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H. 知的財産権の出願・登録状況

特に無し。

厚生労働科学研究費補助金（エイズ対策研究事業）

分担研究報告書

血友病の遺伝子治療用ウイルスベクターの作製に関する研究

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研究要旨

血友病の遺伝子治療において治療遺伝子を標的細胞に効率よく導入し安定的に維持させて当該遺伝子を発現させることが必要である。血液凝固因子は肝臓で合成されるので、肝臓細胞に遺伝子を安定的に維持させる方法としてC型肝炎ウイルス（HCV）をもとにして遺伝子ベクターを開発することを目的とする。劇症肝炎由来のHCVに由来するＲＮＡレプリコンを作製し、培養肝臓細胞に導入した。マーカー遺伝子としてネオマイシン耐性遺伝子を用いた場合にはで長期にわたって維持された。しかし緑色蛍光タンパク質遺伝子をマーカー遺伝子として用いた場合には維持されなかった。今後マーカーによる違いのメカニズムを明らかにし、血液凝固因子を安定的に維持させる方法を開発する必要がある。

A. 研究目的

血友病Bにおける変異遺伝子を相補するために野生型IX因子遺伝子のcDNAを肝幹細胞に導入し長期的に野生型凝固因子を発現させることを考えた。凝固因子は肝臓で合成されるので、最も自然な遺伝子治療戦略であると考えたからである。そのようなベクターをC型肝炎ウイルス（HCV）をもとにして開発することを目的とする。今年度は凝固因子遺伝子の代わりに検出が容易なネオマイシン耐性遺伝子または緑色蛍光タンパク質（GFP）遺伝子を遺伝子導入のマーカーとして利用して実験を行うことにした。

B. 研究方法と結果

HCVレプリコンRNAを作製した。C型肝炎ウイルス（HCV）によって劇症肝炎を起こした患者から脇田らが樹立したJFH1株をもとにネオマイシン耐性（*neo*）遺伝子でコア・エンベロープ遺伝子を置き換えた。このレプリコンRNAを試験管内転写によって合成しヒト肝癌Huh7細胞に電気的にトランスフェクトした。1ug当たり１００−５００個程度のG418耐性コロニーが出現した。このコロニーを継代しても少なくとも２ヶ月はG418耐性であった。ネオマイシン耐性遺伝子をGFP遺伝子に変更した場合、緑色蛍光を発する細胞は１週間は認められるもののそれ以降には認められなくなった。さらに、*neo*遺伝子にGFP遺伝子を融合させ

てマーカーとしたときにはG418耐性細胞コロニーはまったく出現しなかった。

（倫理面での配慮）
該当しない

C. 考察
*neo*遺伝子を有するHCVレプリコンは長期にわたって細胞で維持された。しかし、さらに長期の維持と発現が可能かは不明である。
また、GFP遺伝子を有するHCVレプリコンは維持されない。この理由は不明であるが、RNAの配列がHCVレプリコンの複製にtoxicに働いている可能性があると思われる。

D. 結論
培養肝臓細胞で長期にわたって維持されるRNAレプリコンが作製できた。

E. 健康危険情報
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REGULAR ARTICLE

Phenotype correction of hemophilia A mice with adeno-associated virus vectors carrying the B domain-deleted canine factor VIII gene☆

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KEYWORDS
Hemophilia;
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Factor VIII

Abstract Adeno-associated virus (AAV) vectors carrying the B domain-deleted canine FVIII (BDD cFVIII) gene utilizing the β-actin minimum promoter (167b) pseudotyped with serotype 1 (AAV1-β-actin-cFVIII) and serotype 8 (AAV8-β-actin-cFVIII) were developed to express cFVIII in hemophilia A mice. FVIII clotting activities measured by the APTT method increased in hemophilia A mice with intramuscular injection of AAV1-β-actin-cFVIII in a dose-dependent manner. Therapeutic FVIII levels (2.9 ± 1.0%) in hemophilia A mice with the AAV1-β-actin-cFVIII dose of 1×10^{12} gc/body were achieved, suggesting partial correction of the phenotype with AAV1-β-actin-cFVIII vectors. FVIII clotting activity levels in hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII also were increased dose-dependently, achieving therapeutic FVIII levels (5–90%) in hemophilia A mice with the AAV8-β-actin-cFVIII doses of $1–3 \times 10^{11}$ gc/body and supernormal FVIII levels (180–670%) in hemophilia A mice with the AAV8-β-actin-cFVIII dose of 1×10^{12} gc/body. Transduction of the liver with AAV8-β-actin-cFVIII is superior to transduction of skeletal

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muscles with AAV1cFVIII regarding the FVIII production and antibody formation. These data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have a potential for hemophilia A gene therapy.
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Introduction

Hemophilia A is an inherited X-linked life-threatening bleeding disorder caused by abnormalities in the factor VIII (FVIII) gene that lead to deficiency of FVIII and bleeding diathesis. Hemophilia is considered suitable for gene therapy because it is caused by a single gene abnormality and therapeutic coagulation factor levels may well be in a wide range (5–100%) without strict gene regulation [1–3]. Gene therapy is expected to provide an alternative to current FVIII supplemental therapy because it may be able to prevent lethal intracranial bleeding episodes and provide a good quality of life without bleeding. Among a variety of vectors, Adeno-associated virus (AAV) vectors are thought to be ideal for transfer of therapeutic genes since they are derived from non-pathogenic viruses and have been demonstrated to provide sustained transgene expression in non-dividing cells with little toxicity, although delivery of the FVIII gene using AAV vectors is limited by their small packaging capacity [4].

The dual AAV vector system that utilized two AAV2 vectors separately carrying the FVIII heavy chain gene and the FVIII light chain gene was successful for expressing functional FVIII molecules and correction of phenotypes of hemophilia A mice [5]. A recent report has shown that construction of single AAV vectors carrying the 4.5-kb B domain-deleted (BDD) canine FVIII (cFVIII) gene can be packaged in AAV vectors using the 543-base (b) DNA fragments composed of the insulin-like growth factor binding protein (IGBP) promoter, an enhancer element, and an intron, although the packaging efficiency for incorporation of the cFVIII gene into the AAV vectors was low [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact, minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy, whereas no liver dysfunction was observed upon injection of the same vector into the skeletal muscles in the same series of clinical trials [7,8]. Although the precise mechanisms of these phenomena have not yet been elucidated, the T cell response to viral capsid was thought to be one of the causes of the liver injuries [8]. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs.

To explore the possibility that skeletal muscles transduced with AAV vectors could produce FVIII resulting in increase of FVIII levels in the circulation, we packaged the BDD FVIII gene in AAV vectors using the same promoter and compare production of FVIII in the skeletal muscles and in the liver that transduced with AAV vectors. We developed AAV vectors carrying the BDD cFVIII gene utilizing the β-actin minimum promoter (167b) and tried to express canine FVIII in hemophilia A mice. Recent studies on recombinant AAV vectors have shown that AAV serotypes have tropism, suggesting that a specific AAV serotype vector can be used for gene delivery to certain organs [3,6,9]. AAV serotype 1 (AAV1) may be the best AAV serotype for transduction of skeletal muscles and the AAV serotype 8 (AAV8) is superior to other AAV serotypes for transduction of the liver [6,9], thus in this study, we constructed AAV1 and AAV8 vectors carrying the BDD cFVIII gene and studied efficacies of these vectors for FVIII transgene expression, long term transgene expression, and neutralizing antibody formation to the transgene products in the hemophilia A mice.

Materials and methods

Vector construction

The full-length human FVIII (hFVIII) cDNA was a generous gift from Dr. J.A. van Mourik (Blood Coagulation, Sanquin, Amsterdam, Netherlands) and the human B domain deleted (BDD) FVIII (hFVIII) cDNA was generated by PCR-based mutagenesis as described [10,11]. The canine FVIII (cFVIII) cDNA was a generous gift from Dr. Brownlee (Chemical Pathology Unit, University of Oxford, UK) and the BDD canFVIII cDNA also was generated by PCR-based mutagenesis. The intervening amino acid sequence of the heavy chain and the light chain of BDD cFVIII was RSFS^{743}-Q^{1630}NPPVSK. The CAG promoter is a chimeric promoter, composed of the CMV enhancer, the chicken β-actin promoter,

and an intron, was derived from pCAGGS [12]. The chicken β-actin minimum promoter (−155~+12, 167 b) was generated by PCR, cloned in pCR2.1 TOPO (Invitrogen), and sequenced. Plasmid vector p1.1c, composed of the CMV promoter, human growth hormone gene intron 1, and the SV40 polyadenylation signal sequences, was kindly supplied by Avigen Inc. The DNA fragments spanning the CMV promoter and the human growth hormone intron of p1.1c were replaced with the CAG promoter, the phosphoglycerokinase 1 (PGK1) promoter, or the β-actin minimum promoter DNA fragments to make plasmid p1.1CAG, p1.1PGK1, or p1.1β-actin, respectively. The DNA fragments encoding the BDD hFVIII cDNA or the BDD cFVIII cDNA were cloned in the downstream of the respective promoter sequences of these plasmids to make p1.1CMV-hFVIII, p1.1CAG-hFVIII, p1.1PGK1-hFVIII, p1.1β-actin-hFVIII, and p1.1β-actin-cFVIII, respectively. The Lac Z gene was cloned in the downstream of the β-actin promoter to make plasmid p1.1β-actin Lac Z. DNA fragments spanning the promoter, the LacZ gene, and the poly adenylation signal sequence of pAAV2 Lac Z (Stratagene) were replaced with DNA fragments spanning the β-actin promoter, the BDD cFVIII cDNA, and the SV40 poly adenylation signal sequences of p1.1β-actin-cFVIII to make the gene transfer vector pAAV2-β-actin-cFVIII in which these DNA fragments were flanked by ITR sequences of AAV serotype 2 (AAV2) as described previously [9,13]. The gene transfer vector pAAV2-β-actin-Lac Z equipped with AAV2 ITRs was also constructed. The chimeric packaging plasmid for AAV8 capsid pseudotyping was a generous gift from Dr. James M. Wilson (Division of Medical Genetics, Department of Medicine, University of Pennsylvania, Philadelphia, PA) [6]. The packaging plasmid composed of the AAV2 rep gene and the cap gene derived from AAV1 for AAV1 capsid pseudotyping was described previously [9].

AAV vector production

Viral vectors were packaged with AAV1 or AAV8 capsid by pseudotyping. The FVIII gene or the Lac Z gene located in the downstream of the β-actin minimum promoter and flanked by AAV2 ITRs was packaged by triple plasmid transfection of human embryonic kidney 293 (HEK 293) cells, kindly supplied by Avigen Inc., with the chimeric packaging plasmid, the adenovirus helper plasmid pHelper (Strategene, La Jolla, CA), and gene transfer plasmid vectors (pAAV2-β-actin-cFVIII or pAAV2-β-actin-Lac Z) as described previously [9,13]. For virus vector purification, the DNAse (Benzonase, Merck Japan, Tokyo, Japan)-treated virus particle containing samples were subjected to two rounds of iodixyanol-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) in the presence of 25 mM EDTA at 21 °C as described [9]. Titration of recombinant AAV vectors was carried out by quantitative dot-blot hybridization using the ^{32}P-labeled probes [9,13].

Analysis of the β-actin minimum promoter activity

Expression of hFVIII in HEK293 cells transfected with plasmid vectors p1.1β-actin-hFVIII, p1.1CMV-hFVIII, p1.1CAG-hFVIII, and p1.1PGK1-hFVIII by the calcium phosphate coprecipitation method was studied to show that the β-actin minimum promoter had a enough FVIII expression activity. After incubation with the DNA containing media for 6 h, HEK 293 cells were incubated further in DMEM/HAM F-12 media supplemented with 10% fetal bovine serum for 48 h at 37 °C in the presence of 5% CO_2. FVIII clotting activities in the conditioned media of HEK 293 cells harvested after 48 h incubation were quantified by the activated partial thromboplastin time (APTT) method using FVIII deficient plasma. FVIII activities were expressed as the percentages of normal control plasma.

Animal experiments

FVIII-deficient mice (Hemophilia A mice) with targeted destruction of exon 16 of the FVIIII gene were previously reported by Bi et al. [14] and generously given to us by Dr. H. H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA). J1 ES cells were used for targeted destruction of the FVIII gene and blastcysts derived from C57BL/6 mice were used to generate chimaeras [14]. C57BL/6 wild-type mice were purchased from SLC Inc. Mice were maintained in a standard lighting condition in a clean room. All surgical procedures were carried out in accordance with guidelines approved by the institutional Animal Care and Concern Committee at Jichi Medical School [15]. Male hemophilia A mice and male wild-type C57BL/6 mice were used in the experiments. Blood was drawn from the cervical vein plexus of mice and mixed with 1 / 10 volume of 3.8% sodium citrate, and then platelet-poor plasma was prepared by centrifugation. AAV8 vectors were injected intravenously into the cervical vein plexus while AAV1 vectors were injected directly to the skeletal muscles of lower extremities of mice under anesthesia with isoflurene [15]. Cyclophosphamide (100 μg/body/day, SIGMA-ALDRICH Japan, Tokyo, Japan) and tachrolimus (12.5 μg/body/day, Fujisawa Pharmaceuticals Co., Tokyo, Japan) were

given (s.c.) to mice daily after vector injection as the immunosuppressant.

Analysis of cFVIII expression in mice

FVIII activities were measured by the activated partial thromboplastin time (APTT) method used for determination of plasma FVIII activities of hemophilia patients utilizing human FVIII-deficient plasma as described [15]. Antigen levels of cFVIII in mouse plasma were determined by ELISA (Asserachrom FVIIIC: Ag, Diagnostica Stago, Parsipanny, NJ) as described [6]. Analyses of neutralizing antibodies against cFVIII developed in mice were performed by the Bethesda method as described using FVIII deficient plasma and normal canine plasma. Detection of the transcripts of cFVIII transgene was performed by RT-PCR [10,11]. RNA was isolated from the mouse organs using an RNA isolation kit (RNeasy Protect kit; Qiagen Inc., Valencia, CA). DNAse I (Amplification grade, Invitrogen, Carlsbad, CA)-treated and heat-treated RNA samples were subjected to RT-PCR using a pair of primers (sense: 5′-GTTGGAGCACAAACTGACTTCC-3′, antisense: 5′-CAATTGAGAAGGTGTCATCATACTC-3′) for cFVIII and an RT-PCR kit (SuperScript One-Step RT-PCR System, Invitrogen). PCR amplification (25–30 cycles) for cFVIII was performed as described [10,11]. A primer pair for mouse GAPDH mRNA (R&D Systems, Inc., Minneapolis, MN) was used instead of cFVIII primers in the control RT-PCR experiments. For detection of cFVIII molecules in mouse tissues by immunohistochemistry, the skeletal muscles and the liver were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at 4 °C, incubated with PBS containing sucrose (10–30%), and then frozen in the presence of OCT compound in dry ice/ethanol. Sections were prepared from frozen tissues at −25 °C and attached to poly-lysine coated glass slides. For detection of FVIII, tissue sections were blocked with 1% rabbit serum in PBS containing Triton-X 100 (0.1%) and incubated with sheep polyclonal anti-human FVIII antibodies (Cedarlane Laboratories Ltd, Homby, Ontario, Canada) at 4 °C for 16 h [10,11]. After washing in PBS, sections were incubated with biotin-conjugated rabbit anti-sheep IgG antibody followed by the ABC reagents (Vectastain ABC Elite kit; Vector, Burlingame, CA) and a DAB kit (Vector).

Detection of ß-galactosidase in mouse tissue

To analyze Lac Z gene expression in mice injected with AAV-β-actin-Lac Z, mouse tissues were fixed with 2% paraformaldehyde in PBS for 5 min, washed with PBS, incubated with PBS containing sucrose (10–30%), and frozen with OTC compound (Tissue-Tek; Miles, Inc., Elkhart, IN) in dry ice/ethanol. Sections, prepared from frozen tissues at −25 °C and attached to poly-lysine coated glass slides, were incubated in PBS containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 0.01% Na deoxycholate, 0.1% Triton X-100 at 25 °C for 1 h [10]. Some sections further were processed for the Feulgen reaction (red purple) to visualize nuclei.

Results

Expression of FVIII by the ß-actin minimum promoter

We studied expression of hFVIII in HEK293 cells transfected with plasmid vectors p1.1β-actin-hFVIII, p1.1CMV-hFVIII, p1.1CAG-hFVIII, and p1.1PGK1-hFVIII. FVIII clotting activities detected in the conditioned medium of HEK293 cells are shown in Fig. 1. Expression of FVIII driven by the β-actin minimum promoter (167 b) in the 293 cells was approximately 1/3–1/2 of that by the CMV promoter (1 kb) or the CAG promoter (1.7 kb). Although the β-actin minimum promoter is weaker than the CMV promoter and the CAG promoter, it

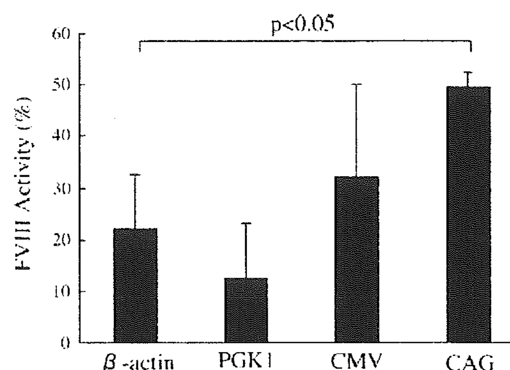

Figure 1 FVIII expression by the β-actin minimum promoter in vitro. FVIII clotting activities expressed in the conditioned media of HEK 293 cells transfected with p1.1CMV-FVIII, p1.1 CAG-FVIII, p1.1 PGK1-FVIII, or p1.1 β-actin-FVIII after 48 h incubation are shown. FVIII clotting activities in the conditioned media were quantified by the APTT method using FVIII deficient plasma and FVIII clotting activities were expressed as the percentages of normal control plasma. There were no FVIII activities in the conditioned media of HEK 293 cells with mock transfection. There was a significant difference of FVIII activity levels in the conditioned media of HEK 293 cells transfected with p1.1 CAG-FVIII and those in the conditioned media of HEK 293 cells transfected with p1.1 β-actin-FVIII ($n=4$, Student's t-test, $p<0.05$).

was stronger than the PGK1 promoter (515 b) by 1.2-fold in terms of FVIII expression activity. Since the β-actin minimum promoter was stronger than the PGK1 promoter and was short enough to construct 5.1-kb AAV vectors carrying the BDD FVIII cDNA, we used the β-actin minimum promoter to produce AAV vectors carrying the BDD FVIII gene.

Expression of Lac Z gene by the ß-actin minimum promoter in vivo

To confirm that the β-actin minimum promoter can express a transgene in vivo, AAV vectors carrying the Lac Z gene located in the downstream of the β-actin minimum promoter (AAV1-β-actin-Lac Z, AAV8-β-actin-LacZ) were injected to wild-type mice and expression of the Lac Z gene was studied by X-gal staining. When AAV1-β-actin-Lac Z was injected to the skeletal muscles of lower extremities of wild-type mice, Lac Z gene expression was observed in muscle fibers as shown in Fig. 2A. No apparent Lac Z gene expression was observed in other organs in the AAV1-β-actin-Lac Z injected mice (not shown), suggesting that transgene expression in other organs was minimum. Lac Z gene expression of mice with intravenous injection of AAV8-β-actin-Lac Z mainly was observed in the liver as shown in Fig. 2B. Lac Z gene expression also was observed in other organs including the heart, lung, and skeletal muscles in accordance with the previous report [16]. The liver could be transduced with intravenously injected AAV8-β-actin-Lac Z almost as efficiently as intraportally injected vectors (not shown).

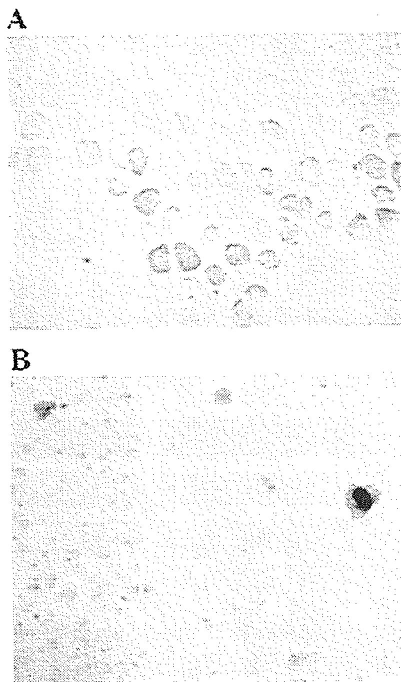

Figure 2 Expression of the Lac Z gene in mice transduced with AAV vectors carrying the Lac Z gene located downstream of the β-actin minimum promoter. X-gal staining of the skeletal muscles of mice with intramuscular injection of AAV1-β-actin-Lac Z (A) and of the liver of mice with intravenous injection of AAV8-β-actin-Lac Z (B) is shown.

Expression of FVIII by AAV vectors carrying the BDD cFVIII gene

AAV1-β-actin-FVIII vectors were injected into skeletal muscles of hemophilia A mice and AAV8-β-actin-FVIII vectors were intravenously injected into the cervical vein plexus of hemophilia A mice. FVIII clotting activities of citrated plasma drawn from mice were measured by the APTT method using FVIII-deficient human plasma.

FVIII clotting activities in mouse plasma increased on days 14 and 28 after AAV1 vector injection. The increase of FVIII clotting activities on day 28 after injection was dose-dependent. The FVIII activity levels in peripheral blood increased to 2.9 ± 1.0% in hemophilia A mice with the AAV1-β-cFVIII dose of 1×10^{12} gc/body (Fig. 3), suggesting partial correction of the phenotype with AAV1-β-cFVIII vectors. After these periods, FVIII activities decreased to the basal levels of mice before vector injection. FVIII antigen levels increased in parallel with levels of FVIII activity, confirming expression of cFVIII transgene in mice (not shown). Analyses for antibody against transgene products showed that neutralizing antibodies developed in 4 out of 6 tested mice by week 12 after vector injection, although the antibody titers were not high (Table 1). The RT-PCR analysis and the immunohistochemistry study suggested the presence of the transgene transcripts and products in the vector-injected muscles, suggesting that decrease of FVIII levels may be accounted for by the presence of neutralizing antibody to cFVIII.

FVIII clotting activity levels in hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII also were increased dose-dependently on day 28, achieving therapeutic FVIII levels (5–90%) in hemophilia A mice with the AAV8-β-actin-cFVIII doses of $1{-}3 \times 10^{11}$ gc/body and supernormal FVIII levels (180~670%) were achieved in hemophilia A mice with the AAV8-β-actin-cFVIII dose of 1×10^{12} gc/body (Fig. 4). These data on AAV8 vector-transduced FVIII expression were almost comparable with the results of the previous study using the single AAV8 vector carrying the BDD cFVIII gene [6], suggesting that β-actin minimum promoter almost

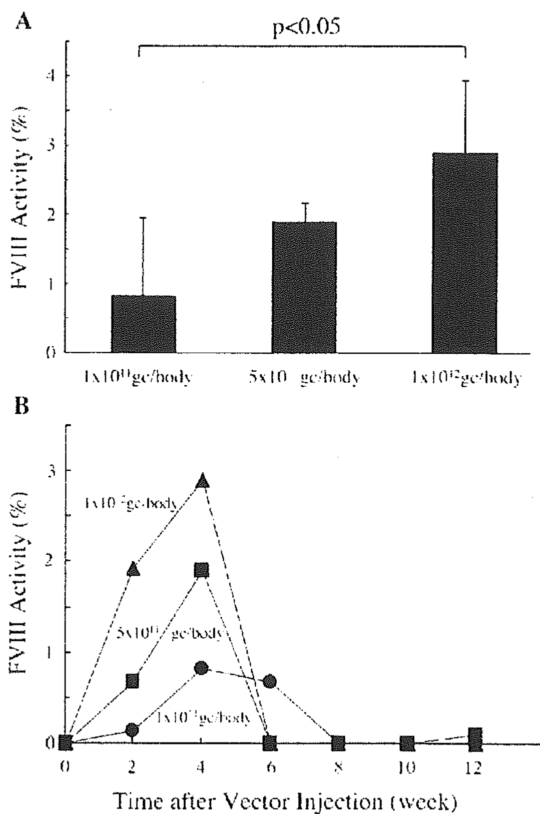

Figure 3 FVIII levels in plasma of hemophilia A mice after intramuscular injection of AAV1-β-actin-cFVIII. FVIII clotting activity levels expressed in plasma of hemophilia A mice ($n=4$) on day 28 after intramuscular injection of AAV1-β-actin-cFVIII are shown in panel A. Activity levels of cFVIII in peripheral blood of hemophilia A mice with injection of AAV1-β-actin-cFVIII (circles, 1×10^{11} gc/body: squares, 5×10^{11} gc/body: triangles, 1×10^{12} gc/body) are shown in panel B.

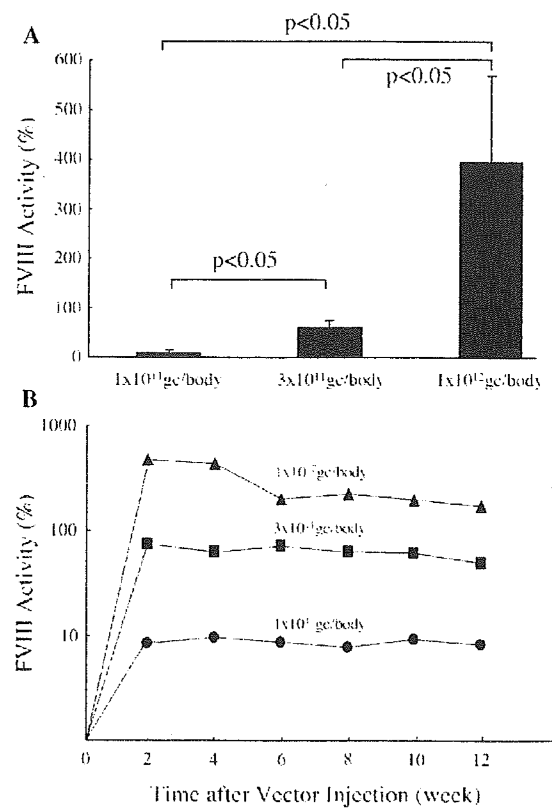

Figure 4 FVIII levels in plasma of hemophilia A mice after intravenous injection of AAV8-β-actin-cFVIII. FVIII clotting activity levels expressed in plasma of hemophilia A mice ($n=4$, each group) on day 28 after intravenous injection of AAV8-β-actin-cFVIII are shown in panel A. Activity levels of cFVIII in peripheral blood of hemophilia A mice ($n=4$, each group) with injection of AAV8-β-actin-cFVIII (circles, 1×10^{11} gc/body; squares, 3×10^{11} gc/body; triangles, 1×10^{12} gc/body) are shown in panel B.

worked as efficiently as the chimeric IGBP promoter complexes. High-level expression of FVIII in the vector-injected hemophilia A mice was sustained for more than 12 weeks. No apparent neutralizing antibody developed during the 12-week period after vector injection (Table 1). FVIII antigen levels also increased in parallel with FVIII activity levels, confirming expression of the cFVIII transgene in mice (not shown). The antigen levels of cFVIII determined by the ELISA for human FVIII were approximately 1/5 of the FVIII activity levels determined by the APTT method. Analyses for cFVIII transcripts suggested that the cFVIII gene mainly was expressed in the liver (Fig. 5) together

Table 1 Neutralizing antibodies against cFVIII developed in hemophilia A mice

	Inhibitor positive mouse	Bethesda units/mL
AAV1cFVIII	4/6 (66.7%)	9.4 ±9.5[a]
AAV8cFVIII	0/9 (0%)	Not detected

[a] Neutralizing antibodies detected by week 12 after vector injection.

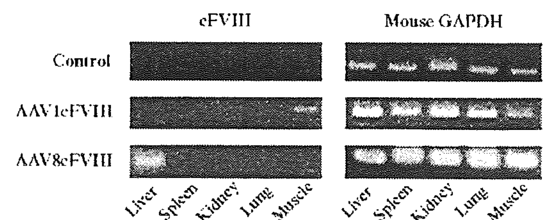

Figure 5 Analysis for cFVIII transcripts in mice. The RT-PCR analyses for the transcripts derived from the cFVIII gene (cFVIII) of RNA isolated from hemophilia A mouse organs without (control) or with intramuscular injection of AAV1-β-actin-cFVIII vectors (AAV1cFVIII) or intravenous injection of AAV8-β-actin-cFVIII vectors (AAV8cFVIII) are shown. For the control, the RT-PCR analysis for mouse GAPDH (Mouse GAPDH) of RNA isolated from hemophilia A mice with or without injection of AAV-β-actin-cFVIII vectors was performed simultaneously.

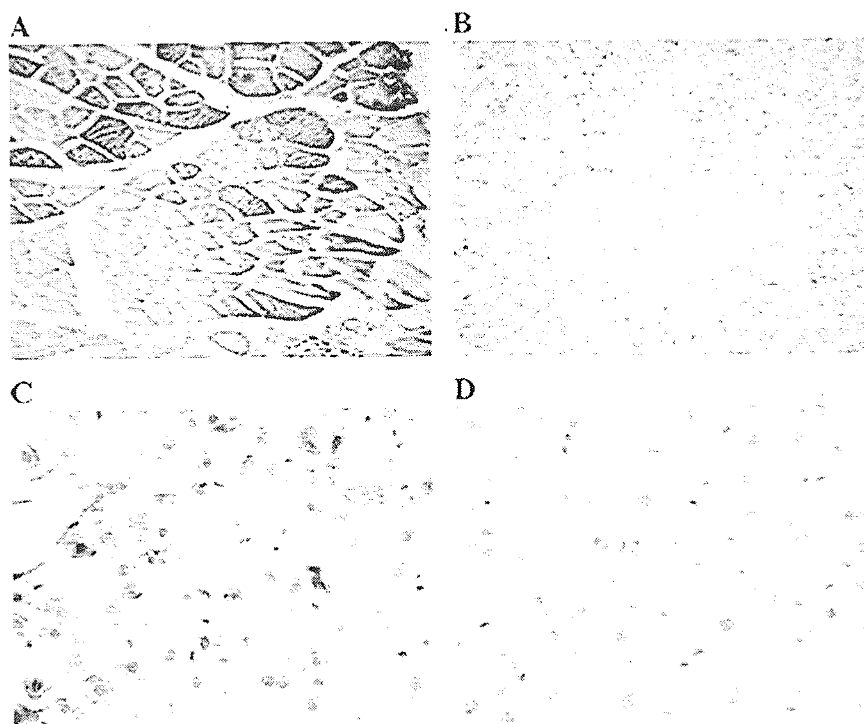

Figure 6 Immunohistochemical Analysis for cFVIII transgene products. Immunohistochemistry for cFVIII of the skeletal muscles of hemophilia A mice with intramuscular injection of AAV1-β-actin-cFVIII vectors (A) and the liver of hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII vectors (C) is shown (positive stain: brown). For the control, sections of the skeletal muscles (B) and the liver (D) obtained from hemophilia A mice without vector injection were processed simultaneously with anti-FVIII antibodies.

with the traceable expression in the heart, lung, and spleen (not shown). In accordance with the data on cFVIII transcripts, cFVIII molecules were immunohistochemically detected in the skeletal muscles of AAV1-β-actin-cFVIII injected mice and in the liver of mice with intravenous injection of AAV8-β-actin-cFVIII (Fig. 6).

Discussion

Because of the size and nature of the FVIII gene (cDNA), there were difficulties in hemophilia A gene therapy compared with gene therapy for hemophilia B. These difficulties were solved by efforts of many investigators that allowed use of a modified FVIII gene such as BDD FVIII cDNA, improved vector systems, and new strategies. Based upon these studies, a few clinical trials of hemophilia A gene therapy were conducted [17–19]. Increase of FVIII activities in the circulation and clinical improvements were observed in patients who received vector injection or transplantation of genetically modified cells. However, long-term expression of FVIII from the transgenes was not achieved in these studies. Thus, reexamination of the vector systems, the target organs for transduction, and the promoters may be required.

The recombinant AAV vectors are thought to be one of the better vectors in terms of its capability to transduce non-dividing cells and long-term transgene expression, although delivery of the FVIII gene using AAV vectors were limited by its small packaging capacity [4]. The dual AAV vector system utilizing separate AAV2 vectors independently carrying the FVIII heavy chain gene and the FVIII light chain gene could express functionally active FVIII [5]. However, there was an imbalance in the expression levels of the FVIII heavy chain and FVIII light chain, suggesting that over-expressed free FVIII light chain molecules might be more immunogenic than the native molecules. The BDD FVIII gene could be packaged in AAV2 or AAV8 vectors in the previous studies and these vectors could efficiently transduce the liver with intraportal injection of the vectors [6]. Transduction of the liver with peripheral vein injection of AAV8 vectors was as efficient as portal vein injection of vectors, although that of AAV2 vectors was not [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ, so FVIII

synthesis in hepatocytes and its subsequent secretion into the circulation may be warranted. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs. AAV1 vector-based transduction of the skeletal muscles has beneficial characteristics of removing the transgenes. This is the first report of sufficient expression of FVIII in the skeletal muscles transduced with AAV vectors and suggests that skeletal muscle-directed FVIII expression has a potential for hemophilia A gene therapy.

Compared with synthesis and secretion of FVIII into the circulation from the liver, transport of sufficient FVIII into the circulation from the skeletal muscle fibers is not assured. Based upon our data, it is apparent that transduction of the liver with AAV8-β-actin-cFVIII is superior to transduction of skeletal muscles with AAV1-β-actin-cFVIII regarding FVIII production. The difference between FVIII levels in the peripheral blood of these vector-injected mice may be due to how the transduced cells secrete FVIII molecules into the circulation. Hepatocytes actively secrete a variety of molecules including FVIII into the circulation. Since recombinant cFVIII is in a BDD form, its expression in and secretion from hepatocytes is expected to be better than native FVIII [20], accounting for the high cFVIII expression in mice with intravenous injection of AAV8 vectors carrying the cFVIII gene though cFVIII expressing hepatocytes were not abundant. Although muscle fibers are surrounded by capillaries, transport of recombinant FVIII molecules from muscle fibers to capillaries would not be as efficient as that from hepatocytes.

In terms of the immune reaction to transgene products, muscle stem cells have been shown to function as antigen-presenting cells, suggesting that expression of the transgene by the ubiquitous promoter in the skeletal muscles might lead to development of antibodies against the transgene products if there is no immune tolerance to the transgene products [21]. This was confirmed by Wang et al. [22]. Neutralizing antibody formation was observed in 66.7% of mice with AAV1cFVIII injection even with administration of immunosuppressant, while it was not observed in mice with AAV8-β-actin cFVIII injection by week 12 after vector injection, supporting the potential advantage of AAV8 vector-based transduction of the liver over the muscle-directed transduction by AAV1 vectors.

Each vector system has advantages and disadvantages in these respects. We may need to confirm the results obtained in hemophilia mice using dogs and non-human primates that genetically are more close to humans because there may be differences in transduction efficiency of various serotypes between mice and humans [23]. Taken together, we may need to perform a comparative study using another animal models such as hemophilic dogs and non-human primates that are more genetically close to humans than mice to address these questions. Additionally, use of tissue-specific promoters to minimize neutralizing antibody formation may be a better strategy for expressing transgenes in a tissue- and organ-specific manner. These experiments will be performed in future studies.

In conclusion, our data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have the potential for hemophilia A gene therapy. Our present studies have provided important insight about selecting the appropriate target for delivery of the therapeutic genes and the vector system for the hemophilia A gene therapy.

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Severe secondary deficiency of von Willebrand factor–cleaving protease (ADAMTS13) in patients with sepsis-induced disseminated intravascular coagulation: its correlation with development of renal failure

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Deficiency of ADAMTS13 is found in patients with thrombotic thrombocytopenic purpura (TTP), and the genetic defects in the *ADAMTS13* gene or the autoantibody against ADAMTS13 is thought to be responsible for the development of TTP. The clinical correlation and mechanisms of secondary ADAMTS13 deficiency in other disease states were investigated. In addition to TTP, ADAMTS13 levels were severely decreased in patients with sepsis-induced disseminated intravascular coagulation (DIC). The incidence of acute renal failure and serum creatinine levels in patients with ADAMTS13 activity levels lower than 20% (incidence, 41.2%; creatinine, 160 ± 150 μM [1.81 ± 1.70 mg/dL]) ($P < .05$) were significantly higher than they were in patients with ADAMTS13 activity levels higher than 20% (incidence, 15.4%; creatinine, 84 ± 67 μM [0.95 ± 0.76 mg/dL]) ($P < .01$). Additionally, unusually large von Willebrand factor multimers were detected in 26 (51.0%) of 51 patients with ADAMTS13 activity levels lower than 20%. Lower molecular weight forms of ADAMTS13 were found in the plasma of patients with sepsis-induced DIC, suggesting that the deficiency of ADAMTS13 was partially caused by its cleavage by proteases in addition to decreased synthesis in the liver. These data suggested that severe secondary ADAMTS13 deficiency can be associated with sepsis-induced DIC and may contribute to the development of renal failure. (Blood. 2006;107:528-534)

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Introduction

Deficiency of the von Willebrand factor (VWF)–cleaving protease,[1-5] ADAMTS13 (a disintegrin-like metalloprotease with thrombospondin type 1 repeats) is found in most patients with thrombotic thrombocytopenic purpura (TTP), and this deficiency is thought to be responsible for platelet aggregation and microthrombi formation in the circulation, which in turn cause typical thrombotic microangiopathies (TMAs) to develop.[6-9] Deficiency of ADAMTS13 in patients with TTP is caused by genetic defects in the *ADAMTS13* gene (familial TTP, Upshaw-Schulman syndrome) or by autoantibodies against ADAMTS13. Although hemolytic uremic syndrome (HUS) is clinically similar to TTP, the role of ADAMTS13 deficiency in the development of HUS is controversial because reports conflict about whether ADAMTS13 activity remains unchanged[6-8] or decreases.[10-13] It also is possible that secondary deficiency of ADAMTS13 may account for the development of microthrombi formation in disease states other than TTP. To search for the clinical correlation of secondary ADAMTS13 deficiency in disease states, we measured ADAMTS13 activity levels by the standard method[14] and determined antigen levels by our newly developed monoclonal antibody–based enzyme-linked immunosorbent assay (ELISA) for ADAMTS13 in patients with TTP and in patients with sepsis-induced disseminated intravascular coagulation (DIC). We found that severe secondary ADAMTS13 deficiency could occur in patients with sepsis-induced DIC and that it had a clinical correlation with the development of renal failure.

Patients, materials, and methods

Blood samples

All samples were obtained with informed consent from patients according to the Declaration of Helsinki. Blood was drawn from 113 patients (65 men, aged 17-83; 44 women, aged 21-81; idiopathic TTP, 3 patients; Upshaw-Schulman syndrome, 1 patient; sepsis-induced DIC, 109 patients). The diagnosis of TTP was made with note of the presence of typical clinical features (fever, bleeding tendency, neurologic symptoms) laboratory examination results (thrombocytopenia, hemolytic anemia with red blood cell fragmentation, increased levels of LDH, increased levels of serum creatinine), and effectiveness of plasma exchange treatment. Patients with definite infection, such as bacteremia, pneumonia, urinary tract infection, biliary tract infection, or pathogenic *Escherichia coli* O-157 infection, were excluded from the TTP group. The patient with Upshaw-Schulman syndrome had TTP, and plasma transfusion was effective in preventing recurrence.

The diagnosis of DIC was made according to the criteria established in 1988 by the Japanese Ministry of Health and Welfare. Criteria for DIC were

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reported previously.[15] Briefly, the presence of underlying disease—such as infection and malignancies—specific clinical conditions (bleeding symptoms, organ dysfunction), and results of laboratory examinations (platelet counts, prothrombin time, fibrinogen, fibrin degradation products) were quantified based on score. If the score was 7 or more, the diagnosis of DIC was made. In patients with hematologic malignancy, scores on the bleeding symptom and platelet counts were excluded, and the diagnosis of DIC was made if the total score was 4 or more.

The diagnosis of sepsis was made according to the guidelines of the Society of Critical Care Medicine Consensus Conference Committee.[16] Briefly, patients had to meet at least 3 of the 4 criteria for systemic inflammatory response and had to have a known infection or a suspected infection, as evidenced by one or more of the following: bacteremia, pathologic microorganisms or white blood cells in a normally sterile body fluid such as urine or joint fluid; purulent sputum; radiographic evidence of pneumonia; clinical signs associated with high risk for infection (eg, cholangitis, peritonitis) or increased levels of endotoxin, β-D-glucan, or *Candida* antigen.

Thirty-nine patients with DIC were shown to have bacteremia, as evidenced by their blood cultures. Twelve patients, whose bacteremia was not evidenced by blood culture, had increased levels of endotoxin, β-D-glucan, or *Candida* antigen. Twenty-eight patients who were negative for bacteria in blood culture or who did not have increased levels of endotoxin, β-D-glucan, or *Candida* antigen, had pneumonia as evidenced by radiography, 11 patients had urinary tract infection, 4 patients had wound infection during postoperative periods, 1 patient had biliary tract infection, 1 patient had bacterial arthritis, 1 patient had bacterial osteomyelitis, and 12 patients had suspected respiratory infection with the presence of pathogenic microorganisms, such as methicillin-resistant *Staphylococcus aureus* in sputum cultures.

Citrated platelet-poor plasma samples were prepared and stored at −80°C until use. Blood was also drawn from 12 healthy volunteers (7 men, aged 25-53; 5 women, aged 25-48) for the preparation of normal pooled plasma. Laboratory analyses of patients' blood were performed by the standard methods using automated analyzers. Complete blood cell counts, serum creatinine (normal range, 35-97 μM [0.4-1.1 mg/dL]), serum bilirubin (normal range, 3-21 μM [0.2-1.2 mg/dL]), aspartate aminotransferase (AST; normal range, 8-35 IU/L), alanine aminotransferase (ALT; normal range, 5-40 IU/L), serum albumin (normal range, 39-51 g/L [3.9-5.1 g/dL]), and C-reactive protein (CRP; normal range, less than 5 mg/L [0.5 mg/dL]) were measured in this study.

Determination of ADAMTS13 antigen and activity levels

The human *ADAMTS13* cDNA used in this study was described previously.[5] Human ADAMTS13 was expressed in human embryo kidney 293 cells stably transfected with pCAG-ADAMTS13 Neo and was purified. Murine monoclonal antibodies (mAbs) to human ADAMTS13 were generated by the standard method[17] after immunization of BALB/c mice with recombinant human ADAMTS13. Two mAbs, WH10 and WH2-22-1A, were selected for ELISA, which was shown to bind to the third TSP-1 motif and to the disintegrin domain of ADAMTS13 by the binding study to recombinant ADAMTS13 mutants, respectively.[5,14,18] WH10 (2 μg/mL) was used for microtiter plate coating (Maxi Sorp plate; Nalge Nunc International, Rochester, NY). After blocking with 1% casein, plasma samples from healthy subjects and patients were diluted in phosphate-buffered saline, pH 7.2/0.1% casein, and then incubated in WH10-coated plates. ADAMTS13 bound to the microtiter plates was detected by peroxidase-conjugated WH2-22-1A. Purified recombinant ADAMTS13 was used as the standard to determine ADAMTS13 antigen levels in normal plasma. The ADAMTS13 level in each patient's plasma was expressed as the percentage of that in normal pooled plasma. ADAMTS13 activity levels in plasma were measured according to the previously described method.[14] Briefly, 10 μL plasma was mixed with purified VWF (1 μg) in 100 μL reaction buffer (5 mM Tris [pH 8.0]/1.5 M urea/10 mM $BaCl_2$/0.4 mM Pefabloc SC [Roche Diagnostics, Mannheim, Germany]) at 37 °C for 24 hours. Reaction was terminated by the addition of 10 μL of 500 mM EDTA, pH 8.0.[14] Portions of samples were subjected to 1.4% sodium dodecyl sulfate–agarose gel electrophoresis to determine the extent of VWF degradation. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes, and VWF multimers were detected by peroxidase-labeled rabbit anti–human VWF antibodies (Dako, Glostrup, Denmark).[12-14]

Quantification of molecular markers of DIC

Plasma levels of fibrin degradation products (FDPs) were quantified with commercial kits (Roche Diagnostics, Tokyo, Japan) used for laboratory examinations. Given that the quantification of free thrombin concentration in plasma is technically difficult, we used ELISA (Sysmex, Kobe, Japan) to quantify plasma levels of thrombin/antithrombin III (TAT) complexes. Similarly, plasma levels of plasmin/α2 plasmin inhibitor complexes (PICs) were measured using ELISA with commercial kits (Sysmex) used for laboratory examinations. Plasma plasminogen activator inhibitor 1 (PAI-1) levels were quantified by the latex photometric immunoassay by using a commercial kit (Mitsubishi Kagaku Iatron, Tokyo, Japan), as described previously.[19] The granulocyte elastase digests of cross-linked fibrin (granulocyte elastase–dependent fibrin degradation products [E-XDPs]) were measured by the automated latex photometric immunoassay using IF-123 monoclonal antibody, which is specific for the fibrin fragment D species generated by granulocyte-elastase digestion.[20] Monoclonal antibody IF-123–bound latex particles (Mitsubishi Kagaku Iatron) were used for the assay. A 2.4-μL aliquot of sample plasma was mixed with 32 μL latex reagents in 250 μL Tris-buffered saline, and then absorbance changes were analyzed with an automated analyzer for latex photometric immunoassay (model LPIA-NV7; Mitsubishi Kagaku Iatron). The standard E-XDP was purified according to the method of Kohno et al.[20] The normal range of plasma E-XDP levels is less than 3 U/mL.

Effect of granulocyte elastase on ADAMTS13

Recombinant ADAMTS13 (250 nM) was incubated in 20 μL Tris-buffered saline, pH 7.4, in the absence or presence of granulocyte elastase (Elastin Products, Owensville, MO) at 5 nM and 50 nM. Aliquots (5 μL each) were harvested after incubation at 37°C for 5, 15, and 30 minutes. The reaction of each aliquot was terminated by addition of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS. Samples were then analyzed by SDS-PAGE followed by Western blotting with anti-ADAMTS13 monoclonal antibody WH2-22-1A and peroxidase-labeled anti–mouse IgG.

Detection of ADAMTS13 molecular forms in plasma

Western blot analysis of ADAMTS13 in plasma by mAb WH2-22-1A was performed after immunoprecipitation with anti-ADAMTS13 polyclonal antibody immobilized to protein G-Sepharose.

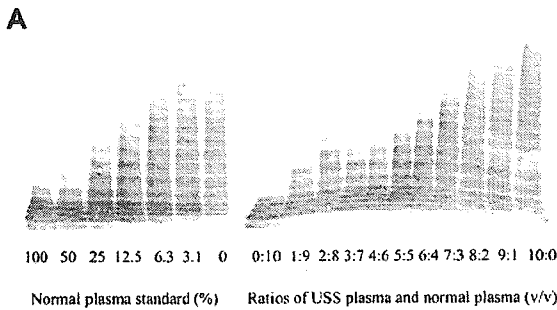
Analysis of VWF multimers in patient plasma

VWF multimers in patient plasma were analyzed by SDS-agarose gel electrophoresis according to the method described previously.[12-14]

Results

ELISA for ADAMTS13

We generated mAbs against recombinant human ADAMTS13 and used them to develop an mAb-based ADAMTS13 ELISA. To determine the specificity of this assay, plasma obtained from a patient with Upshaw-Schulman syndrome was mixed with normal plasma at various ratios, and the ADAMTS13 activity and antigen levels were measured. As shown in Figure 1, ADAMTS13 activity and ADAMTS13 antigen levels in the plasma of the patient with Upshaw-Schulman syndrome were less than 1%, and the ADAMTS13 antigen level in the patient plasma increased linearly in parallel with the ADAMTS13 activity in the presence of increasing amounts of normal plasma. The correlation coefficient



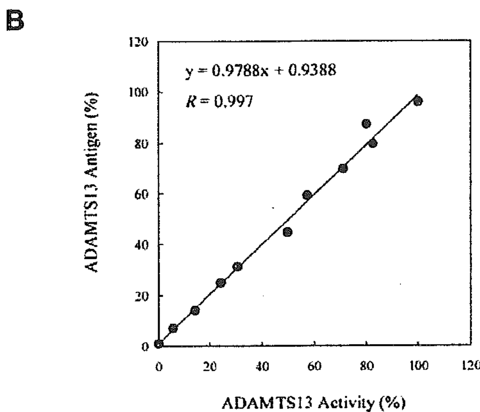


Figure 1. Analysis of ADAMTS13 activity and antigen levels in plasma of patients with Upshaw-Schulman syndrome. ADAMTS13 activity and antigen levels were determined in the plasma of a patient with Upshaw-Schulman syndrome (USS) mixed with normal pooled plasma at various ratios. (A) Result of ADAMTS13 activities in the plasma of the USS patient mixed with normal plasma at various ratios (0:10-10:0). (B) Correlation of ADAMTS13 activity and antigen levels in these samples.

between ADAMTS13 antigen and ADAMTS13 activity was 0.997. The ADAMTS13 level in normal pooled plasma was 1.57 μg/mL when recombinant human ADAMTS13 was used as the standard. The calibration curve was linear (r = 0.999), and the ELISA could distinguish absorbance changes of ADAMTS13 at 0.3% of the normal plasma level from ADAMTS13-depleted plasma. Interassay variability in samples containing 50% and 100% of ADAMTS13 were 7.9% and 5.2%, respectively.

ADAMTS13 levels in disease states

ADAMTS13 antigen and activity levels in the plasma of patients with sepsis-induced DIC or TTP were studied (Figure 2). The

Table 1. Correlation between the ADAMTS13 levels and molecular markers of DIC in patients with sepsis-induced DIC

	ADAMTS13*		
	Activity	Antigen	Activity-antigen ratio
Fibrinogen	−0.347	−0.244	−0.219
FDP	0.354	0.242	0.274
TAT	0.246	0.379	0.367
PIC	0.370	0.357	0.327
PAI-1	−0.230	−0.300	−0.006
Platelet	0.260	0.245	0.239
E-XDP	−0.399†	−0.404†	−0.229

n = 109 patients.

*Values are rs determined by Spearman rank correlation test.

†Statistically significant (P < .01).

correlation coefficient of ADAMTS13 antigen and ADAMTS13 activity was 0.80. As shown in Figure 2A, discrepancies between ADAMTS13 antigen levels and activity levels were observed in many samples. These discrepancies mainly were caused by the decreased level of specific ADAMTS13 activity compared with the ADAMTS13 antigen level. Some samples had higher specific activity of ADAMTS13. To explore the possibility that decreased levels of the ADAMTS13-specific activity correlated with disease states, Western blot analysis of ADAMTS13 molecular forms in patient plasma was performed. Low molecular–weight ADAMTS13 species were observed in DIC patient plasma by Western blotting (Figure 2B), indicating that proteolytic cleavage of ADAMTS13 could occur in this disease state. The recent report showed that ADAMTS13 could be digested in vitro by proteases such as thrombin and plasmin.[21] Because thrombin and plasmin can be generated in DICs, we tested the correlation between ADAMTS13 levels and molecular markers of coagulation and fibrinolysis. There was no correlation of ADAMTS13 activity, antigen, or specific activity level with levels of fibrinogen, FDP, TAT, PIC, PAI-1, or platelet counts (Table 1). We could only find a negative correlation between activity levels and antigen levels of ADAMTS13 and plasma levels of granulocyte elastase digests of fibrin (E-XDP) (Table 1; Figure 3A-B). Based on these results, we studied the effects of granulocyte elastase on ADAMTS13 in vitro. In accordance with previous reports, recombinant ADAMTS13 was determined to migrate at approximately 190 kDa by SDS-PAGE, followed by Western blotting.[14,21] As shown in Figure 3C, recombinant ADAMTS13 migrating at approximately 190 kDa was converted to the 120-kDa and 100-kDA fragments and finally to the approximately 40-kDa fragment on incubation with granulocyte

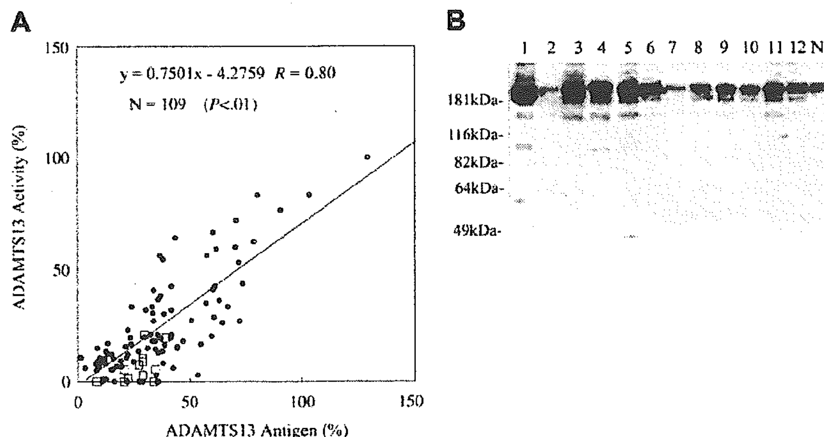


Figure 2. Analysis of ADAMTS13 activity, antigen, and molecular forms in plasma of patients with sepsis-induced DIC. (A) ADAMTS13 activity and antigen levels in the plasma of patients with sepsis-induced DIC were determined as described in "Patients, materials and methods." Samples (□) were subjected to immunoprecipitation followed by Western blotting to investigate the cleavage of ADAMTS13, as described in "Patients, materials, and methods." (B) Typical Western blot of degraded ADAMTS13 found in the patients' plasma indicated in panel A (□) is shown. Western blotting of ADAMTS13 antigen in normal pooled plasma (N) is shown as the control. ADAMTS13 molecules in normal plasma migrated at approximately 190 kDa.

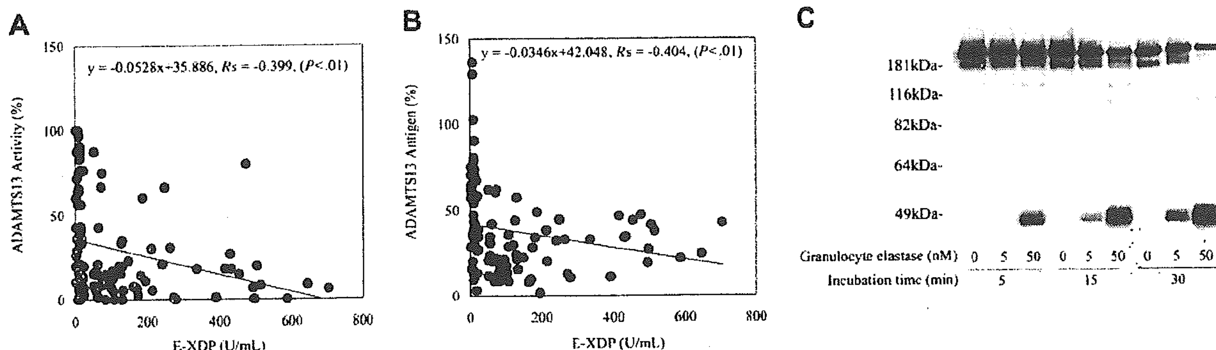


Figure 3. Correlation between the ADAMTS13 levels and the granulocyte elastase digests of cross-linked fibrin (E-XDP) levels in plasma of patients with sepsis-induced DIC and the effect of granulocyte elastase on ADAMTS13 in vitro. Correlations between the activity levels of ADAMTS13 and the plasma levels of granulocyte-elastase digests of fibrin (E-XDP) (A) and between the antigen levels of ADAMTS13 and the plasma levels of granulocyte-elastase digests of fibrin (E-XDP) (B) in patients with sepsis-induced DIC are shown. Values were analyzed by Spearman correlation coefficient by rank test. Recombinant ADAMTS13 was incubated with granulocyte elastase at 5 nM or 50 nM, and degradation of ADAMTS13 by granulocyte elastase was studied after the indicated time and analyzed as described in "Patients, materials, and methods" (C).

elastase in a dose-dependent and a time-dependent manner in vitro. A variety of lower molecular–weight ADAMTS13 fragments were detected in DIC patient plasma by Western blot (Figure 2B). According to the previous report, the ADAMTS13 fragments migrating approximately 150 to 170 kDa could be generated by thrombin.[21] ADAMTS13 fragments migrating approximately 120 kDa and 100 kDa in patient plasma might correspond to the granulocyte elastase digests of ADAMTS13. However, the 120-kDa ADAMTS13 fragment and the 100-kDa ADAMTS13 fragment could be generated by thrombin and plasmin, respectively.[21] It also is possible that thrombin-cleaved ADAMTS13 or plasmin-cleaved ADAMTS13 could be digested by granulocyte elastase or vice versa. These data may suggest that granulocyte elastase, together with other proteases (thrombin and plasmin plays a role in ADAMTS13 cleavage under certain pathologic conditions), may partially account for the decrease of the ADAMTS13-specific activity observed in DIC patients.

ADAMTS13 deficiency in disease states

ADAMTS13 antigen and activity levels in patient groups and in healthy subjects are shown in Figure 4. The plasma ADAMTS13 antigen and activity levels in untreated patients with TTP (no plasma exchange treatment, no fresh frozen plasma transfusion) were 13.5% ± 7.1% (range, 5.1%-19.6%) and 6.3% ± 5.7% (range, 0%-12.5%), respectively (idiopathic TTP 3, Upshaw-Schulman syndrome 1). Decreased levels of ADAMTS13 antigen and activity were observed in patients with sepsis-induced DIC compared with healthy subjects ($P < .01$) in this study, and severe decreases of ADAMTS13 activity and antigen levels were observed in patients with sepsis-induced DIC. Of the 109 patients with sepsis-induced DIC, decreases in ADAMTS13 activity levels (less than 5%) were found in 17 (15.6%) patients; clinical features and laboratory data of these patients are summarized in Table 2. Consciousness disturbance, thrombocytopenia, decreased hemoglobin levels, and increased LDH levels were commonly found in these patients. Clinical features were indistinguishable from those of patients with TTP, though patients with sepsis-induced DIC had evidence of the infection. Given that the highest ADAMTS13 activity level in patients with TTP without plasma exchange or blood transfusion was 12.5%, patients with sepsis-induced DIC were divided into 2 groups. One included patients with decreased ADAMTS13 activity levels (less than 20%; n = 51), and the other included patients with ADAMTS13 activity levels greater than 20% (n = 52). Patients with chronic renal failure before infection were excluded from this analysis. Patients were in severe condition; 25 (49.0%) of 51 patients in the former group and 35 (67.3%) of 52 patients of the latter group received transfusions of fresh frozen plasma, platelet concentrates, or both within 5 days of the determination of ADAMTS13 levels. This might have affected the activity and antigen levels of ADAMTS13.

Correlation between secondary ADAMTS13 deficiency and organ failure

Analyses of clinical and laboratory data showed that the patients with severe ADAMTS13 deficiency (ADAMTS13 activity less than 20%) had elevated serum creatinine levels (Figure 5) that were

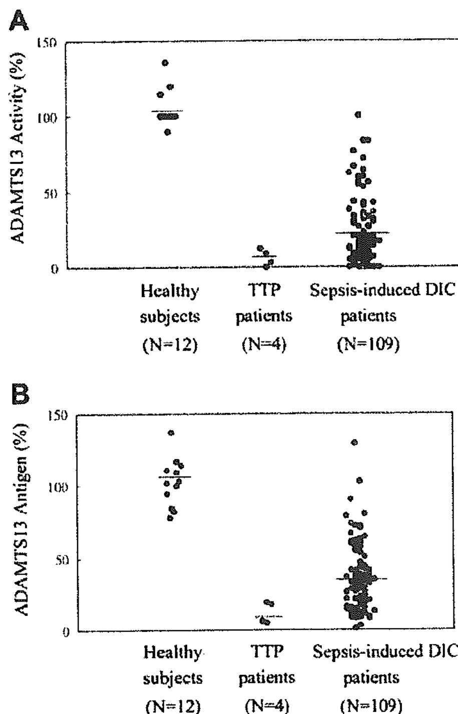


Figure 4. Plasma ADAMTS13 levels in patients and healthy subjects. ADAMTS13 activity levels (A) and antigen levels (B) of healthy subjects, patients with TTP (idiopathic TTP, 3; Upshaw-Schulman syndrome, 1) before plasma exchange treatment, and patients with sepsis-induced DIC (n = 109) are shown. Differences in the mean values (horizontal lines) between the healthy subject group and patient groups were statistically significant (nonrepeated measures ANOVA and Dunnett test; $P < .01$).

Table 2. Clinical profiles of patients with sepsis-induced DIC whose ADAMTS13 activity levels were lower than 5%

Characteristic	Value
Age, y	56.9 ± 21.3
Consciousness disturbance, no. (%)	8 (47.1)
Blood transfusion, no. (%)	11 (64.7)
ADAMTS13 antigen, %	25.5 ± 13.6
Creatinine, mg/dL	1.86 ± 2.06
Albumin, g/dL	2.2 ± 0.5
WBC count, cells/μL	11 200 ± 7 500
RBC count, × 10^4/μL	260 ± 86
Hemoglobin, g/dL	8.3 ± 2.0
Platelet count, × 10^4/μL	6.7 ± 5.3
LDH, IU/L	2481.3 ± 4107.8
CRP, mg/dL	18.11 ± 13.41

n = 17 patients.

Values for all categories except consciousness disturbance and blood transfusion are mean ± SD.

To convert creatinine from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 88.4.

To convert albumin from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

To convert WBC count from cells per microliter to × 10^9 cells per liter, divide cells per microliter by 1000.

To convert RBC count from × 10^4 cells per microliter to × 10^{12} cells per liter, divide × 10^4 cells per microliter by 100.

To convert hemoglobin from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

To convert platelet count from × 10^4 platelets per microliter to × 10^9 per liter, multiply × 10^4 platelets per microliter by 10.

To convert CRP from milligrams per deciliter to milligrams per liter, multiply milligrams per deciliter by 10.

significantly higher than those in patients with ADAMTS13 levels higher than 20% (Table 3). The incidence of renal injuries in patients with severe ADAMTS13 deficiency (ADAMTS13 activity less than 20%) was significantly higher than in patients with ADAMTS13 activity levels higher than 20% (Table 3). However, there were no differences in the incidence of liver dysfunction or serum levels of bilirubin, AST, and ALT among these groups (Table 3), suggesting that severe ADAMTS13 deficiency in these patients may be linked to the development of renal injuries. There was a significant difference in serum albumin levels between both groups, suggesting that the decrease of ADAMTS13 activity and antigen levels in patients was at least partially caused by reduced synthesis in the liver.

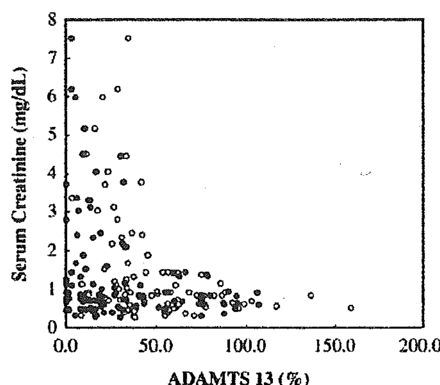


Figure 5. Correlation between the plasma ADAMTS13 levels and the serum creatinine levels. Correlation between serum creatinine levels and ADAMTS13 activity (●) levels or antigen (○) levels in patients with sepsis-induced DIC is shown (n = 103). Patients with a history of chronic renal failure were excluded from the study.

Table 3. Correlation between ADAMTS13 levels and organ injury in patients with sepsis-induced DIC

	ADAMTS13 activity less than 20%, n = 51	ADAMTS13 activity greater than 20%, n = 52	P
Creatinine, mg/dL	1.81 ± 1.70	0.95 ± 0.76	< .01*
AST, IU/L	106 ± 128	182 ± 290	NS
ALT, IU/L	72 ± 109	122 ± 160	NS
Bilirubin, mg/dL	2.70 ± 3.13	2.20 ± 2.53	NS
Albumin, g/dL	2.3 ± 0.4	2.9 ± 0.7	< .05*
CRP, mg/dL	13.50 ± 10.51	6.90 ± 8.61	< .01*
Organ injury, no. (%)			
Renal injury	21 (41.2)	8 (15.4)	< .05†
Liver injury	40 (78.4)	38 (73.1)	NS

Values for all categories except organ injury are mean ± SD. Renal injury: serum creatinine greater than 1.2 mg/dL.

Liver injury: elevation of bilirubin (> 2.0 mg/dL), AST (> 40 IU/L), or ALT (> 40 IU/L).

To convert creatinine from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 88.4.

To convert bilirubin from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 17.1.

To convert albumin from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

To convert CRP from milligrams per deciliter to milligrams per liter, multiply milligrams per deciliter by 10.

NS indicates not significant.

*Statistically significant (Welch *t* test)

†Statistically significant (Fisher exact probability test).

Analysis of VWF multimers in patients with severe secondary ADAMTS13 deficiency

Additionally, unusually large VWF multimers were detected in the plasma of patients with severe secondary ADAMTS13 deficiency (ADAMTS13 activity less than 20%), as shown in Figure 6. Serum creatinine levels in patients in whom unusually large VWF multimers and severe ADAMTS13 deficiency were detected were significantly higher than in patients in whom the unusually large VWF multimers were absent (Table 4). There was no significant difference in ADAMTS13 activity (Table 4) and ADAMTS13-specific activity (activity-antigen ratio) between these patient groups (not shown).

There was a significant difference in CRP levels between the ADAMTS13 activity less than 20% group and the ADMATS13 activity greater than 20% group, but their platelet counts were not significantly different (not shown), indicating that the decrease in ADAMTS13 may be related to inflammatory responses. These results are consistent with the data showing a negative correlation between the activity and antigen levels of ADAMTS13 and the plasma levels of granulocyte elastase digests of fibrin (E-XDP).

Discussion

ADAMTS13 has been shown to play an important role in VWF processing.[1-14,22,23] As shown previously, ADAMTS13 may cleave the unusually large multimers of VWF on the endothelial cell surface, preventing entrance of such unusually large multimers into the circulation.[8,24] Without this processing of VWF multimers, the unusually large multimers of VWF secreted from endothelial cells would enter the circulation and initiate platelet thrombus formation, which in turn would cause the development of TMA.[8,24] Patients with primary ADAMTS13 deficiency caused by defects in the *ADAMTS13* gene or with autoantibodies against ADAMTS13 have been shown to develop TTP, suggesting the important physiologic role of ADAMTS13-catalyzed cleavage of these