forward or reverse PCR primers, according to the manufacturer's protocol. The sequencing products were then precipitated with 0.15 M NaOAc (pH 8.0) and cold ethanol, washed once with 70% ethanol, dried, resuspended in 25 μL of Template Suspension Reagent (Applied Biosystems), and analysed by an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Analysis of FV mRNA

To investigate the presence of FV transcripts from the mutant allele in platelets, we analysed platelet RNAs from the proband by mRNA-based PCR-restriction fragment length polymorphisms (RFLPs). In brief, total RNA extracted from the platelets was reverse-transcribed using the respective gene-specific primers: 12GSP (5′-TCTGTTCTGGTAATCA TAGT-3′) for 1943insC or 15GSP (5′-GTGCTG TTTATTGCCATTTT-3′) for A5279G, and Super Script II RT reverse transcriptase (Invitrogen Japan, Tokyo, Japan). To detect the 1943insC mutation, a nested PCR was performed using the following primers: 12rPCR-UP (5′-CCCTATAGCATTTAC CCTCA-3′) and 12GPS for the first PCR, and 12mut-UP (5′-ACTTCTGTAGTGTGGGGggCC-3′; bold lower case characters are mismatched nucleotides) and 12 M-LW (5′-TTCATCATCATCTGGG-ATAC-3′) for the second PCR, introducing a new *Apa*I restriction site in the mutant PCR products, as a single PCR using the first or second PCR primer set failed to amplify authentic PCR products. The 1943insC mutant RT-PCR products would yield 19- and 221-bp fragments, whereas the wild-type products would not be digested (239 bp). To detect the A5279G mutation, PCR was performed with the following primers: 15 M-UP (5′-AAAAATCATCA GAGGGAAAG-3′) and 15mut-LW (5′-CTGGGT TCACAGCTGAcTAG-3′) introducing a *Spe*I restriction site in the wild-type PCR products. Thus, the wild-type RT-PCR products would yield 18- and 159-bp fragments, while the A5279G mutant products would not be digested (177 bp). These fragments were run on a 4% agarose gel and stained with ethidium bromide. We evaluated the allele-specific mRNA levels by the quantitative densitometric analyses using the NIH image software (version 1.62) (National Institutes of Health, Bethesda, MD, USA).

Preparation of mutant FV expression vectors

We prepared individual FV expression vectors bearing the identified mutations, 1943insC (FS592X; the initial Met residue is denoted amino acid +1) and A5279G (Y1702C), based on pMT2 containing a full-length cDNA of human FV (pMT2-FV). Both mutations were introduced individually into the pMT2-FV expression vector using the recombinant PCR method described elsewhere [14]. After recombinant PCRs, each DNA fragment encoding the 1943insC or A5279G mutation was isolated as *Bsp*361-*Bsp*EI or *Bsp*MI-*Sna*BI fragments, and separately replaced into the appropriate position for the pMT2/FV expression vector. DNA sequencing confirmed that no unexpected mutation was found in any of the whole mutant inserts in either construct.

Transient expression of recombinant FVs in COS-1 cells

African green monkey kidney COS-1 cells were cultured in a 5% CO_2 humidified atmosphere at 37°C in Dulbecco modified Eagle medium (DMEM; Invitrogen) supplemented with fetal calf serum (10%), glutamine (1%), and antibiotics (penicillin and streptomycin, 100 IU mL^{-1} and 100 μg mL^{-1} respectively). Cells in 30-mm dishes were transfected with either wild type or individual mutant plasmids using the Fu-GENE6™ transfection reagent (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer's instructions. After 48-h culture of the transfected cells in serum-free DMEM, conditioned media containing the secreted recombinant proteins were collected, then concentrated using Centrisart I (cut off MW 20000; Sartorius, Goettingen, Germany), and subjected to one-stage clotting assay as well as ELISA (Cedarlane Lab. Ltd) for recombinant FV antigen measurements as described above.

Results and discussion

Case report

The patient (individual II-1, Fig. 1) is a 39-year-old Japanese woman who had recurrent episodes of bleeding such as epistaxis, joint region haematoma and hypermenorrhea, which were treated with FV replacement therapy by transfusion of fresh frozen plasma. When the patient was 4 years old, she had been diagnosed as having coagulation FV deficiency, since laboratory tests revealed that the prothrombin time and the activated partial thromboplastin time were prolonged, and FV activity was below the measurable limit. There was no history of bleeding tendencies in her other family members tested, since FV activities in plasma of both her mother and a sibling were 65%, suggesting that they might be

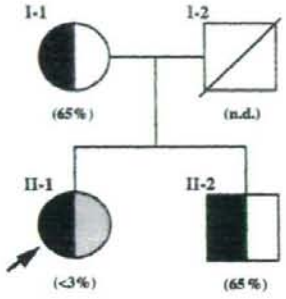

Fig. 1. Pedigree of the factor V-deficient family. The propositus is subject II-1 (arrow). Circle and square indicates male and female respectively. Values in parentheses represent plasma factor V activities (n.d., not done). Subjects with 1943insC and A5279G mutations are demonstrated with solid and shaded areas respectively.

heterozygous for FV-deficiency causing mutation. Consanguinity in the family was excluded.

DNA sequencing

In order to identify causative FV gene mutation(s) in such an FV-deficient patient, we analysed nucleotide sequences of all 25 exons and exon–intron boundaries of the FV gene. Results from direct sequencing of the FV gene revealed that the patient had a C insertion in three consecutive cytosine nucleotides [^{589}Thr(ACC)–^{590}Gln(CAG)] in exon 12 at nucleotide positions 1940–1942 (1943insC), and an A–G transition in exon 15 at nucleotide position 5279 (A5279G) (Fig. 2). DNA samples from her mother and brother also showed heterozygosity for the 1943insC mutation, but no A5279G mutation (data not shown), which are consistent with the data of plasma FV activity, i.e. about half that of normal subjects; 1943insC is a novel mutation, which can cause a frame-shift resulting in a substitution of the amino acids after ^{590}Gln with two abnormal residues (^{590}Pro–^{591}Glu) followed by a stop codon (FS592X). The A5279G will cause the amino acid substitution Y1702C, which was previously designated FV Seoul 2 [15]. The A5279G FV gene mutation has also been found in Italian and Slovenian subjects [16,17], and is thought to be a very ancient and/or recurrent mutation. In this study, we demonstrated that this mutation also occurred in a Japanese subject, suggesting that the A5279G might be a hot-spot mutation rather than a founder mutation.

mRNA analysis (RT-PCR RFLPs)

We analysed the expression of mutant FV gene transcripts from the patient's platelets by mRNA-mediated PCR-RFLPs (RT-PCR RFLPs). For 1943insC (FS592X-FV mRNA), the nested RT-PCR followed by *Apa*I digestion yielded 239- and 221-bp bands, representing transcripts from the normal and mutant alleles, respectively, although the mutant signal was markedly reduced (Fig. 3a). For A5279G (Y1702C-FV mRNA), the RT-PCR products digested with *Spe*I yielded 159- and 177-bp bands, representing transcripts from the normal and mutant alleles respectively (Fig. 3b). Thus, both mutant transcripts were present in the patient's platelets. However, the FS592X-FV mRNA signal was markedly reduced to 12% of the wild type in the quantitative densitometric analysis, whereas the Y1702C-FV mRNA signal was more intense (250% of the wild type). These data suggest that the patient could be compound heterozygous for these mutations, and that her RNA surveillance system would eliminate most of the FV mRNA derived from the mutant allele encoding a premature termination by the frame-shift mutation, FS592X [18]. On the other

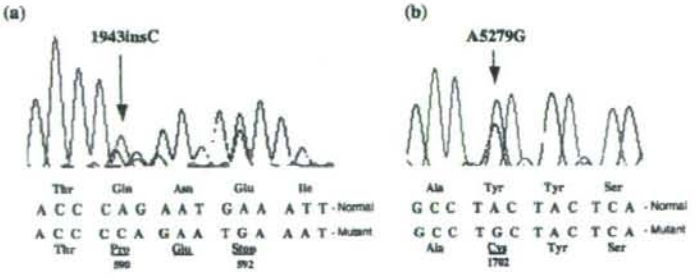

Fig. 2. Patient's nucleotide and amino acid sequences surrounding the mutations. (a) Nucleotide and amino acid sequences surrounding 1943insC. Arrow indicates mutation point. The mutation predicts an abnormal sequence of two amino acid residues and a stop codon. (b) Nucleotide and amino acid sequences surrounding A5279G. Arrow denotes mutation point. Patient's heterozygous sequencing pattern is shown.

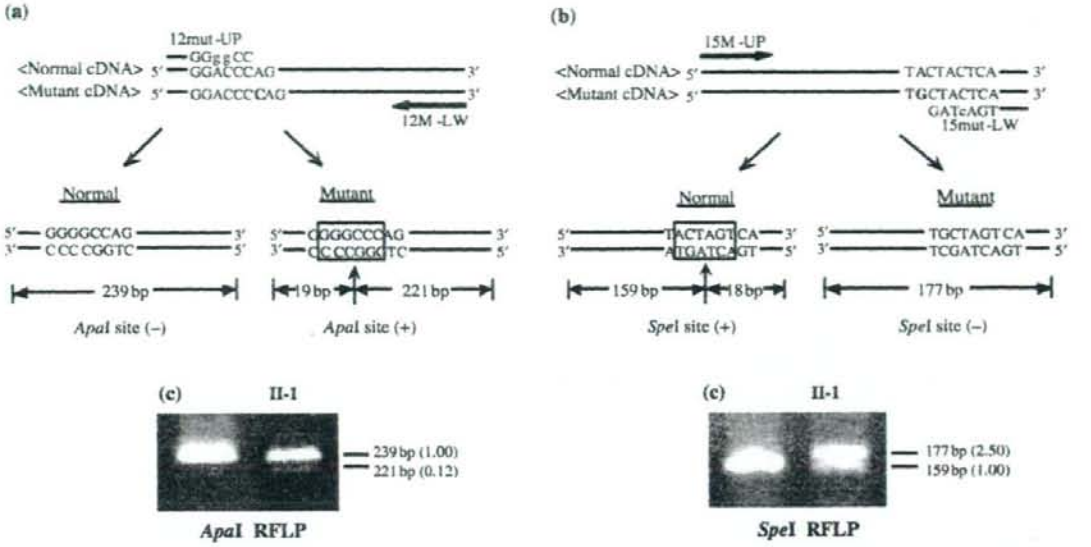

Fig. 3. Detection of mutant factor V mRNAs in patient's platelets. (a) Reverse transcriptase polymerase chain reaction (RT-PCR) products (239 bp) using primers 12mut-UP and 12M-LW were digested with *Apa*I, then electrophoresed on 4% NuSieve 3:1 agarose gel. Wild-type RT-PCR product migrated as an uncleaved 239-bp band, while FS592X (1943insC) RT-PCR product is represented by an *Apa*I cleaved 221-bp band. II-1, proband; C, control donor. Numbers in parentheses are relative amounts of signals measured by the quantitative densitometric analysis (wild type = 1.00). (b) RT-PCR products (177 bp) using primers 15M-UP and 15mut-LW were digested with *Spe*I, then electrophoresed on 4% NuSieve 3:1 agarose gel. Wild-type RT-PCR product migrated as *Spe*I cleaved 159-bp band, whereas Y1702C (A5279G) RT-PCR product is represented by an uncleaved 177-bp band. II-1, proband; C, control donor. Numbers in parentheses are relative amounts of signals measured by the quantitative densitometric analysis (wild type = 1.00).

hand, both the FV antigen and activity in her plasma were below the detectable limit, suggesting that the mutant Y1702C-FV might be impaired during the post-transcriptional process of protein synthesis and/or in secretion. Indeed, it has also been previously reported that the FV allele bearing the Y1702C mutation was expressed normally at the mRNA level, but not at the protein level in plasma [15].

Expression of wild type and mutant recombinant FVs in COS-1 cells

It is important to determine the patient's phenotype on Met1736Val polymorphism, as it will exert a great influence on the expression level of the recombinant FV [19]. Sequence analysis revealed that the patient was homozygous for 1736Met, which is the same phenotype encoded in the pMT2-FV, and thus its influence may not be revealed in expression experiments for her Y1702C-FV.

We investigated the effects of the identified mutants on FV secretion by conducting transient transfection experiments in COS-1 cells, which do not express endogenous FV. We observed that the wild type recombinant FV proteins were secreted efficiently into culture media with an adequate specific activity (0.94), but that the mutant Y1702C-FV showed an impaired secretion (1.8% of the wild type) and inadequate FV procoagulant activity (0.56) (Fig. 4). These data tend to support the conclusion that the Y1702C mutation could be causative for the FV deficiency as reported previously [15]. Indeed, plasma levels of FV activity in her mother and brother, who had only the Y1702C mutation in heterozygous, were reduced to 65% of normal. The 1702Y is a highly conserved amino acid not only in FV molecules among various species, but also in human FVIII and ceruloplasmin [15]. Moreover, an X-ray crystal structure analysis of wild type FV has demonstrated that the FV Y1702C mutation leads to the disappearance of two hydrogen bonds with P1618, and that its structure was significantly altered by a new hydrogen bond bridge formed between this cysteine and one of the other free cysteines [15]. These data suggest that 1702Y may play an important role in maintaining the structure and function of the FV molecule.

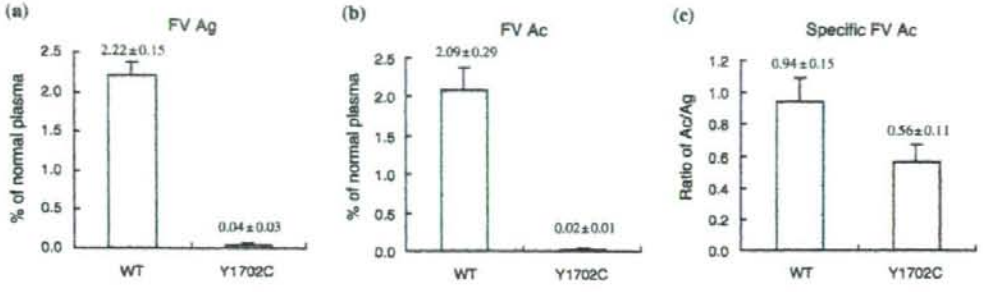

Fig. 4. Transient expression of wild-type factor V (FV) and 1702C mutant FV in COS-1 cells. Plasmids containing wild type (pMT2/FV) or mutant (pMT2/FV-Y1702C) FV cDNA were transiently transfected in COS-1 cells using FuGene reagent. Antigen and activity levels of recombinant FVs were measured in conditioned media 48 h after transfection (a, FV antigen; b, FV activity; c, FV-specific activity). Bars represent mean values ± SD of three independent experiments, each performed in duplicate. FV levels were expressed as percentage of normal pooled plasma from 25 healthy individuals.

On the other hand, recombinant FS592X-FV molecule was not detected in cultured media of the transfected COS-1 cells (data not shown). The transcripts of FS592X-FV were detected in the patient's platelets, but were found to be markedly reduced compared with normal allele transcripts (Fig. 3a). Moreover, as the FS592X-FV is a truncated molecule in the A2 domain, it would not be processed normally as reported for other mutant coagulation factors [20,21].

Conclusion

In this study, we investigated the molecular basis of a severe coagulation FV deficiency in a Japanese woman and identified two distinct mutations (1943insC/FS592X and A5279G/Y1702C) in her FV gene. The data indicated that both mutant FV molecules would be impaired, at least in part, during the post-transcriptional process of protein synthesis and/or in secretion. Taken together with the above observations, it seems to suggest that each mutation could be separately responsible for severe FV deficiency, while this phenotype is due to the in-trans combination of the two defects.

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Review Article

Intravenous Immunoglobulin Therapy for Acquired Coagulation Inhibitors: A Review

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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 for 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

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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. Against 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†						
OlSullivan 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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Unblinded Pilot Study of Autologous Transplantation of Bone Marrow Mononuclear Cells in Patients With Thromboangiitis Obliterans

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Background—The short-term clinical benefits of bone marrow mononuclear cell transplantation have been shown in patients with critical limb ischemia. The purpose of this study was to assess the long-term safety and efficacy of bone marrow mononuclear cell transplantation in patients with thromboangiitis obliterans.

Methods and Results—Eleven limbs (3 with rest pain and 8 with an ischemic ulcer) of 8 patients were treated by bone marrow mononuclear cell transplantation. The patients were followed up for clinical events for a mean of 684±549 days (range 103 to 1466 days). At 4 weeks, improvement in pain was observed in all 11 limbs, with complete relief in 4 (36%). Pain scale (visual analog scale) score decreased from 5.1±0.7 to 1.5±1.3. An improvement in skin ulcers was observed in all 8 limbs with an ischemic ulcer, with complete healing in 7 (88%). During the follow-up, however, clinical events occurred in 4 of the 8 patients. The first patient suffered sudden death at 20 months after transplantation at 30 years of age. The second patient with an incomplete healing of a skin ulcer showed worsening of the lesion at 4 months. The third patient showed worsening of rest pain at 8 months. The last patient developed an arteriovenous shunt in the foot at 7 months, which spontaneously regressed by 1 year.

Conclusions—In the present unblinded and uncontrolled pilot study, long-term adverse events, including death and unfavorable angiogenesis, were observed in half of the patients receiving bone marrow mononuclear cell transplantation. Given the current incomplete knowledge of the safety and efficacy of this strategy, careful long-term monitoring is required for future patients receiving this treatment. (***Circulation***. **2006;114:2679-2684.**)

Key Words: angiogenesis ■ collateral circulation ■ endothelium ■ peripheral vascular diseases

The clinical consequences of severe peripheral arterial disease or critical limb ischemia include rest pain and the loss of tissue integrity in the distal limb.[1–3] Therapeutic options for such patients are limited. These conditions are often refractory to conservative measures and are typically unresponsive to drug therapy. When vascular obstruction involves a long segment or is widespread, percutaneous revascularization may not be feasible. Surgical therapy, consisting of arterial bypass or amputation, is complicated by variable morbidity and mortality, and its effectiveness depends on the short- and long-term patencies of the conduit employed. Therapeutic angiogenesis thus constitutes a potential alternative treatment strategy for such patients.[4,5]

Previous investigators have suggested that endothelial progenitor cells, originating from bone marrow, circulate in adult peripheral blood and participate in postnatal neovascularization.[6–8] Subsequent experiments have shown that bone marrow or bone marrow–derived cells have the potential to stimulate angiogenesis and thereby modulate the hemodynamic deficit in ischemic limbs in vivo.[9,10] The Therapeutic Angiogenesis by Cell Transplantation (TACT) study first demonstrated that the magnitude of angiogenesis stimulated by these cells is sufficient to constitute a therapeutic benefit in patients with critical limb ischemia.[11] In that study, the investigators injected bone marrow mononuclear cells (BM-MNCs) into the ischemic limb of patients and documented a significant improvement in the hemodynamic deficit as well as the relief of ischemic symptoms. Although the TACT

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study established the concept of using BM-MNCs for therapeutic angiogenesis, limited information is available about the long-term safety and efficacy of this strategy.

The purpose of the present study was to determine the long-term safety and clinical impact of BM-MNC transplantation for "no-option" patients with thromboangiitis obliterans.

Methods

Patients

Eight patients with thromboangiitis obliterans were treated with an autologous transplantation of BM-MNCs between March 2002 and September 2004. The diagnosis of thromboangiitis obliterans was based on the criteria proposed by Olin[12]: (1) onset before age 45; (2) current (recent) history of tobacco use; (3) the presence of distal-extremity ischemia (infrapopliteal or infrabrachial) indicated by claudication, rest pain, ischemic ulcers, or gangrene; (4) exclusion of autoimmune or connective tissue diseases, hypercoagulable states, and diabetes mellitus; (5) exclusion of a proximal source of emboli by echocardiography and arteriography; and (6) consistent arteriographic findings in the clinically involved and noninvolved limbs.

Patients qualified for cell transplantation if they had chronic limb ischemia, with rest pain or a nonhealing ischemic ulcer, present for a minimum of 4 weeks without evidence of improvement in response to conventional drug therapy; showed angiographic evidence of vasculopenia in the affected limb; and were not candidates for percutaneous or surgical revascularization. The exclusion criteria included severe concurrent illness, the presence of proliferative diabetic retinopathy, and a history or clinical evidence of a malignant disorder.

All the patients involved in the present study received continuous medical therapy for >2 months before BM-MNC transplantation to confirm that conventional measures would be insufficient to achieve improvement in rest pain or skin ulcer/gangrene. During this period, no surgical therapies such as bypass grafting, extensive debridement, skin grafting, or limb amputation were performed. In addition, the patients were admitted to the hospital for a minimum of 1 month before BM-MNC transplantation to exclude the likelihood of spontaneous improvement in ischemic symptoms resulting from an enrollment bias. It should be also pointed out that the patients remained in the hospital and received the same therapy for at least 1 month after BM-MNC transplantation to avoid changes in their treatment.

BM-MNC Transplantation

While the patients were under general anesthesia, marrow cells were aspirated from the ileum. BM-MNCs were sorted on an AS-104 blood-cell separator (Fresenius HemoCare, Redmond, Wash) and concentrated to a final volume of ≈50 mL. After bone marrow cells were sorted on the AS-104 blood-cell separator, a small fraction of the cells was used for BM-MNC counting; the concentration of BM-MNCs in the final product was determined by using a microscope counting chamber after May-Giemsa staining. By using another fraction of cells, the number of $CD34^+$ cells in the BM-MNCs was also determined by fluorescence-activated cell sorting (FACS SCAN flow cytometer; Becton Dickinson, San Jose, Calif). The cells were incubated with the FITC-conjugated mouse monoclonal antibody against human CD34 (clone 581; Becton Dickinson) according to manufacturer's instructions.

For each patient, ≈100 aliquots of BM-MNCs (0.5 mL per aliquot) were administered via a syringe with a 27-gauge needle. Injection was performed into 9 lower limbs in 7 of the patients and the bilateral hands in 1. Injection sites were arbitrarily selected according to angiographic findings (ie, the degree of vasculopenia) and included calf muscles such as the soleus and gastrocunemius muscles as well as the sole muscles of the foot. For the patient with hand ischemia, injection was performed in palm muscles.

Assessment of Short-Term Outcome

Ischemic pain was assessed with a visual analog pain scale (VAS) with 10 levels. Ischemic ulcers were documented by color photography. Resting ankle-brachial pressure index (ABI) was calculated as the quotient of absolute ankle pressure and brachial pressure (the patient who received BM-MNC transplantation in his hands was excluded from ABI analysis). Angiographic assessment was performed with magnetic resonance angiography, computed tomographic angiography, or digital subtraction angiography. Adverse events were defined as death, limb amputation, pathological angiogenesis, recurrence/worsening of ischemic symptoms (ie, rest pain, skin ulcer, gangrene), myocardial infarction, stroke, and malignant disease.

Assessment of Long-Term Outcome

The mean length of follow-up was 684±549 days (range 103 to 1466). Patients were followed up by history analysis, physical examination, routine blood testing, ABI, and angiography at prescribed intervals during the first year, after which they were contacted at an outpatient clinic or by telephone to track events.

Data Analysis

All data are presented as mean±SD (range) or frequencies (percentage).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Diagnosis

The diagnosis of thromboangiitis obliterans was made according to the criteria described above. Among the 8 patients, only patient 6 did not completely fulfill the criteria; ie, this patient had no history of tobacco use (Table 1). Laboratory screening excluded the possibility of other underlying diseases, however, including autoimmune and connective tissue diseases. It should be also pointed out that patient 6 had diabetes mellitus at the time of cell transplantation but not at the onset of thromboangiitis obliterans. With the typical characteristic angiographic findings of thromboangiitis obliterans, such as multiple segmental arterial involvement (skip lesions) and "cork-screw" collateral vessels, we diagnosed patient 6 as having thromboangiitis obliterans, even though the patient did not have a history of tobacco use.

Patient Characteristics

The demographic and clinical data of the 8 patients are shown in Table 1. The mean age of the patients enrolled was 46±14 years (range 28 to 63). Seven patients (88%) were male. One patient had undergone prior femoral-tibial artery bypass grafting, and 1 had undergone sympathetic ganglion block. These treatments were performed >1 year before BM-MNC transplantation. Seven patients (88%) had a history of smoking, all of whom stopped smoking at least 1 month before transplantation.

Short-Term Outcome

The total volume of cells aspirated from the ileum was 728±72 mL (range 600 to 800) per patient, and the total volume of injected BM-MNCs was 45±7 mL (range 30 to 50) per patient. Total number of injected BM-MNCs was $3.5\pm0.8\times10^8$ (range 2.0 to 4.7×10^8), and that of $CD34^+$ cells was $6.8\pm2.6\times10^7$ (range 2.4 to 9.7×10^7).

TABLE 1. Patient Characteristics

Patient	Age	Sex	Fontaine Stage	Previous Treatment	DM	HT	HLP	Smoking	BM-MNC ($\times 10^8$)	$CD34^+$ in BM-MNC ($\times 10^7$)	ABI, Baseline	ABI, 1 Month	VAS, Baseline	VAS, 1 Month
1	63	M	III(lt)	Bypass graft	−	−	+	+	3.0	6.6	0.34	0.55	5	0
2	31	M	IV(rt)	Medical	−	−	−	+	4.7	9.7	0.49	0.39	5	1
3	52	M	IV(lt)	Medical	−	−	−	+	4.1	9.0	0.65	0.67	7	2
4	28	M	IV(lt)	Sympathetic ganglion block	−	−	−	+	2.0	6.8	0.50	0.26	5	0
5	32	M	IV(rt)	Medical	−	−	−	+	3.8	2.4	−	−	5	2
			IV(lt)								−	−	5	2
6	55	F	IV(lt)	Medical	+	+	−	−	3.4	4.0	0.53	0.51	4	3
7	63	M	III(rt)	Medical	−	+	−	+	3.0	9.1	1.10	0.91	5	3
			IV(lt)								0.76	0.94	5	3
8	43	M	IV(rt)	Medical	−	+	−	+	3.6	6.8	1.00	1.04	5	0
			III(lt)								1.00	1.07	5	0

DM indicates diabetes mellitus; HT, hypertension; and HLP, hyperlipidemia.

Angiographic assessment at 4 weeks after transplantation revealed an apparent increase in limb vascularity in 3 of the 8 (38%) patients (4 of the 11 limbs) (Figure 1). Hemodynamic assessment also failed to document evidence of improved collateral development. Specifically, an increase in ABI (>0.1) was observed in 2 of 7 (29%) patients (2 of 8 limbs), whereas a decrease in ABI (>0.1) was observed in 2 of 7 (29%) patients (2 of 8 limbs). As a result, mean ABI measured at 4 weeks (0.71 ± 0.30) did not differ from that at the baseline (0.70 ± 0.27). Because 2 patients had sites of arterial occlusion distal to the ankle, they showed normal ABIs before treatment. Even after the exclusion of these 2 patients, ABI showed no changes between before (0.55 ± 0.15) and after transplantation (0.55 ± 0.24).

In contrast to the angiographic and hemodynamic results, improvement in limb status was observed in all 8 patients (100%). Improvement in VAS was observed in all 11 limbs, with a decrease from a mean of 5.1 ± 0.7 to 1.5 ± 1.3. Furthermore, complete pain relief was achieved in 4 of the 11 limbs (36%). Improvement in skin ulcers was also observed in all 8 limbs (100%), with complete healing in 7 (88%). Although surgical amputations of the distal limb were performed in 2 patients at 1 month, these operations were intentionally scheduled to be performed after transplantation with the expectation of sufficiently improving the limb perfusion to distally advance the site of amputation (Table 2; Figure 2A and 2B).

Figure 1. Digital subtraction angiography at (A) baseline and (B) 1 month after cell transplantation. Arrows indicate newly visible collateral vessels at the calf level.

Long-Term Outcome

The mean follow-up period was 684 ± 549 days (range 103 to 1466). At the final follow-up, VAS score remained unchanged from that observed at 1 month after transplantation in 5 of the 8 patients (63%). The mean VAS score at follow-up also remained low (2.3 ± 1.9) compared with that observed at baseline (5.1 ± 0.7).

In contrast to the pain scale results, adverse events were observed in as many as 4 patients (50%) (Table 2). At age 30

TABLE 2. Adverse Outcomes After Autologous Transplantation of BM-MNCs in Patients With Thromboangiitis Obliterans

Adverse Outcomes	30 Days	Final Follow-Up
Death	0	1 (13)
Major amputation	0	0
Minor amputation	2 (25)*	0
Unexpected angiogenesis	0	1 (13)
Recurrence/worsening of skin ulcer/gangrene	0	2 (25)†
Recurrence/worsening of pain	0	1 (13)
Cardiovascular event	0	0
Cerebrovascular event	0	0
Malignancy	0	0

Values are expressed as n (%).

*Amputation was intentionally scheduled to be performed at 1 month after transplantation.

†One patient was the same one who developed unexpected angiogenesis.

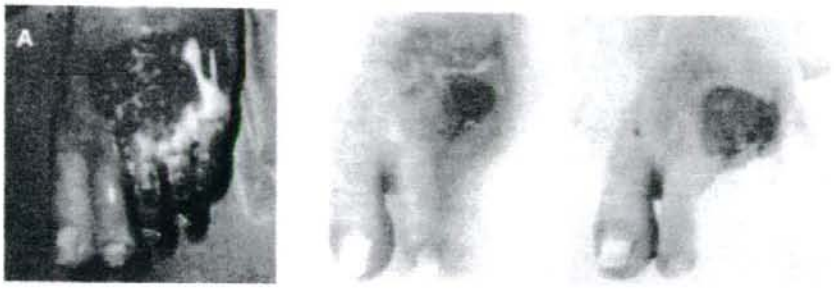

Figure 2. Skin ulcer at (A) 1 month, (B) 2 months, and (C) 4 months after cell transplantation. The patient had far-advanced gangrene, and total limb integrity could not be fully preserved. The patient received a prescheduled amputation of the distal limb at 1 month, and the skin ulcer continued to improve thereafter. At 4 months, however, the skin lesion began to enlarge.

years, patient 4 suddenly died of an unknown cause at 20 months after transplantation. This patient had previously been a smoker, but had stopped smoking before cell transplantation. He had no history of diabetes, hypertension, or hyperlipidemia. Furthermore, ^{201}thallium myocardial scan performed before BM-MNC transplantation showed no signs of myocardial ischemia. After cell transplantation, his limb pain disappeared within 1 week and his skin ulcer resolved by 1 month. Thereafter, he was completely free of limb symptoms. Twenty months after cell transplantation, however, he was found dead at his home. He had never experienced chest pain up to the time of his death. Because no autopsy was performed, the cause of his death remains unknown.

Patient 6 showed worsening of an ischemic ulcer at 4 months. The patient had far-advanced gangrene, and total limb integrity could not be fully preserved. The patient underwent a prescheduled amputation of the distal limb at 1 month (see Short-Term Outcome) (Figure 2A), and the skin ulcer continued to improve thereafter (Figure 2B). At 4 months, however, the skin lesion began to increase in size (Figure 2C). The patient subsequently received a second round of cell therapy.

In patient 7, despite complete healing of the skin ulcer, rest pain did not completely resolve after transplantation, with a VAS score of 3 at 1 month. At 8 months, the patient experienced worsening of rest pain (VAS score=4). After a combination of exercise training and maximal drug therapy, the pain improved and became well tolerated.

Patient 8 experienced swelling and recurrence of the skin ulcer in his foot at 7 months. Computed tomographic angiography documented an early venous return of contrast material in his right limb (Figure 3B) that was not observed at the baseline (Figure 3A). Ultrasound examination disclosed an arterialized waveform in the dorsal vein at the base of his third toe, suggesting the presence of an arteriovenous shunt. By 1 year, the swelling and skin ulcer had spontaneously regressed. The systolic pulsatile component in the venous waveform was found to be diminished on ultrasound examination, and early venous filling had disappeared on computed tomographic angiography (Figure 3C).

Discussion

In the present unblinded and uncontrolled pilot study, we documented that the transplantation of BM-MNCs was associated with an improvement in ischemic symptoms for up to 4 years. Indeed, VAS scores improved from 5.1 ± 0.7 to 2.3 ± 1.9 at follow-up. Furthermore, skin ulcers remained completely healed in 6 of 7 patients. In this regard, the present findings extend previous observations[11] by establishing the potential long-term benefit of BM-MNC transplantation for the treatment of arterial insufficiency.

It should be noted, however, that half of the patients suffered adverse events during follow-up. Such a high rate of adverse events cannot be explained by the natural course of the disease itself. In general, the prognosis of patients with thromboangiitis obliterans is directly related to tobacco

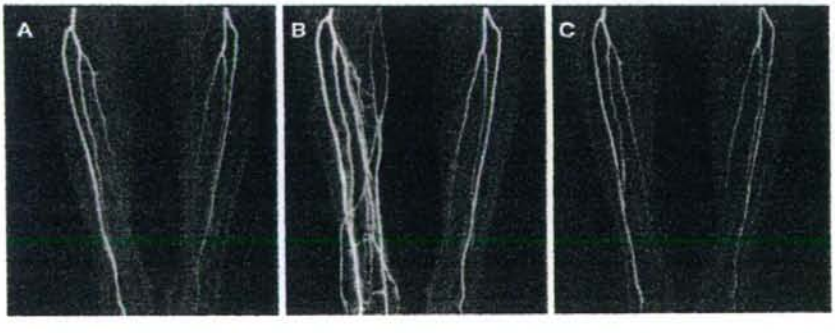

Figure 3. Computed tomographic angiography at (A) baseline, (B) 7 months, and (C) 1 year after cell transplantation. Early venous return of contrast material observed at 7 months spontaneously regressed by 1 year.

use.[12,13] Patients who are able to stop smoking avoid the recurrence of the disease and amputation.[14] In addition, unlike those with atherosclerosis of the extremities, these patients rarely show involvement of visceral vessels and do not appear to be at an increased risk of stroke or myocardial infarction. The mortality rates are thus not higher than those of age- and sex-matched populations.[15,16] It is entirely possible that the high rate of adverse events observed in our patients may have been directly related to BM-MNC transplantation rather than to the progression of the disease itself.

Patient 4 suddenly died at 20 months after transplantation at the age of 30 years. The deaths of patients receiving BM-MNC transplantation were previously reported in the TACT study, in which 2 of 25 patients died of acute myocardial infarction within 24 weeks after transplantation.[11] The patients' backgrounds in the TACT study, in terms of age and comorbidity, may have been totally different from those of our study, in which only patients with thromboangiitis obliterans were recruited. As mentioned above, in patients with thromboangiitis obliterans, coronary involvement is rare, and they usually do very well as long as they discontinue smoking.[12–16] Patient 4 had no risk factors for atherosclerosis and stopped smoking before BM-MNC transplantation. Furthermore, ^{201}thallium scintigraphy performed before transplantation documented no sign of myocardial ischemia. Considering the patient's background and the natural course of the disease,[12–16] the possibility that his death was related to BM-MNC transplantation cannot be excluded. In this regard, several studies have suggested the possible role of BM-MNCs in atherogenesis. A recent report by Silvestre et al,[17] for example, demonstrated that the transplantation of BM-MNCs into ischemic limbs of apolipoprotein E–knockout mice led to a significant increase in atherosclerotic plaque size at a distant site. More recently, George et al[18] have also shown that an intravenous injection of bone marrow cells into apolipoprotein E–knockout mice results in an increase in atherosclerotic lesion size, whereas an injection of endothelial progenitor cells influences plaque stability. These reports indicate that attempts to enhance neovascularization by using BM-MNCs could also enhance unwanted plaque growth and instability, thus suggesting the possibility that our young patient died of an acute coronary event due to accelerated atherogenesis after BM-MNC transplantation.

We also encountered the development of an arteriovenous shunt, which could be a potential consequence of BM-MNC transplantation. Indeed, concerns have been raised about the potential adverse effects of cell transplantation, ie, unregulated differentiation and proliferation. Wakitani et al[19] reported that teratoma formation could occur after embryonic stem cell transplantation. Yoon et al[20] documented intramyocardial calcification after the transplantation of bone marrow cells in rats. Our observations may provide another cautionary example of unregulated differentiation and proliferation. Although the arteriovenous shunt in our case was self-limited, it may represent unwanted angiogenesis; thus, careful monitoring is warranted for future patients who receive BM-MNC transplantation.

Worsening or recurrence of ischemic symptoms was observed in 3 patients. The short-term outcome at 1 month was poor in these patients except in the patient with an arteriovenous shunt. The improvement in rest pain was not substantial in 1 patient. Healing of the skin ulcer was incomplete in another. It is anticipated that a poor response 1 month after BM-MNC transplantation could result in a poor long-term outcome. It is important to note that, in the latter patient with incomplete healing of the skin ulcer, the angiographic improvement of the collateral network at 1 month remained unchanged at 5 months when worsening of the skin ulcer was observed. It is suggested that the temporal sequence of improvement in ischemic limb status does not necessarily parallel the temporal evolution of collateral development.

Conclusions

In this unblinded and uncontrolled pilot study, long-term adverse events after BM-MNC transplantation, including death and unfavorable angiogenesis, were observed in half of the patients with thromboangiitis obliterans. Given the current incomplete knowledge of the safety and efficacy of this strategy, careful long-term monitoring is required for future patients receiving BM-MNC transplantation.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The favorable short-term outcome of bone marrow mononuclear cell transplantation (BM-MNC) transplantation has been established in patients with critical limb ischemia. However, the long-term outcome of this treatment strategy has not been determined yet. In our case series, we documented that long-term adverse events, including death and unfavorable angiogenesis, were observed in 4 of 8 patients receiving BM-MNC transplantation. The first patient suffered sudden death at 20 months after transplantation at 30 years of age. The second patient with incomplete healing of a skin ulcer showed worsening of the lesion at 4 months. The third patient had worsening of rest pain at 8 months. The last patient developed an arteriovenous shunt in the foot at 7 months, which spontaneously regressed by 1 year. Given the current incomplete knowledge on the safety and efficacy of this strategy, it is suggested that careful long-term monitoring is required in patients receiving BM-MNC transplantation. To our knowledge, this is the first report on the long-term outcome of transplantation of BM-MNCs for critical limb ischemia, and the first that documents the development of unfavorable angiogenesis and sudden death after therapeutic angiogenesis.

Complete deficiency in ADAMTS13 is prothrombotic, but it alone is not sufficient to cause thrombotic thrombocytopenic purpura

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ADAMTS13 is a plasma metalloproteinase that regulates platelet adhesion and aggregation through cleavage of von Willebrand factor (VWF) multimers. In humans, genetic or acquired deficiency in ADAMTS13 causes thrombotic thrombocytopenic purpura (TTP), a condition characterized by thrombocytopenia and hemolytic anemia with microvascular platelet thrombi. In this study, we report characterization of mice bearing a targeted disruption of the *Adamts13* gene. ADAMTS13-deficient mice were born in the expected mendelian distribution; homozygous mice were viable and fertile. Hematologic and histologic analyses failed to detect any evidence of thrombocytopenia, hemolytic anemia, or microvascular thrombosis. However, unusually large VWF multimers were observed in plasma of homozygotes. Thrombus formation on immobilized collagen under flow was significantly elevated in homozygotes in comparison with wild-type mice. Thrombocytopenia was more severely induced in homozygotes than in wild-type mice after intravenous injection of a mixture of collagen and epinephrine. Thus, a complete lack of ADAMTS13 in mice was a prothrombotic state, but it alone was not sufficient to cause TTP-like symptoms. The phenotypic differences of ADAMTS13 deficiencies between humans and mice may reflect differences in hemostatic system functioning in these species. Alternatively, factors in addition to ADAMTS13 deficiency may be necessary for development of TTP. (Blood. 2006;107:3161-3166)

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening systemic disease, characterized by anemia, thrombocytopenia, and microvascular thrombosis.[1-4] Hemolysis, the cause of the anemia, generates pointed red cell fragments, schistocytes. Thrombocytopenia is caused by the consumption of platelets in thrombi, which cause renal and neurologic dysfunction. Without treatment, the mortality rate of affected patients exceeds 90%, but plasma exchange reduces the death rate to approximately 20%.[5]

Our understanding of TTP pathophysiology increased considerably with the identification of ADAMTS13, which specifically cleaves the Tyr^{1605}-Met^{1606} peptidyl bond of von Willebrand factor (VWF).[6-10] VWF is a large glycoprotein that mediates platelet adhesion to vascular lesions. It is mainly synthesized in endothelial cells and secreted into the blood as "unusually large" VWF (UL-VWF) multimers, the highly active forms for platelet adhesion and aggregation.[11,12] ADAMTS13 cleaves UL-VWF multimers into smaller forms under flow, limiting platelet thrombus formation under normal conditions. Severe deficiency in ADAMTS13 activity is observed in most patients with TTP, allowing UL-VWF multimers to persist in the circulation.[1-4] UL-VWF multimers mediate enhanced platelet clumping under shear stress, which is thought to cause the clinical symptoms of TTP. Congenital TTP is associated with mutations in the *ADAMTS13* gene, whereas acquired TTP results from the production of autoantibodies against ADAMTS13. A number of causative mutations for congenital TTP have been identified within the *ADAMTS13* gene.[3,4] In vitro expression studies have confirmed the deleterious effects of mutant ADAMTS13 on proteolytic activity or secretion.[13-15]

Here, we generated a mouse model of ADAMTS13 deficiency by a gene-targeting approach, to further understand the pathophysiologic process of TTP. We found that the complete deficiency in ADAMTS13 is not sufficient to produce in mice the typical TTP phenotype. Other triggers may be needed to provoke the disease.

Materials and methods

Generation of ADAMTS13-deficient mice

The isolation of λ phage genomic clones containing *Adamts13* has been previously described.[16] The targeting vector was constructed from a 12.3-kb fragment including exons 3-12, in which the 3.6-kb *SalI-Eco*RI region containing exons 3-6 was replaced by a neomycin resistance cassette. A

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