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The mild phenotype in severe hemophilia A with Arg1781His mutation is associated with enhanced binding affinity of factor VIII for factor X

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Summary

The clinical severity in some patients with haemophilia A appears to be unrelated to the levels of factor (F)VIII activity (FVIII:C), but mechanisms are poorly understood. We have investigated a patient with a FVIII gene mutation at Arg[1781] to His (R1781H) presenting with a mild phenotype despite FVIII:C of 0.9 IU/dl. Rotational thromboelastometry using the patient's whole blood demonstrated that the clot time and clot firmness were comparable to those usually observed at FVIII:C 5–10 IU/dl. Thrombin and FXa assays using plasma samples also showed that the peak levels of thrombin formation and the initial rate of FXa generation were comparable to those observed at FVIII:C 5–10 IU/dl. The results suggested a significantly greater haemostatic potential in this individual than in those with severe phenotype. The addition of incremental amounts of FX to control plasma with FVIII:C 0.9 IU/dl in clot waveform analyses suggested that the enhanced functional tenase assembly might have been related to changes in association between FVIII and FX. To further investigate this mechanism, we prepared a stably expressed, recombinant, B-domainless FVIII R1781H mutant. Thrombin generation assays using mixtures of control plasma and FVIII revealed that the coagulation function observed with the R1781H mutant (0.9 IU/dl) was comparable to that seen with wild-type FVIII:C at ~5 IU/dl. In addition, the R1781H mutant demonstrated an ~1.9-fold decrease in K_m for FX compared to wild type. These results indicated that relatively enhanced binding affinity of FVIII R1781H for FX appeared to moderate the severity of the haemophilia A phenotype.

Keywords

Haemophilia A, FVIII, clinical phenotype, FX, association

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Introduction

Haemophilia A results from a deficiency or defect of the coagulant protein, factor (F)VIII, and is the most common of the severe, inherited bleeding disorders. Clinical phenotype in haemophilia generally correlates well with the level of FVIII activity (FVIII:C), and on this basis, patients are classified into three categories: severe (FVIII:C <1 IU/dl), moderate (1-5 IU/dl), and mild type (>5 IU/dl). Patients with severe haemophilia A typically develop spontaneous haemorrhage into joints or muscles that require frequent FVIII replacement (1, 2). The severity and frequency of bleeding may be different, however, in haemophiliacs with similar plasma levels of FVIII, and a mild bleeding phenotype has been reported in 10-15% of individuals with severe plasma FVIII deficiency (1, 2). The reasons for this heterogeneity of clinical expression in severe haemophilia A remain to be fully clarified. A recent study by Santagostino et al. (3) using multivariable logistic regression analysis suggested that non-null mutations (for example missense mutations) of FVIII genes (*F8*) might represent the main determinant for bleeding tendency. Other coagulation factors, including natural anticoagulants, platelets, and fibrinolytic proteins appear to have negligible roles as modulators of disease severity (4, 5).

Accurate measurements of blood coagulation *in vitro* are essential for complete clinical assessment of clotting function. Conventional one-stage clotting assays (prothrombin time; PT and activated partial thromboplastin time; APTT) are useful for routine laboratory examination, but they only partially reflect coagulation in a non-physiological environment and are based on the classical concepts of intrinsic and extrinsic cascade mechanisms. It might be, therefore, that the use of this basic methodology might contribute to the discrepancy between the coagulant activity and clinical phenotype. More recently, interest has focused on global coagulation assays, developed from a better understanding of the coagulation reaction involving tissue factor (TF)-triggered, cell-based mechanisms generating thrombin on activated platelets. Tests of this nature such as thromboelastography, specific throm-

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bin generation tests, and clot waveform analysis have been established (6-8).

FVIII, which circulates in plasma in complex with von Willebrand factor, functions as a cofactor in the tenase complex, and is responsible for phospholipid (PL)-dependent conversion of FX to FXa by FIXa (9). FVIII is synthesised as a single chain molecule consisting of 2,332 amino acid residues with a molecular mass of ~300 kDa arranged into three domains, A1-A2-B-A3-C1-C2, based on amino acid homology. FVIII is processed into a series of metal ion-dependent heterodimers by cleavage at the B-A3 junction, generating a heavy chain consisting of the A1 and A2 domains together with heterogeneous fragments of proteolysed B domain linked to a light chain consisting of the A3, C1, and C2 domains (10). Assembly of the tenase complex is markedly enhanced by conversion of FVIII to FVIIIa following limited proteolysis by thrombin (or FXa) (11). The coagulant activity of the tenase complex is dependent on direct interaction of FVIIIa with both FIXa and FX on the PL vesicles (12).

We have identified a patient with severe haemophilia A (FVIII:C 0.9 IU/dl) with a mild bleeding tendency and with an *F8* point mutation at Arg^{1781} to His (R1781H). The haemophilia A database (HAMSTERs [13]) indicates that haemophiliacs with this mutation are clinically heterogeneous and reflect phenotypes ranging from severe to mild/moderate. We have investigated mechanisms of FVIII deficiency in this individual using several global coagulation assays and a recombinant FVIII mutant. The assays demonstrated that coagulation function of the patient's native plasma (0.9 IU/dl) with the R1781H mutant was comparable to that usually obtained at levels of 5-10 IU/dl FVIII:C. This represented a 5-10-fold increased haemostatic potential. In addition, the R1781H mutant showed an ~1.9-fold decrease in K_m for FX compared to wild type. The results suggested that the mild bleeding tendency in this patient with the R1781H genetic defect appeared to be related to the relatively enhanced binding affinity of FVIII for FX.

Material and methods

Reagents

Recombinant FVIII was a generous gift from Bayer Corp. Japan (Osaka, Japan). Recombinant lipidated TF (Innovin®; Dade Behring, Marburg, Germany), ellagic acid (Sysmex, Kobe, Japan), thrombin-specific fluorogenic substrate (Bachem, Bubendorf, Switzerland), and thrombin calibrator (Thrombinoscope, Maastricht, Netherlands) were obtained from the indicated vendors. Human thrombin, FIXa, FX, FXa (Hematologic Technologies Inc. Essex, VT, USA), recombinant hirudin (Calbiochem, San Diego, CA, USA), FXa substrate S-2222 (Chromogenix, Milano, Italy) and plasma-derived FVIII-deficient plasma (George King Biomedical, Overland Park, KS, USA) were commercially purchased. PL vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, 30% phosphatidylethanolamine (Sigma-Aldrich, St Louis, MO, USA) were prepared as previously described (14). The B-domainless FVIII expression construct RENeo FVIII and baby hamster kidney cells were gifts kindly provided by Dr. Pete Lollar.

Patients' plasma

Whole blood was obtained by venipuncture into tubes containing 1:9 volume of 3.8% (w/v) trisodium citrate. After centrifugation for 15 minutes (min) at 1,500 x g, the plasma samples were stored at -80°C, and thawed at 37°C immediately prior to the assays. The levels of FVIII:C and FVIII:Ag were measured as reported previously (15). The blood samples were obtained from patients diagnosed by our research group and enrolled in the Nara Medical University Haemophilia Program. All samples were obtained after informed consent following local ethical guidelines.

DNA extraction and genotyping of F8

Genomic DNA was extracted from white blood cells with fully informed patient's consent. Intron 22 inversion and intron 1 inversion analysis was performed by long distance PCR and by multiplex PCR, and 26 exons and their flanking regions of *F8* were amplified by PCR using specific primers with a simple modification as previously described (16). The PCR products were purified and sequenced with a BigDye® terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). ABI PRISM SeqScape® (Applied Biosystems) software was used for mutation detection. The nomenclature was based on the current recommendations from HGVS (Human Genome Variation Society). The nucleotide number was assigned according to the FVIII cDNA sequence from A of the initiator ATG site as +1. The amino acid sequence numbering assigned the first residue of mature FVIII as +1, and thus the initiator methionine was at the -19 position, as used in the *F8* HAMSTeRS mutation database.

Mutagenesis, expression, and purification of wild-type and variant FVIII

Recombinant wild-type FVIII and FVIII variant Arg1781His (R1781H) were constructed, expressed, and purified as described previously (17). Resultant FVIII forms were typically >90% pure as judged by SDS-PAGE with albumin representing the major contaminant. FVIII concentrations were measured using an enzyme-linked immunosorbent assay, and FVIII:C levels were determined by one-stage clotting assay. FVIII samples were quick-frozen and stored at -80°C

Rotational thromboelastometry (ROTEM)

ROTEM was performed using the Whole Blood Haemostasis Analyzer® (Pentapharm, Munich, Germany) (6). After drawing, citrated whole blood was kept at rest for 30 min at room temperature and was used within 2 hours. At the start of measurement, 20 µl of $CaCl_2$ (final concentration, f.c., 12.5 mM) was added to the whole blood (280 µl). Clot formation was assessed using clotting

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time (CT; the time from the start of measurement until detection of clot firmness of 2 mm amplitude) and clot formation time (CFT; the time from the initiation of clotting until detection of clot firmness of 20 mm amplitude).

FXa generation assays

(i) plasma-based chromogenic assay

Coatest®SP FVIII kit (Chromogenix) was used according to the manufacturer's instructions. Plasma samples were mixed with excess amounts of FIXa/FX/PL in the supplied buffer, and the mixtures were incubated at 37°C as described previously (18). Hirudin (2.5 IU/ml) was added to the mixtures to completely exclude feedback activation induced by the generated thrombin. FXa generation was initiated by the addition of $CaCl_2$ and S-2765. The initial velocity rates were determined at 405 nm using Labsystems Multiskan Multisoft microplate reader (Labsystems, Helsinki, Finland).

(ii) purified FXa generation assay

The rate of conversion of FX to FXa was monitored in a purified system at 37°C (19). FVIII (5 nM) was activated by thrombin (10 nM) in the presence of PL vesicles (20 μM). Thrombin activity was inhibited after 1 min by the addition of hirudin (2.5 IU/ml), and FXa generation was initiated by the addition of FIXa and FX at the indicated concentrations. Aliquots were removed at appropriate times to assess the initial rates of product formation and were mixed with EDTA (f.c. 50 mM) to quench the reactions. Rates of FXa generation were measured by the addition of specific chromogenic substrate, S-2222 (f.c. 0.46 mM). Reactions were read at 405 nm using microplate reader.

Clot waveform analysis

APTT measurements were performed using the MDA-II™Haemostasis System (Trinity Biotech, Dublin, Ireland). The clot waveforms obtained were computer-processed using the commercial kinetic algorithm (7). The minimum value of the first derivative (min1) was calculated as an indicator of the maximum velocity of coagulation achieved. The second derivative of the transmittance data reflects the acceleration of the reaction at any given time point. The minimum value of the second derivative (min2) was calculated as an index of the maximum acceleration of the reaction achieved. Since the minimum of min1 and min2 are derived from negative changes, the data were expressed as |min1| and |min2|, respectively. The clot time was defined as the time until the start of coagulation.

Thrombin generation assay

The calibrated automated thrombin generation assay (Thrombinoscope) was performed as previously described (8). Although a small amount of TF is generally used as a trigger reagent, the sensitivity of this original assay was relatively low and differences in coagulation function at low levels of FVIII:C (<2-3 IU/dl) were not seen (8). The addition of a small amount of ellagic acid to the mixtures containing TF little affects the lag-time (representing FVIIa/TF-induced activation of the extrinsic pathway), but mediates higher levels of peak thrombin and ETP (representing the subsequent activation of intrinsic pathway). This modified thrombin generation assay, therefore, sensitively reflects global coagulation in intrinsic as well as extrinsic, cell-based pathways, and enables the evaluation of coagulation function at very low levels of FVIII:C (lower limit <0.4 IU/dl [8]). Briefly, plasma samples (80 μl) were preincubated for 10 min with 20 μl of trigger reagent containing TF, PL, and ellagic acid (final concentration (f.c.) 0.5 pM, 4 μM, and 0.3 μM, respectively). Measurements were then recorded after the addition of 20 μl reagent containing $CaCl_2$ and fluorogenic substrate (f.c. 16.7 mM and 2.5 mM, respectively). The development of fluorescent signals was monitored using a Fluoroskan Ascent microplate reader (Thermo Fisher Scientific, Boston, MA, USA). Data analyses were performed using the manufacture's software, and the standard parameters; peak thrombin, time to peak, and endogenous thrombin potential, were derived.

Data analyses

All experiments were performed on at least four separate occasions; the mean values and standard deviations are shown. Nonlinear least squares regression analysis was performed using Kaleidagraph (Synergy, Reading, PA, USA). The K_m and V_{max} values for FVIIIa/FIXa-catalysed activation of FX were calculated from the Michaelis-Menten equation.

Results

Patient's profile

A 27-year-old male had been diagnosed at 2 years of age following uncontrolled bleeding from a post-traumatic frontal cutaneous incision, and was characterised as severe haemophilia A with FVIII:C level <1.0 U/dl in a one-stage clotting assay. There was no family history or past history of bleeding. The current levels of FVIII:C and FVIII:Ag were 0.9 IU/dl and 1.8 IU/dl, respectively, showing negative cross-reactive material. The case showed FVIII:C level 4-5 IU/dl in FXa generation chromogenic assay, however. The levels of all other plasma procoagulant, anti-coagulant, and fibrinolytic proteins were within normal range (data not shown). To date, bleeding episodes have been rare, regular prophylaxis has not been necessary and consequently he has required extremely small total amounts of FVIII concentrate products as compared to typical patients with severe haemophilia A. He has no chronic complications (e.g. arthropathy) commonly associated with the severe phenotype.

The *F8* genotype of this patient demonstrated a missense mutation comprising a single nucleotide conversion of G5399 to A in exon 16, resulting in an amino acid substitution from arginine to histidine at codon 1781. This mutation has been already enrolled in the haemophilia A database (HAMSTERs [13]), and some clinical and laboratory profiles of these patients had been described,

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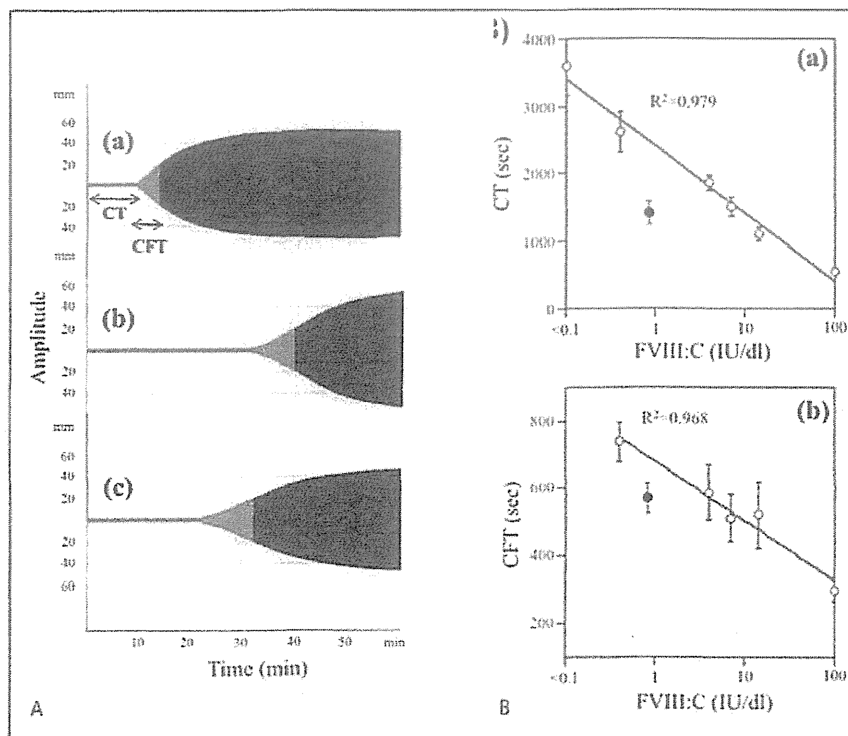

Figure 1: ROTEM in the haemophilia A patient with R1781H. A) $CaCl_2$ was added to FVIII-deficient whole blood (<0.2 IU/dl) mixed with the indicated concentrations of FVIII (a: 100 IU/dl, b: 1.0 IU/dl) and to patient's whole blood with R1781H (c) at the start of the assay. The thromboelastograms were recorded using ROTEM as described in *Methods*. Representative data are illustrated. B) Parameters (CT (a) and CFT (b)) obtained at various concentrations of FVIII (open circles) and those obtained from patient's sample (closed circles). The solid line illustrates the linear regression fitting the data. The dashed lines show the FVIII:C levels corresponding to the parameters obtained from the patient sample. All experiments were performed at least four separate times, and the average values and standard deviations were calculated.

but there has been any little functional and expression studies on FVIII with R1781H mutation.

ROTEM analysis of whole blood from the patient with the R1781H mutation

It was evident that the plasma FVIII:C level (0.9 IU/dl) in this patient with the R1781H mutation did not correlate with his clinical phenotype. To investigate this discrepancy, we first evaluated whole blood coagulation function using ROTEM. A representative thromboelastogram and the derived parameters (CT and CFT) are shown in ▶Figure 1A-c and B, respectively. The values obtained were 1,493 ± 106 seconds (sec) and 580 ± 12 sec, respectively. To assess the level of FVIII:C that could be expected to reflect these parameters, various amounts of FVIII were added to whole blood obtained from a volunteer patient with severe haemophilia A (<0.2 IU/dl), and assayed using same method. Representative data in these control plasmas with added FVIII (100 IU/dl and 1.0 IU/dl) are illustrated in ▶Figure 1A-a,b, and the parameters obtained are shown in ▶Figure 1B. The coagulation function observed in patient's sample with FVIII:C (0.9 IU/dl) was significantly greater than that of the control sample at FVIII:C 1.0 IU/dl, and both parameters obtained were comparable to those observed at FVIII:C 5-10 IU/dl.

Thrombin generation in the presence of FVIII R1781H

Specific thrombin generation assays have been developed to examine global coagulation function based on the principles of cell-based clotting. Representative thrombograms in the patient's plasma or control samples (prepared from FVIII-deficient plasma mixed with various amounts of FVIII) are illustrated in ▶Figure 2A, and levels of peak thrombin derived from these curves are shown in ▶Figure 2B. The levels of peak thrombin in patient's plasma and control plasma with FVIII:C 1.0 IU/dl were 300 ± 15 nM and 169 ± 14 nM, respectively, indicating that thrombin generation in the patient's plasma (0.9 IU/dl) was much greater than that in control with similar FVIII:C level. This result was comparable to that obtained with plasma with a FVIII:C of ~10 IU/dl. The other parameters (time to peak and ETP) showed similar tendencies (data not shown). The results suggested a significantly greater coagulation potential in this case with R1781H mutation than in the equivalent control.

To examine whether the properties of this patient's sample were unique, we utilized the same method to examine coagulation function in four cases of mild/moderate haemophilia A with similar FVIII:C levels. These patients had been diagnosed with point mutations at R372H, T295I, G2325R, and R2307Q (FVIII:C 1.0, 3.4, 4.9, and 5.8 IU/dl, respectively). The levels of peak thrombin obtained in all cases were comparable to those in the equivalent control samples with similar levels of FVIII:C measured in a one-stage clotting assay (▶Figure 2B). The findings appeared to reflect the clinical phenotypes in these patients (data not shown), and indicated that the enhanced haemostatic potential was unique to the R1781H mutation.

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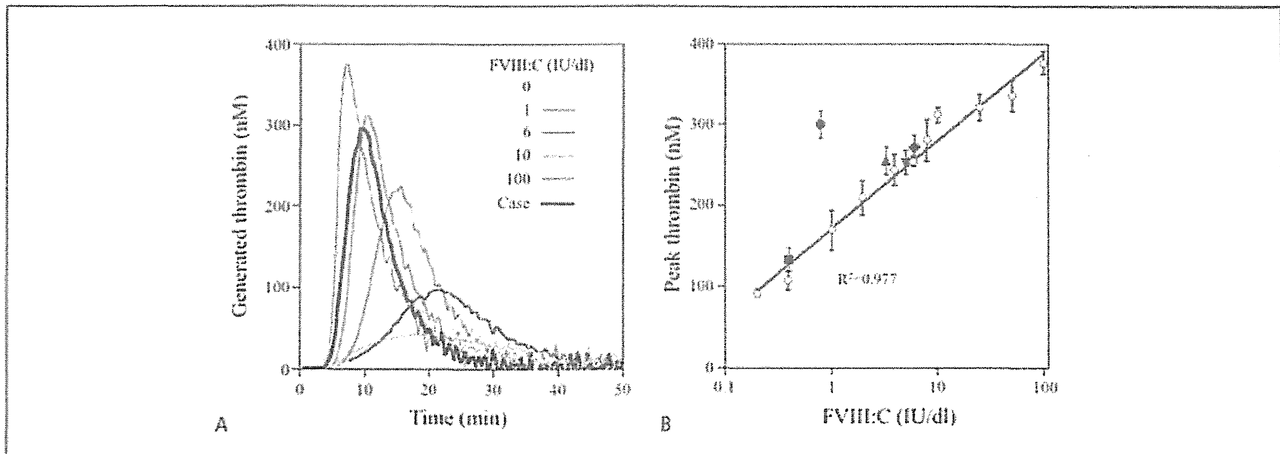

Figure 2: Thrombin generation in the patient with R1781H. A) FVIII-deficient plasma, mixed with various concentrations of FVIII (0-100 IU/dl) was incubated with TF (0.5 pM), ellagic acid (0.3 M) and PL vesicles (4 M), followed by the addition of fluorogenic substrate and CaCl2 at the start of the assay as described in *Methods*. Representative selected curves are illustrated. The bold black line shows the thrombin generation curve obtained from the patient's sample. B) Peak thrombin parameter obtained from the thrombin generation assays. The symbols used are samples with various amounts (0-100 IU/dl) of FVIII (open circles), R1781H (closed circle), R372H (closed square), T295I (closed triangle), G2325R (closed inverted-triangle), and R2307Q (closed diamond). The solid line illustrates the linear regression fitting the data. The dashed line shows the FVIII:C level corresponding to the peak thrombin parameter obtained with the patient's sample. All experiments were performed at least four separate times, and the average values and standard deviations were calculated.

Enhancement of intrinsic FXa generation in the presence of FVIII R1781H

Intrinsic FXa generation, corresponding to the upstream process of thrombin generation, was measured using the chromogenic assay to further investigate the mechanism(s) of enhanced thrombin generation associated with the FVIII R1781H mutation. The initial rate of FXa generation in the presence of R1781H was ~3-fold greater than with control plasma at FVIII:C 1.0 IU/dl (0.240×10^{-3} and 0.082×10^{-3}, respectively), and was comparable to plasma at FVIII:C ~5 IU/dl (▶Figure 3). The findings were similar to those obtained by ROTEM and thrombin generation assays. The results suggested that the mutation R1781H, located in the FVIII A3 domain, governed the formation of the tenase complex consisting of FVIIIa, FIXa, and FX on the PL surface, and accelerated thrombin and FXa generation. Overall, therefore, the coagulation potential estimated by global coagulation assays in this case (corresponding to FVIII:C ~10 IU/dl, rather than 0.9 IU/dl) correlated with the clinical phenotype.

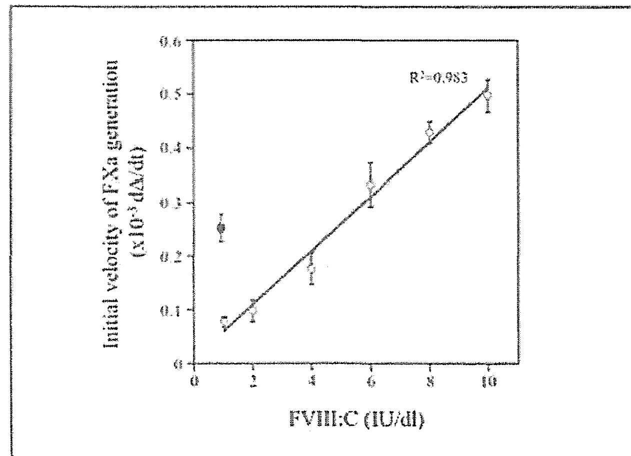

Figure 3: Endogenous intrinsic FXa generation in the patient with R1781H. FVIII-deficient plasma, mixed with small amounts of FVIII (1-10 IU/dl, open circles) was incubated with a mixture of FIXa/FX/PL in the presence of hirudin, followed by the addition of CaCl2 as described in *Methods*. The rates of initial velocity of endogenous FXa generation were determined at 405 nm after the addition of S-2765. All experiments were performed at least four separate times, and the average values and standard deviations were calculated. The solid line illustrates the linear regression fitting the data. The dashed line shows the FVIII:C level corresponding to FXa generation using the patient's sample (closed circles).

Clot waveform analysis in patient's plasma with R1781H

In clot waveform analyses, the data obtained from the waveforms using patient's plasma and control plasmas prepared as described above are illustrated in ▶Figure 4A. The clot time with the patient's plasma was significantly shorter than that with the equivalent control plasma (55.2 ± 0.4 sec and 78.8 ± 1.7 sec, respectively), and was comparable to that of the control with FVIII:C <15 IU/dl (estimated ~10 IU/dl). In contrast, |min2| obtained with the patient's plasma was lower than that with the control at FVIII:C 1.0 IU/dl (0.21 ± 0.01 and 0.26 ± 0.01, respectively). These results were in keeping with the earlier characteristics of the R1781H mutation. As described above in the FXa generation assays (see ▶Figure 3), the moderated haemostatic potential appeared likely to be associ-

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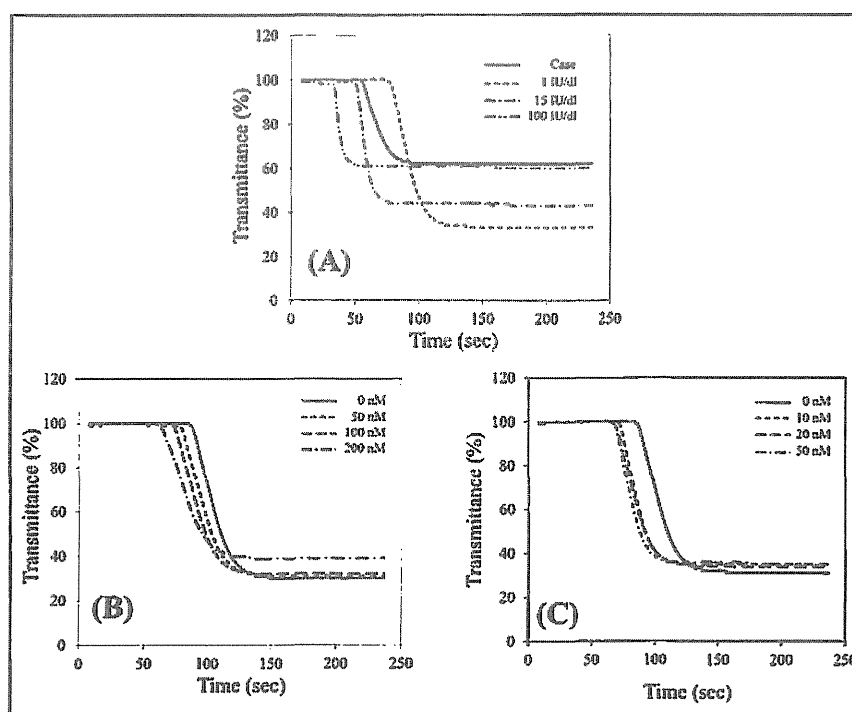

Figure 4: Clot waveform analysis in the patient with R1781H, and in control plasma (FVIII:C, 0.9 IU/dl) with added FVIII or in the presence of exogenous FX or FIXa. $CaCl_2$ was added to FVIII-deficient plasma mixed with various amounts of FVIII (1, 15, and 100 IU/dl) and to patient's plasma (A). Alternatively, various amounts of FX (0-200 nM) (B) or FIXa (0-50 nM) (C) were mixed with FVIII-deficient plasma (FVIII:C, 0.9 IU/dl), followed by the addition of $CaCl_2$. Clot waveforms were visualised in an APTT-based assay as described in *Methods*. Representative data are illustrated in A-C.

ated with the limited reactions of FVIII(a), FIXa, and FX. Furthermore, the clot time was shortened and |min2| was modestly reduced in a dose-dependent manner in the presence of incremental amounts of FX added to control plasma (with FVIII:C 0.9 IU/dl) (▶Figure 4B), and were consistent with the waveform pattern in the presence of the mutant R1781H sample. In contrast, no effects such as FX were evident in the presence of varying amounts of FIXa (▶Figure 4C). It may be speculated that these findings represented an alteration in the association between R1781H FVIII(a) and FX in patient's plasma.

Thrombin generation in the presence of FVIII R1781H mutant

Differences between the coagulation potential of patient's plasma and that of control plasmas might have been related to the concentrations of procoagulant, anticoagulant and/or fibrinolytic components other than FVIII in the test samples. Experiments were repeated, therefore, using a constructed FVIII mutant where Arg[1781] was converted to His by site-directed mutagenesis, and stably expressed in BHK cells as a B-domainless FVIII form. This R1781H mutant exhibited a modest reduction of specific activity (~60% level of wild type) measured in a one-stage clotting assay.

Thrombin generation assays were repeated as described above using FVIII-deficient plasma mixed with FVIII wild type or R1781H mutant. The levels of peak thrombin in the presence of wild type and R1781H mutant at the same level of FVIII:C (1.0 IU/dl) were 233 ± 2 nM and 338 ± 5 nM, respectively, supporting that thrombin generation in the presence of the R1781H mutant was much greater than that in wild-type FVIII at equivalent levels of FVIII:C, and that 1.0 IU/dl R1781H was comparable to 5-6 IU/dl wild-type FVIII. In addition, the other parameters (time to peak and ETP) also showed similar tendencies (data not shown). The findings were consistent with the earlier data and were in keeping with the suggestion that the mutation alone contributed to the significantly greater coagulation potential in our case with FVIII R1781H.

Effect of FVIII R1781H mutant on the K_m for FX activation

The results illustrated above (see ▶Figure 4) indicated a possible significant alteration in the association between FVIII(a) and FX in the R1781H patient. Purified FXa generation assays were performed, therefore, using a tenase complex containing FVIIIa wild type or R1781H mutant. The results are shown in ▶Figure 5. The K_m value obtained from the fitted curve for FX in the presence of R1781H mutant was 32.5 ± 3.8 nM, and was ~1.9-fold lower than to that obtained using wild-type FVIII (58.5 ± 6.1 nM). In contrast, the V_{max} obtained for the two forms of FVIIIa were similar (203 ± 71 min^{-1} and 225 ± 13 min^{-1}, respectively). These results suggested a significant increase (~1.6-fold) in catalytic efficiency (V_{max}/K_m) for the tenase in the presence of R1781H relative to native tenase. The K_m and V_{max} values for FIXa or PL vesicles obtained using the two FVIIIa forms were both similar (data not shown). These results supported the concept that the R1781H mutant affinity for FX, was greater than that of the wild type, and were in keeping with the conclusion that the increase in tenase activity in the presence of FVIII R1781H resulted from a relative enhancement in the association between FVIIIa and FX.

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Discussion

One-stage clotting assays based on the APTT are generally used for the measurement of FVIII:C, and coagulation factor levels generally correlate well with the clinical phenotypes of patients with haemophilia A. Assays of this type, however, only partially reflect coagulation in a non-physiological environment, and the limited sensitivity at lower levels of FVIII:C (<2-3%) could compromise the accuracy between these specific laboratory assays and clinical status. Furthermore, discrepancies in one-stage and two-stage clotting assays of FVIII:C are observed in haemophiliacs with point mutations at Ala[284]/Ser[289] in A1, Arg[527]/Arg[531]/Arg[698] in A2, and His[1954] in A3, which are functionally crucial residues for the interactive surfaces in the FVIII A domains (20). One-stage clotting assays may have limited use, therefore, in the evaluation of coagulation function *in vivo* in haemophiliacs. In consequence, a number of global coagulation functional assays have been established and are utilised for clinical diagnosis (6-8). We have previously reported that both clot waveform and thrombin generation assays are useful for clinical assessment in patients with very low levels of FVIII (<1%) (7, 8). In the present investigation, global coagulation assays demonstrated that the R1781H mutant exhibited enhanced coagulation potential, in accordance with clinical phenotype, whilst one-stage clotting assays failed to identify this association. The findings suggested that the global tests rather than the one-stage clotting assays reflected coagulation potential in the patient with FVIII R1781H. It seems likely that comprehensive appraisal of coagulation function in haemophilia A could depend on both conventional clotting assays and modern global coagulation assessment.

The results of the different coagulation measurements appear likely to depend on the final concentration of the substrate FX in the assay systems. In the current investigations, ROTEM, FXa and thrombin generation assays were performed at near physiological concentrations of FX (~120 nM). These FX concentrations were significantly greater than the K_m values of FX for tenase, comprising FVIII wild type and R1781H (by 2.1- and 3.7-fold, respectively), and were near the saturating level of FX for FVIIIa binding (FVIII; ~1 nM in plasma). In the one-stage assay, however, the plasma is diluted at least four-fold, and the limiting amount of FX is lower than that for the K_m for FX (by 0.50- and 0.93-fold, respectively), and provides a limiting level of FX. This markedly depresses both the rate of tenase activity and the subsequent reactions leading to clot formation. The differences in K_m for FX and FVIII-variant association could account for the discrepancies between coagulation activities seen using different methodologies, and may be similar to the mechanism governing the dampening of tenase activity observed when comparing FVIII:C levels of FVIIIa and inactivated FVIIIa lacking the A1[337-372] region. In these circumstances, significant differences are found between one-stage clotting assays and FXa generation assays (21).

The addition of incremental amounts of exogenous FX, but not FIXa, to control, FVIII-deficient plasma (FVIII:C 0.9 IU/dl) mediated a pattern of clot waveform similar to that seen with native R1781H plasma. It could be that the concentration of FVIII in

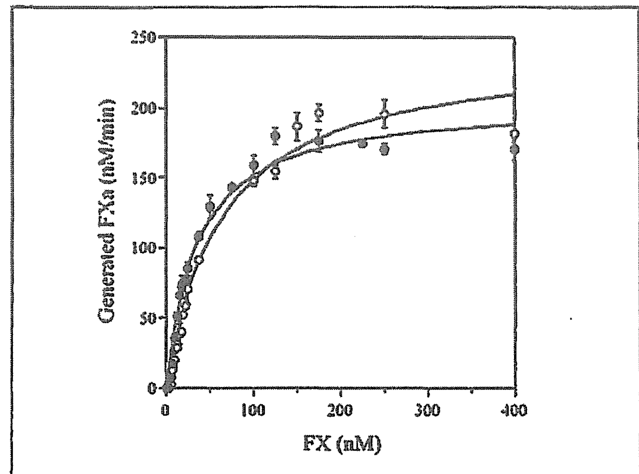

Figure 5: Michaelis-Menten analysis of the tenase complex formed using R1781H. FVIII preparations (5 nM) were activated with thrombin (30 nM) for 1 min in the presence of PL vesicles (20 μM). FXa generation was initiated by the addition of FIXa (0.5 nM) and various concentrations of FX (0-400 nM) as described in *Methods*. The symbols used are as follows: FVIII wild type (open circles), R1781H (closed circles). Initial rates of FXa generation are plotted as a function of FX concentration and fitted to the Michaelis-Menten equation by non-linear least squares regression. All experiments were performed at least four separate times, and the average values and standard deviations were calculated.

plasma in these experiments was very low (~1 nM), and that the physiological amount of FX in plasma was insufficient for interaction with FVIIIa. In the presence of excess amounts of FX, however, FVIIIa would have been completely saturated, and the clot waveform obtained under these conditions was indeed similar to that observed with R1781H. It seems likely, therefore, that the relatively high affinity of R1781H for FX in plasma compared to that of wild-type FVIII could have accounted for the enhanced interaction between these two components of the tenase complex, and hence mediated the greater coagulation potential of R1781H.

A FX-interactive site on FVIII is localised within the acidic region in the A1 domain, especially in the clustered acidic residues Asp[361-363] (22). This region interacts with the heparin-binding exosite (in particular Arg[240]) in the catalytic domain of FX. In addition, Fay and colleagues have recently reported (23) that FX interacts with the residues 2007-2016 (in particular Thr[2012] and Phe[2014]) in the A3 domain, which contributes to the K_m of tenase complex for FX. Studies on the X-ray crystal structure of the FVIII molecule (24) indicated that Arg[1781] is unlikely to be in close proximity to the 2007-2016 region, although the 337-372 acidic region in A1 is not defined in this model. It might be, therefore, that Arg[1781] represents a yet unidentified FX-interactive site in FVIII. Alternatively, Arg[1781] might not directly participate in FX binding, but rather might indirectly affect the interaction of FVIII with FX through a possible conformational change by the substitution of Arg[1781] to His.

To date, 28 haemophilia A patients, indentified with the R1781H mutation, have demonstrated clinical heterogeneity, rang-

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What is known about this topic?

- The clinical severity in haemophilia A patients with similar FVIII:C levels may be different. A mild bleeding phenotype has been reported in ~10% of individuals with severe haemophilia A.
- Non-null mutations of *F8* may represent the main determinant for bleeding tendency. Other factors associated with other coagulation abnormalities appear to be negligible as modulators of disease severity. The heterogeneity of clinical symptoms in severe haemophilia A remains to be fully explained.
- Some severe haemophilia A patients with the specific R1781H gene mutation present with a mild/moderate clinical phenotype, but the mechanism remains to be fully investigated.

What does this paper add?

- The plasma FVIII:C in a patient with haemophilia A and the R1781H mutation was ~0.9 IU/dl in one-stage clotting assays. Comprehensive global coagulation tests showed significantly greater haemostatic potential, corresponding to a FVIII:C level of 5–10 IU/dl.
- Thrombin generation tests demonstrated that haemostatic function in the presence of recombinant R1781H FVIII (~0.9 IU/dl) was comparable to that observed at concentrations of ~5 IU/dl native FVIII:C.
- The recombinant R1781H mutant demonstrated an ~1.9-fold decrease in K_m for FX in the tenase complex compared to FVIII wild type. This enhancement of binding affinity of FVIII R1781H for FX appeared to reflect the severity of the haemophilia A phenotype.

ing from a severe to mild/moderate phenotype (13). Among these patients, only three patients with FVIII:C ≤1 IU/dl have been classed as severe. Some of these cases seem to contradict our conclusion based on enhanced coagulation potential of R1781H relative to wild-type FVIII. Complete profiles of these patients (type and/or degree of life activity and arthropathy, etc) have not been reported, however, and further clarification is required. In addition, 17 patients with this mutation had FVIII:C >1 IU/dl and presented with a mild/moderate clinical phenotype. Our findings indicate that enhanced coagulation potential mediated by the R1781H mutant would contribute to the phenotype of these individuals, although it is well known that in general patients with FVIII:C >1 IU/dl have mild/moderate haemorrhagic symptoms.

A mutation of the Arg residue to His is a relatively conservative amino acid change. Interestingly, another causative mutation (R1781C) described in several reports, also seems to mediate variable phenotypes, ranging from severe to mild/moderate. It may be, therefore, that the mutation of Arg at 1781 by itself, and not the nature of the amino acid substitution, influences the mechanisms governing the clinical presentation. Experiments using a range of amino acid substitutions at this site might help to clarify if the bulkier imidazole side chain of the His residue is a fundamental contributor to the structural change in the FVIII molecule.

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Conflicts of interest
None declared.

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ORIGINAL ARTICLE

Genotypic and phenotypic features of Japanese patients with mild to moderate hemophilia A

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Abstract Hemophilia A is the most common inherited bleeding disorder. To better understand the genotypic and phenotypic features of Japanese patients with mild to moderate hemophilia A, we studied 29 unrelated patients with more than 1 % FVIII activity (FVIII:C). Differences were observed in nine of 21 patients in measured FVIII:C levels between the one-stage clotting and chromogenic assays. We identified a mutation in *F8* in 28 of the 29 patients. Mutations in two amino acids, Y492 and R550, were detected at a much higher frequency in our patients than in the international hemophilia A mutation database. We demonstrated that all five patients with the Y492C mutation have an identical *F8* haplotype that is unique to them, suggesting that the mutation may have originated from a common ancestor. Because non-severe, moderate to mild, hemophilia patients have a longer lifespan, mutations that cause non-severe phenotypes tend to persist in the population. We believe that the Y492C mutation is a distinctive feature of Japanese patients with mild hemophilia A. The identification of a high frequency of R550 mutation that underlies the discrepancies in FVIII:C measurements in the present study suggests that Japanese patients with mild hemophilia may require careful characterization.

Keywords Hemophilia A · Founder effect · Mutation · Discrepancy

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Introduction

Hemophilia A (MIM +306700) is the most common inherited bleeding disorder caused by a quantitative or qualitative abnormality of the blood coagulation factor VIII (FVIII). The clinical severity of hemophilia A correlates well with the residual factor VIII activity (FVIII:C) in circulating blood, and the presence of small amounts of FVIII:C markedly reduces the clinical severity. Mild to moderate hemophilia A, generally presenting with mild or negligible bleeding symptoms, is defined as FVIII:C of more than 1 % [1]. About 60 % of hemophilia A patients are classified into this category [2]. The factor VIII gene (*F8*) is located on the most distal band of chromosome X (Xq28) and spans over 186 kb. This large gene consists of 26 exons and 25 introns, and encodes 2,351 amino acids. Since the cloning of *F8* in 1984 [3–6], numerous analyses regarding *F8* have been performed to identify the mutation(s) responsible for hemophilia A. To date, about 1,500 unique mutations have been identified and registered in a worldwide mutation database [HADB; http://hadb.org.uk, also known as HAMSTeRS (The Haemophilia A Mutation, Structure, Test and Resource Site)]. According to HADB, almost all the mutations that cause a mild to moderate hemophilia A are a missense mutation.

There are some racial differences in the allele frequency of single nucleotide polymorphisms (SNPs) and the frequency of mutations that cause hemophilia A. For example, the frequency of *F8* inversion, which is known as the most common mutation responsible for severe hemophilia A, is possibly lower in the Japanese population than in the Caucasian population [7]. Some founder effects detected at a high frequency only in a limited geographical area have been reported thus far [8–10]. Therefore, it is important to understand the features of genetic abnormality of

hemophilia A in a particular region or race. In the present study, we analyzed and characterized the genotypic and phenotypic features of 29 Japanese patients with mild to moderate hemophilia A.

Materials and methods

Patients

We analyzed 29 unrelated Japanese patients with moderate to mild (FVIII:C > 1 %) hemophilia A. All the patients characterized in this study live in the Tokyo metropolitan area. The study was approved by the ethical committee of Tokyo Medical University, and written informed consent was obtained from all patients. This study was conducted in accordance with the ethical principles of the declaration of Helsinki, revised at Seoul in 2008.

Coagulation assay

FVIII:C assay

We measured the FVIII:C level by one-stage clotting assay and chromogenic assay.

The one-stage clotting assay was performed on an ACL-9000 automatic coagulation analyzer (Instrumentation Laboratory, Bedford, MA, USA) using 2 commercially available APTT reagents, HemosIL™ APTT-SP reagent (Instrumentation Laboratory) and Thrombo-Check APTT(S) (Sysmex, Kobe, Japan).

The chromogenic assay was carried out using a Coatest SP FVIII kit (Chromogenix, Milan, Italy) according to the manufacturer's instructions.

Factor VIII antigen (FVIII:Ag) assay

We measured the FVIII:Ag level using an Asserachrom VIII:Ag kit (Diagnostica Stago, Asnieres, France) according to the manufacturer's instructions.

Gene analysis

Genomic DNA was extracted from peripheral blood cells according to the standard methods with proteinase K and phenol/chloroform, or using an EZ1 DNA Blood 350 μL kit (Qiagen, Hilden, Germany) on a BioRobot EZ1 workstation (Qiagen). *F8*, including the entire coding sequence, exon–intron junctions, and part of the 5′ and 3′ untranslated regions were amplified by PCR using 36 sets of primers. Although we designed most PCR primers used in this study, some were designed as described previously [11]. The M13 consensus sequence was added to the 5′ end of all primers for direct sequencing. The amplified products were electrophoresed on a 3 % agarose gel and were extracted using a QIAquick Gel Extraction kit (Qiagen). The purified PCR products were directly sequenced by the dideoxy chain termination method using the M13 consensus sequence as the primer, and analyzed with an SQ-5500 or SQ-5500E sequencer (Hitachi, Tokyo, Japan). Nucleotide sequences obtained by sequencing were compared with the *F8* reference sequence (ENSG00000185010) in the Ensembl.

F8 haplotype analysis

Seven different SNPs [rs6649625 (C/T), rs1470586 (C/T), rs1800291 (C/G), rs4898352 (A/T), rs4074307 (C/T), rs1050705 (A/G), and rs6571266 (A/G)] in *F8* were analyzed for 79 unrelated Japanese male patients (51 hemophilia A patients including 29 patients in this study, 26 hemophilia B patients, and 2 normal individuals). The regions including each SNP were amplified by PCR using specific primer pairs that we designed and the products were digested by appropriate restriction endonucleases. The location of the SNPs, nucleotide primer sequences, and restriction endonucleases used in this study are shown in Table 1. The short tandem repeats (STR) in IVS-13 of *F8* [12] were also analyzed. The region including the STR was amplified by PCR using specific primer pairs (fwd: 5′-CAC TTT AAA AAT GCC GCT CC-3′; rev: 5′-CAA GAG CTG TGT GAC AAA ATT GA-3′) and the number of the CA repeats was determined by direct sequencing of the PCR products.

Bioinformatic analysis

The effects of missense mutations were predicted using the PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and the SIFT (http://sift.jcvi.org/). The 3D structure of factor VIII was obtained from the crystallographic data (3CDZ) [13] registered in the Protein Data Bank (http://www.rcsb.org/pdb/) and was built with the UCSF Chimera program.

Results

Coagulation study

In this study, we examined the FVIII:C level in 21 of the 29 patients (Table 2). Because an appropriate plasma sample was not obtained to evaluate FVIII:C level in this study, the phenotypes of the remaining 8 patients were decided by the historically lowest FVIII:C level in the medical record. No significant differences were observed between the FVIII:C

Table 1 SNPs used for haplotype analysis of *F8*

SNP	Location	Direction	Primer sequence (5′→3′)	Restriction enzyme	Informative
rs6649625	IVS-1	Fwd	ATC TGG TGG GTG AAA GCA AT	*Alu*I	Yes
		Rev	GCA TCA CAC TTA TAA AAT ACA CAG AGA		
rsl470586	IVS-1	Fwd	GAT TCA ATA TAG AAA TCC TGC CAA A	*Aci*I	Yes
		Rev	ATG CTG ATT AAC AGG ATA AGC TGA C		
rsl800291	Exon 14	Fwd	GCC TCA GAT ACA TAC AGT GAC	*Mnl*I	Yes
		Rev	CTG CTG GCT TGT ATT AGG AG		
rs4898352	IVS-18	Fwd	ATG GTC TAG GCA CTG GGA AC	*Bcl*I	No
		Rev	GTG CCC TAT GGG ATT TGA GA		
rs4074307	IVS-19	Fwd	TTC CTT TCT GGA ATG GTT GC	*Hind*III	No
		Rev	TTC GAG CTT TAC CAA GTT GTG A		
rsl050705	3′-UTR	Fwd	CCC TGT GAA GTT CTT AAA GT	*Bcl*I	No
		Rev	GGG AAA AGA ATG CCA AAA TAA GAT GAT		
rs6571266	3′-UTR	Fwd	GCC TCA ATC CAG GAG AAC AG	*Bsl*I	No
		Rev	TTA TTA GCG ACG GGA TTT CG		

Seven SNPs located in a wide range of *F8* were analyzed. However, only 3 SNPs (i.e., rs6649625, rs1470586 and rs1800291) were informative for the analysis. There is a strong linkage disequilibrium between rs1470586 and 4 other SNPs (i.e., rs4898352, rs4074307, rs1050705, and rs6571266) in this analysis

levels obtained by the one-stage clotting assay using HemosIL™ APTT-SP, which we usually use in our laboratory as the APTT reagent, and those obtained by the chromogenic assay. However, the FVIII:C levels obtained by the one-stage clotting assay using Thrombo-Check APTT(S) as the APTT reagent were clearly higher than those obtained by the chromogenic assay in some of the 21 patients. Six patients (1 case of A303P, 1 case of G498R, 2 cases of R550C, 1 case of R550H, and 1 case of W707L) who showed TC/Coa ratios higher than 2.0 (Table 2) were concluded to have FVIII:C discrepancy. Three additional patients (patients #12, 14 and 16 in Table 2) had TC/Coa ratios of <2.0, but they showed the same R550C and R550H mutations; thus, we conclude that these three patients similarly have the FVIII:C discrepancy.

Gene analysis

We identified mutations in *F8* in 28 of the 29 patients (Table 2). In the remaining patient who showed a moderately severe phenotype, we could not find any genetic abnormality that may be responsible for hemophilia A. The mutations identified in this study were all missense mutations. Five mutations (W707L, T1793N, H1878P, S1907N, and Q2106H) were novel and not yet registered in the HADB database as of August 2012.

The missense mutations were distributed over the entire length of *F8*. However, there was a high frequency of mutations in 2 amino acid residues: 5 (17.2 %) mutations were found in Y492 in exon 10, and 6 (20.7 %) in R550 in exon 11.

An intragenic analysis of 7 SNPs and 1 STR was carried out to determine the haplotype of patients with the Y492C mutation. Seven SNPs that were considered to work effectively for haplotype analysis by referring to the allelic frequency from the HapMap project data were selected. However, there was a strong linkage disequilibrium between rs1470586 (C/T) SNP and 4 other SNPs: rs4898352 (A/T), rs4074307 (C/T), rs1050705 (A/G), and rs6571266 (A/G). Therefore, only 3 SNPs [rs6649625 (C/T), rs1470586 (C/T), and rs1800291 (C/G)] were informative for the analysis. *F8* from 79 Japanese males were classified into 4 haplotypes using 3 informative SNPs (Table 3) and 9 of them showed the CTC haplotype. All 5 *F8* with the Y492C mutation showed the CTC haplotype. IVS-13 STR analysis was additionally performed in the 9 *F8* with the CTC haplotype. These were divided into 2 groups: 4 with 21 CA repeats, and 5 with 22 CA repeats. The latter group is composed of only *F8* with the Y492C mutation. Taken together, all 5 *F8* with the Y492C mutation showed the same unique haplotype (CTC and 22 CA repeats).

Discussion

We set out to study 29 unrelated Japanese patients with mild to moderate hemophilia A to analyze their genotypic and phenotypic features.

We have identified a high frequency of mutations in R550 and Y492 in these patients. The R550 residue includes the CpG dinucleotide in the codon. The CpG

Table 2 Phenotype and genotype of 29 Japanese patients with mild to moderate hemophilia A

No.	One-stage clotting (IU/mL)			FVIII:C Coa (IU/mL)	TC/ Coa	Discrepancy	FVIIIAg (IU/mL)	Severity	Exon	Mutation		HADB
	FVIII:C SP	FVIII:C TC	Medical record							Nucleotide change[a]	Amino acid change[b]	
1	0.080	0.085		0.084	1.01		0.238	Mild	3	c.328A > G	p.M110V (91)	Yes
2	NA	NA	0.031	NA				Moderate	5	c.606T > G	p.S202R (183)	Yes
3	0.054	0.051		0.073	0.7		0.060	Mild	5	c.606T > G	p.S202R (183)	Yes
4	0.180	0.265		0.109	2.43	Yes	0.218	Mild	7	c.907G > C	p.A303P (284)	Yes
5	NA	NA	0.088	NA				Mild	10	c.1475A > G	p.Y492C (473)	Yes
6	0.079	0.082		0.082	1		0.086	Mild	10	c.1475A > G	p.Y492C (473)	Yes
7	0.078	0.087		0.095	0.92		0.100	Mild	10	c.1475A > G	p.Y492C (473)	Yes
8	NA	NA	0.110	NA				Mild	10	c.1475A > G	p.Y492C (473)	Yes
9	0.063	0.084		0.088	0.95		0.094	Mild	10	c.1475A > G	p.Y492C (473)	Yes
10	0.053	0.109		0.037	2.95	Yes	0.234	Moderate	10	c.1492G > A	p.G498R (479)	Yes
11	0.080	0.119		0.045	2.64	Yes	0.186	Mild	11	c.1648C > T	p.R550C (531)	Yes
12	0.084	0.133		0.074	1.8	Yes	0.182	Mild	11	c.1648C > T	p.R550C (531)	Yes
13	0.053	0.110		0.047	2.34	Yes	0.170	Mild	11	c.1648C > T	p.R550C (531)	Yes
14	0.272	0.362		0.225	1.61	Yes	0.402	Mild	11	c.1648C > T	p.R550C (531)	Yes
15	0.199	0.320		0.134	2.39	Yes	0.481	Mild	11	c.1649G > A	p.R550H (531)	Yes
16	0.232	0.366		0.250	1.46	Yes	0.464	Mild	11	c.1649G > A	p.R550H (531)	Yes
17	0.083	0.209		0.096	2.18	Yes	0.372	Mild	14	c.2120G > T	p.W707L (688)	No
18	0.408	0.448		0.417	1.07		0.638	Mild	14	c.2149C > T	p.R717W (698)	Yes
19	0.298	0.364		0.295	1.23		0.262	Mild	14	c.2149C > T	p.R717W (698)	Yes
20	NA	NA	0.034	NA				Moderate	14	c.2167G > A	p.A723T (704)	Yes
21	NA	NA	0.070	NA				Mild	14	c.5122C > T	p.R1708C (1689)	Yes
22	NA	NA	0.074	NA				Mild	14	c.5122C > T	p.R1708C (1689)	Yes
23	0.178	0.196		0.214	0.92		0.211	Mild	16	c.5378C > A	p.T1793N (1774)	No
24	NA	NA	0.060	NA				Mild	17	c.5633A > C	p.H1878P (1859)	No
25	0.159	0.165		0.162	1.02		0.106	Mild	17	c.5720G > A	p.S1907N (1888)	No
26	0.372	0.310		0.425	0.73		0.302	Mild	22	c.6318G > C	p.Q2106H (2087)	No
27	0.070	0.055		0.062	0.89		0.216	Mild	23	c.6506G > A	p.R2169H (2150)	Yes
28	0.052	0.052		0.058	0.9		0.080	Mild	26	c.6977G > A	p.R2326Q (2307)	Yes
29	NA	NA	0.034	NA	1.01			Moderate				

FVIII:C SP, FVIII activity obtained from a one-stage clotting assay using the HemosIL™ APTT-SP reagent as the APTT reagent; FVIII:C TC, FVIII activity obtained from a one-stage clotting assay using the Thrombo-Check APTT(S) as the APTT reagent; FVIII:C Coa, FVIII activity obtained from a chromogenic assay using the Coatest SP FVIII kit. Eight patients (# 2, 5, 8, 20, 21, 22, 24 and 29) from whom an appropriate plasma sample was not obtained to evaluate FVIII:C level in this study provided the historically lowest FVIII:C level in the medical record

NA not available

[a] The nomenclature of mutation is based on the cDNA sequence with nucleotide +1 corresponding to A of the ATG initiation codon

[b] Amino acids are numbered from the initial Methionine 1. The numbers in parentheses indicate amino acid numbers of mature processed FVIII

dinucleotide is a well-known hotspot for mutations [14] and a large number of missense and nonsense mutations were identified in the Arg(CGN) residues in the *F8* of hemophilia A patients. The mutation of C-to-T transition in Arg(CGN) accounted for 990 (35 %) of 2,830 point mutations in the HADB database. The mutation of C-to-T transition in the R550 residue is one of the most frequent point mutations in mild to moderate hemophilia A and accounted for 2.5 % in the HADB database. The R550 mutation was identified with a high frequency (20.7 %) in this study and this frequency was considerably higher than the frequency of R550 mutation found in the international database. When analyzing the mutations of the R550 residue, it is necessary to note that the measured FVIII:C values differ significantly among assay methods, particularly between the one-stage clotting assay and either the

Table 3 Haplotype analysis of *F8* in 79 Japanese male patients

SNP (1,2,3) haplotype[a]	Frequency	IVS-13 VNTR	Y473C
CCC	54/79 (69 %)	NE	0/54
CTC	9/79 (11 %)	21	0/4
		22	5/5
CTG	7/79 (9 %)	NE	0/7
TCC	9/79 (11 %)	NE	0/9

NE not examined

[a] Haplotype: rs6649625 (C/T), rs1470586 (C/T), rs1800291 (C/G)

chromogenic assay or the two-stage assay [15, 16]. It has been reported that the dissociation rate of the A2 domain from the FVIII molecule, which leads to the inactivation of the active FVIII, may be involved in the measurement discrepancies [17, 18], and that the dissociation rate of the A2 domain may be related to some reaction conditions, particularly the pH [19, 20]. We previously reported that there is a weak negative correlation between the pH of the APTT reagent and the measured FVIII:C values in the one-stage clotting assay [21]. In that report, we analyzed FVIII:C of the R550H mutation identified from a hemophilia A patient with a very mild phenotype. When this patient's FVIII:C was measured by the one-stage clotting assay using 14 different APTT reagents, the results were markedly different with each reagent. The highest and the lowest values for FVIII:C were about 50 and 25 %, respectively, showing almost twice the difference. Although all the FVIII:C levels obtained with a variety of APTT reagents reflect a mild phenotype, there is a possibility that the patient cannot receive an optimized treatment based on the numerical value. Furthermore, this may lead to a misdiagnosis of mild hemophilia. The identification of a high frequency of R550 mutation in this study suggests that Japanese patients with mild hemophilia might require more attention and care than the Caucasian population.

Y492 does not include the CpG dinucleotide in the codon. Y492C mutation registers only 6 (0.21 %) of point mutations in the HADB [22–25]. One of these registered cases was from the analysis of a Japanese patient [24], and 2 out of the 5 remaining cases were possibly Japanese subjects [23]. Surprisingly, we have identified 5 cases (17.2 %) of Y492C mutation in this study. Although it is not possible to declare with certainty because of the large difference in the number of patients between this study and the HADB, it is possible that one of the major genotypic features of Japanese patients with mild to moderate hemophilia A is the high frequency of Y492C mutation. The patients identified with Y492C mutation were apparently unrelated Japanese individuals. However, the results of the 3 SNPs and IVS-13 STR analysis indicated that all 5 patients with this mutation had an identical *F8* haplotype that is unique to them. These results strongly suggest a founder effect. The Y492C mutation possibly originated from a common ancestor and then spread into the Japanese population.

Five novel missense mutations were identified in this study. As for 4 out of these 5 mutations, the possibility that they may be the causes of mild to moderate hemophilia A was predicted by either the PolyPhen-2 or SIFT prediction method. However, T1793N was not predicted by either methods (Table 4) although, this mutation was also detected from another Japanese hemophilia A patient. An expression study is necessary to clarify that this mutation is also an etiology of mild to moderate hemophilia A.

We could not identify any mutation from 1 patient in this study. Because some genetic abnormality probably exists in the patient's *F8*, we should perform a different type of analysis such as multiplex ligation-dependent probe amplification.

Measurement of FVIII:C is necessary to determine the phenotype of a hemophilia A patient. However, we must be aware of the characteristics of the method that is used to assay FVIII:C. In some particular cases, such as a mutation identified in R550 as described above, the FVIII:C level possibly differs markedly according to the measurement methods. Mild cases of hemophilia A particularly require more attention [17, 26]. Furthermore, in the case of a mutation located in the interface of each of the A domains,

Table 4 Novel missense mutations identified in the present study

Mutation	Conservation[a] (h/p/m/c)	Polyphen-2[b] (HumDiv score)	SIFT[c] (score)
p.W707(688)L	W/W/W/W	Probably damaging (1.000)	Damaging (0.01)
p.T1793(1774)N	T/T/T/T	Benign (0.061)	Tolerated (0.36)
p.H1878(1859)P	H/R/H/R	Probably damaging (0.988)	Damaging (0.03)
p.S1907(1888)N	S/S/S/S	Benign (0.189)	Damaging (0.00)
p.Q2106(2087)H	Q/Q/Q/Q	Probably damaging (1.000)	Damaging (0.00)

[a] Factor VIII amino acid line up for human, porcine, murine and canine

[b] PolyPhen-2 prediction (http://genetics.bwh.harvard.edu/pph/)

[c] SIFT prediction (http://sift.jcvi.org/)

there is a great possibility of discrepant FVIII:C levels. In the present study, 9 cases showed clearly different FVIII:C levels between the one-stage clotting assay using Thrombo-Check APTT(S) and the chromogenic assay or the one-stage clotting assay using HemosIL™ APTT-SP. The amino acid residues of all 9 cases (1 case of A303P, 1 case of G498R, 4 cases of R550C, 2 cases of R550H, and 1 case of W707L) were previously reported as residues which show different FVIII:C levels.

The discrepancy in FVIII:C measurement was originally reported as the difference between the one-stage clotting assay and the chromogenic assay. Subsequently, it was reported that the discrepancy was also observed within the one-stage clotting assays depending on the assay conditions, particularly when different APTT reagents were used [27]. The discrepancy leads to difficulties in understanding the exact phenotype of the patient. However, this can also bring useful information to the analysis of relationships between phenotypes and genotypes. That is, if the discrepancy can be confirmed before gene analysis, the type of mutation that is likely to exist in the interface of A domains can be predicted. Moreover, if it is a case where a novel mutation is identified, the importance of A2 domain dissociation with respect to the mutation site can also be grasped by confirming the discrepancy. Therefore, intentional confirmation of the discrepancy may become an effective analytical tool. We usually measure the FVIII:C using ACL9000 and an APTT reagent (HemosIL™ APTT-SP). In this analysis, the results obtained by the one-stage clotting assay were almost the same as those obtained by the chromogenic method (Coatest SP FVIII), and it was suggested that the methods can be substituted interchangeably. Because the information about A2 domain dissociation is easily obtained by the one-stage clotting assay using an appropriate APTT reagent even if a chromogenic method is not performed, we recommend to attempt the one-stage clotting assay with two or more APTT reagents to analyze patients, except for a severe hemophilia case.

In conclusion, we have described the genotypic and phenotypic features of Japanese patients with mild to moderate hemophilia A. The identification of a high frequency of Y492C, which is thought to be the founder effect of the Japanese population, is one of the genetic features of Japanese patients with mild to moderate hemophilia A. The identification of a high frequency of R550 mutation that causes the discrepancy in FVIII:C measurement in this study suggests that Japanese patients with mild hemophilia might need more attention and care than the Caucasian population.

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Conflict of interest K. Fukutake has received a research grant and lecture honoraria from Baxter Healthcare, Bayer HealthCare, Pfizer Inc., and Novo Nordisk Pharma Ltd. K. Shinozawa is the holder of an endowed chair at the Department of Molecular Genetics of Coagulation Disorders at Tokyo Medical University, which received funding from Baxter Healthcare. The other authors declare that they have no interests that might be perceived as posing a conflict of interest associated with this study.

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Global brain delivery of neprilysin gene by intravascular administration of AAV vector in mice

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Accumulation of amyloid-β peptide (Aβ) in the brain is closely associated with cognitive decline in Alzheimer's disease (AD). Stereotaxic infusion of neprilysin-encoding viral vectors into the hippocampus has been shown to decrease Aβ in AD-model mice, but more efficient and global delivery is necessary to treat the broadly distributed burden in AD. Here we developed an adeno-associated virus (AAV) vector capable of providing neuronal gene expression throughout the brains after peripheral administration. A single intracardiac administration of the vector carrying neprilysin gene in AD-model mice elevated neprilysin activity broadly in the brain, and reduced Aβ oligomers, with concurrent alleviation of abnormal learning and memory function and improvement of amyloid burden. The exogenous neprilysin was localized mainly in endosomes, thereby effectively excluding Aβ oligomers from the brain. AAV vector-mediated gene transfer may provide a therapeutic strategy for neurodegenerative diseases, where global transduction of a therapeutic gene into the brain is necessary.

Aggregation and deposition of amyloid-β peptide (Aβ) in the brain are triggering events of the long-term pathological cascade of Alzheimer's disease (AD), and are closely associated with the metabolic balance between Aβ anabolic and catabolic activities[1,2]. As almost all familial AD mutations cause an increase in the anabolism of a particular form of Aβ, $A\beta_{1-42}$, leading to Aβ deposition and accelerating AD pathology, a chronic reduction in the catabolic activity would also promote Aβ deposition[1,2]. Neprilysin (EC 3.4.24.11) is a rate-limiting peptidase involved in brain Aβ catabolism, as proven by *in vivo* experiments tracing the catabolism of radiolabeled Aβ in brain and by reverse genetics studies for candidate peptidases in mice[3,4]. Neprilysin gene-disruption caused a gene dosage-dependent elevation of endogenous Aβ levels in mouse brain, suggesting that a subtle but long-term reduction in neprilysin activity would contribute to AD development by promoting accumulation of Aβ[2].

Mounting evidence that expression levels of neprilysin are decreased in the hippocampus and cerebral cortex of AD patients from the early stages of disease development and also with aging in humans, as well as mice, suggests a close association of neprilysin with the etiology and pathogenesis of AD[2]. Indeed, reduced activity of neprilysin in mouse brain elevates the levels of highly toxic Aβ oligomers at the synapses, and leads to impaired hippocampal synaptic plasticity and cognitive function even before apparent amyloid deposition is observed in the brain[5]. Thus, a decline in neprilysin activity appears to be at least partly responsible for the memory-related symptoms of AD, and up-regulation of neprilysin is considered to be a promising strategy for therapy and prevention of AD.

Experimental gene therapy to transfer neprilysin gene into the brains of AD model mice has been reported, and for this purpose various kinds of recombinant viral vectors carrying wild-type neprilysin or its variants that are truncated at the transmembrane region and can be released to extracellular space have been utilized[6,7]. Viral vector-mediated delivery of neprilysin gene successfully retarded amyloid deposition in the brains of AD model mice[6,7]. Beneficial potential of gene therapy has also been shown in other neurodegenerative diseases, including Parkinson's disease (PD). Gene transfer of dopamine-synthesizing enzymes into the putamen alleviated motor

symptoms in PD patients[8,9]. However, infusion of viral vectors via stereotaxic surgery is not necessarily appropriate if the therapeutic gene should be delivered to broad areas of the brain.

In this study we have successfully developed a new gene delivery system by employing the combination of rAAV9 with a neuron-specific promoter, and we have shown that this system can provide functional gene expression throughout the brains of mice after intracardiac administration. The AAV vector can achieve comprehensive gene expression of neprilysin in the brain of young neprilysin-deficient mice, eventually decelerating Aβ accumulation and alleviating cognitive dysfunction based on a water maze test in aged APP transgenic (tg) mice. We show that the majority of the exogenous neprilysin is localized in late and early endosomes, where newly generated Aβ is concentrated, and this may be the reason why Aβ can be effectively excluded from the brain.

Results

Expression profile of neprilysin in the brain after AAV-mediated gene transfer. To deliver an AAV vector from circulating blood to the brain, we employed intracardiac administration, i.e., injection into the left ventricle of the heart, because this provides a direct route to the brain. To evaluate gene expression of neprilysin, we injected rAAV9 vectors that encode either an active or an inactive form of neprilysin in neprilysin-deficient mice[10] and examined the outcome by means of specific immunochemical staining for neprilysin. This staining generated specific signals of endogenous neprilysin in wild-type mice, but not in neprilysin-deficient mice without vector treatment (Fig. 1a,b). Expression of exogenous neprilysin after a single injection of rAAV9-NEP vector (4×10^{11} vector genome [v.g.]) into the left ventricle of the heart of neprilysin-deficient mice was spread over the limbic region on the neprilysin-null background (Fig. 1c,d), and presented a scattered distribution, but with locally intense signals. The total amount of exogenous neprilysin expression was dependent on amount of vector injected into the mice over a range of 0.5–4.0×10^{11} v.g., as far as we examined (data not shown). On the other hand, intracardiac administration of rAAV9-NEP vector did not cause prominent gene expression of neprilysin in heart, lung, kidney or liver (Supplementary Fig. 1).

Next, we examined the localization of neprilysin in the brain by confocal double immunostaining for neprilyin and several marker proteins, after the injection of rAAV9-NEP vector into neprilysin-deficient mice. Neprilysin was present in vesicular structures of NeuN-positive neurons (Fig. 2a–c), but not in glial fibrillary acidic protein (GFAP)-positive astrocytes (data not shown). In addition, we found that exogenous neprilysin is colocalized with late endosomal marker proteins Ras-related protein 7 (Rab7) (Fig. 2d–f) and Rab9 (Fig. 2g–i), and also in part with early endosomal markers Rab5 (Fig. 2j–l) and early endosome antigen 1 protein (EEA1) (Fig. 2m–o), but not with presynaptic markers SV2 (Fig. 2p–r) and syntaxin 1, secretory vesicle marker Rab3a, clathrin-coated vesicle marker clathrin heavy chain, somato-dendritic marker microtubule-associated protein 2 (MAP2), or postsynaptic marker PSD-95 (data not shown).

Functional expression of neprilysin. We investigated functional expression of neprilysin and subsequent reduction of Aβ levels in the brain. Four weeks after the single intracardiac injection of rAAV-NEP_{WT} vector into neprilysin-deficient mice, neprilysin activity in the limbic region including the neocortex and hippocampus was significantly increased compared to that after injection of rAAV-NEP_{MT} vector, although the increased level of neprilysin activity was less than 10% of the level observed in intact wild-type mice (Fig. 3). The injection of rAAV-NEP_{WT} vector into neprilysin-deficient mice significantly reduced Aβ40, Aβ42 and total Aβ levels in the limbic region compared to those in the mice injected with rAAV-NEP_{MT}. The partially compensated neprilysin activity was sufficient to achieve a 50% reduction of the elevated Aβ levels in the neprilysin-deficient mice.

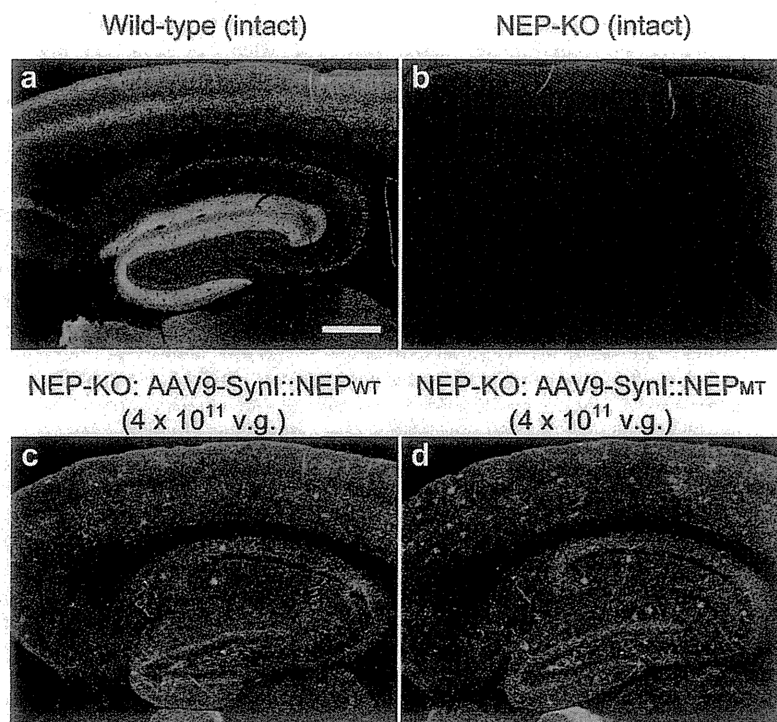
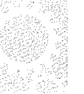


Figure 1 | Intracardiac injection of rAAV9 with SynI promoter leads to widespread gene transduction of neprilysin in the brain. Brain sections from intact wild-type mice (a), intact neprilysin-deficient mice (b), and neprilysin-deficient mice 14 days after intracardiac injection of 4×10^{11} genome vectors of rAAV9-SynI::NEP_{WT} (c) or rAAV9-SynI::NEP_{MT} (d). Scale bars, 200 μm.



marker protein | NEP | merged

NeuN (neuron)

Rab7 (late endosomes)

Rab9 (late endosomes)

Rab5 (early endosomes)

EEA1 (early endosomes)

SV2 (presynapse)

a b c d e f g h i j k l m n o p q r

Figure 2 | **Localization of the exogenous neprilysin in the brain.** Brain sections from neprilysin-knockout mice 14 days after intracardiac injection of 4×10^{11} genome vectors of rAAV9-SynI::NEP_{WT}. Exogenous neprilysin was localized in NeuN-positive neurons (a–c), and was also observed in endosomes as confirmed by colocalization with Rab7 (d–f), Rab9 (g–i), Rab5 (j–l), EEA1 (m–o), and SV2 (p–r). Scale bars, 20 μm.