Expression and characterization of a mutant recombinant blood coagulation factor VIII (rFVIII^m)

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Abbreviations: rFVIII^m, a mutant recombinant blood coagulation factor VIII; FX, factor X; FIXa, activated factor IX; APC, activated protein C; FVIII-B, B domain deleted factor VIII; vWF, von Willebrand factor; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; ECL, enhanced chemiluminescence.

Abstract

In an earlier study, a site directed mutant rFVIII (rFVIII^m, Arg³³⁶ → Gln³³⁶) expressed in baculovirus-insect cell (Sf9) system was found to sustain high level activity during incubation at 37°C for 24 h while the cofactor activity of normal plasma was declined steadily. In this study, a mutant B-domain deleted rFVIII^m, Arg³³⁶ → Gln³³⁶ expressed in baculovirus-insect cell (Sf9) system was characterized for its enzymatic and chemical properties. The expressed rFVIII^m and plasma FVIII (pFVIII) were purified by immunoaffinity column chromatography and identified by Western blot analysis. The partially purified rFVIII^m exhibited cofactor specific activity of 2.01 x 10³ units/mg protein. The molecular weight of rFVIII^m ranged between 40 to 150 kDa with a major band at 150 kDa. Treatment of both rFVIII^m and pFVIII with thrombin increased their cofactor activity in a similar pattern. Treatment of both the activated rFVIII^m and native FVIII with APC decreased their cofactor activities, however, the former exhibited a slower decrease than the latter, although no significant difference was present. rFVIII^m formed a complex with vWF, resulting in a stabilized form, and the lag period of thrombin-mediated activating was extended by vWF association. These results implicated that rFVIII^m expressed in baculovirus-insect cell system had a comparable capacity as FVIII cofactor activity and might be a good candidate for the FVIII replacement therapy for hemophilia A patients.

Keywords: rFVIII^m, baculovirus, expression, thrombin, APC, vWF.

Introduction

Factor VIII (FVIII) is a glycoprotein required for the activation of factor X (FX) by activated factor IX (FIXa) in blood coagulation cascade. Defect in FVIII activity causes hemophilia A, an X-chromosome linked severe bleeding disorder (Anatonarakis *et al.*, 1985), which afflicts 1 in 5,000 male. For the treatment of hemophilia A, replacement of deficient human blood clotting factor VIII molecules in the form of either plasma or recombinant-derived FVIII (pFVIII) preparations is necessary. In an attempt to avoid any risk of unknown hazardous viral contamination from the human donor blood (Chan and Lembach, 1991), there have been enormous efforts to produce a safe and biologically active recombinant FVIII (rFVIII) by genetic engineering technique (Pittman *et al.*, 1993; Fukutake, 2000; Sandberg *et al.*, 2001). FVIII is synthesized as a single peptide of 2,351 amino acid residues (~280 kDa) with the domain structure of A1-A2-B-A3-C1-C2, and it circulates in blood as a series of metal ion-linked heterodimers after limited proteolysis of parental FVIII molecule into heavy (A1-A2) and light (A3-C1-C2) chains by thrombin or FXa during the activation process. Activation of FVIII molecules proceeds with further proteolytic cleavage by thrombin and forms a heterotrimer composed of the A1, A2 and A3-C1-C2 subunits (FVIIIa). Inactivation of FVIII proceeds by proteolysis of A1 subunit by activated protein C (APC) which hydrolyzes subunit peptides at Arg³³⁶ in the heavy chain and at Arg¹⁷²¹ in the light chain (Vehar *et al.*, 1984). Cleavage of FVIII at Arg³³⁶ by APC produces the smaller size of the peptide. However, recent observation suggests that cleavage at Arg¹⁷¹⁹ or Arg¹⁷²¹ in the light chain may be unrelated to FVIII inactivation (Fay, 1993). Full length of FVIII cDNA has been cloned (Toole *et al.*, 1984), and used for the construction of a vector for the expression of rFVIII in mammalian cells. Production of biologically active rFVIII through eukaryotic cell culture system has been demonstrated by several investigators (Woods *et al.*, 1984; Kauffman *et al.*, 1988), however, problems such as low productivity, inhibiting antibody formation and instability of the products still remain. In the previous study, we prepared a mutant cDNA of FVIII heavy chain by site-directed mutagenesis (G¹⁰⁷⁴ → A¹⁰⁷⁴) and substitution of Arg³³⁶ with Gln³³⁶ for the production of rFVIII with high stability (Oh *et al.*, 1999). In the

Figure 1. Construction of FVIII-B^m transfer vector. FVIII-B^m cDNA, a mutant FVIII-B(G¹⁰⁷⁴ → A¹⁰⁷⁴), was inserted into *Xho*I and *Not*I sites of a baculoviral transfer vector(pAcSG2).

present study, the mutant B-domain deleted rFVIII^m expressed in baculovirus-insect cell (Sf9) was purified and its functional characteristics were determined.

Materials and Methods

Construction of FVIII Expression Vector

A mutant B-domain deleted FVIII(FVIII-B^m) cDNA was prepared from FVIII-H^m cDNA of which nucleotide No. 1074 was mutated (G → A) by site-directed mutagenesis for the substitution of Arg³³⁶ to Gln³³⁶ (Oh *et al.*, 1999). FVIII-B^m cDNA was inserted into pAcSG2 baculoviral transfer vector (Figure 1) by subcloning the cDNA into *Xho*I and *Not*I restriction enzyme recognizing sites of pAcSG2 according to the method described by Sambrook *et al.* (1989).

Generation of baculoviruses expressing rFVIII^m in insect cells

For the generation of rFVIII^m expressing recombinant baculoviruses, pAcSG2-FVIII-B^m (G¹⁰⁷⁴→A¹⁰⁷⁴) recombinant transfer vectors (500 ng) were cotransfected into Sf9 insect cells with 5 μl of pBacPAK6 DNA (BsuI digest) using Bacfectin (Clontech Laboratories, Inc., Palo Alto, CA, USA), according to the method described in the manual of Clontech Lab. Inc.. The cells cotransfected with both these vectors and viral DNA were cultured for 5 h at 27°C. At the end of culture, 1.5 ml of Grace's medium supplemented with 10% fetal bovine serum and antibiotics (Gentamicin) was added and was further cultured for 72 h at 27°C. Recombinant baculoviruses released from the cultured cells were harvested after 3 days' culture and saved for plaque assay. For the expression of rFVIII^m in baculovirus expression system, insect cells (Sf9) in 2 ml BacPAK Grace's medium were seeded in 35-mm tissue culture dishes (1 × 10⁶ cells/

dish) and incubated at 27°C for 4 h. The cultured medium was removed from the cells, and 2 ml of fresh BacPAK Grace's basic medium was added to the dishes and the cells were further incubated for 30 min. Baculoviruses harboring the FVIII-B^m were transfected into Sf9 insect cells grown to about 80% confluence (2 × 10⁶ cells/60 mm dish). After 3 days, the cells and culture medium were collected and saved.

Purification of rFVIII^m

rFVIII^m expressed and released into the culture medium of insect cells were harvested and purified by immunoaffinity column chromatography. The media were mixed with equal volume of FVIII buffer (20 mM imidazole/0.15 M NaCl/0.1 M L-lysine/0.02% NaN₃, pH 6.8), and added to the immunoaffinity gel prepared by conjugation of anti-FVIII:C-Ag antibody to CNBr-activated Sepharose-4B gel. The gel was washed with 10 column volumes of 0.1 M Tris-HCl buffer (pH 8.0), and rFVIII^m was eluted with 0.1 M glycine buffer (pH 2.5) into collection tubes containing 1/10 vol of 1M Tris-HCl buffer (pH 8.0). Reconstitution of biologically active rFVIII^m was performed by complexing rFVIII^m (200 nM) with FVIII-free plasma (Sigma Chem Co., St Louis, MO, USA). pFVIII was purified by immunoaffinity column chromatography using the gel prepared by conjugation of anti-FVIII:C-Ag antibody to CNBr-activated Sepharose-4B gel.

FVIII activity Assay

FVIII activity was assayed by the method of Carlebjok *et al.* (1987) using Coamatic FVIII assay kit (Chromogenix AB, Stockholm, Sweden). The principle of the method is the measurement of the FVIII-dependent generation of FXa from FX in the presence of factor FIXa. Diluted sample or plasma (50 μl) was added to the well of microplate and prewarmed to 37°C for 4 min, followed by addition of 50 μl of factor reagent (37°C). The reaction mixture was incubated for another 4 min at 37°C and 50 μl of substrates (S-2765 + I-2581) were added. After 4 min of incubation (37°C) for 2 min, the reaction was stopped by addition of 50 μl of 20% acetic acid and read the absorbance at 405 nm. Factor VIII activity was calculated from the standard curve constructed by standard human plasma supplied in the kit. Total protein content was determined by Bradford method (Bradford, 1976) and quantitative analysis of rFVIII^m was performed by sandwich type ELISA method (Johnston and Thorpe, 1987).

SDS-PAGE and Western blot immunodetection

Immunoaffinity purified rFVIII^m and pFVIII were subjected to SDS polyacrylamide gel (10%) electrophoresis according to the method of Laemmli (1970), and proteins in the gel were electrotransferred onto nitrocellulose membrane for 4 h at 300 mA in transfer chamber (Hoefer Scientific Instrument,

San Francisco, CA, USA) and blotted. The blotted membrane was then treated with PBS containing 5% bovine skim milk by incubating for 20 min to block nonspecific protein binding sites. Identification of rFVIII^m and pFVIII peptides was performed by ECL-mediated western blot immuno-detection kit (Amersham, Buckinghamshire, UK), using a monoclonal anti-FVIIIc antibody (Calbiochem, La Jolla, CA, USA) as primary antibody and peroxidase-conjugated anti-mouse IgG antibody as secondary antibody, according to the method of Burnette (1981).

Treatment of thrombin and APC

rFVIII^m and pFVIII (1 NIH unit per 500 units of rFVIII^m or pFVIII in 1 ml) were treated with human α -thrombin (Sigma Chemical Co.) and the FVIII activity was measured at one min interval for 10 min, and 100 units of rFVIII^m or pFVIII were treated with 1ml of APC (AccucolorTM protein C; Sigma) and their cofactor activity of them were measured by the same procedure as in the thrombin treatment.

Association of rFVIII^m with vWF

vWF was prepared from fresh human plasma by anti-vWF antibody-conjugated immunoaffinity gel (CNBr-activated Sepharose 4B) column chromatography. vWF bound to the gel was eluted with glycine buffer (0.1 M, pH 2.5). vWF (50 g) dissolved in FVIII buffer (20 mM imidazole/0.15 M NaCl/0.1 M L-lysine/0.02% NaN₃, pH 6.8) was mixed with rFVIII^m (2 g), and they were incubated for 20 min at 37°C and their association was tested by the shift of original protein (rFVIII^m) peak to a peak of higher molecular weight, when the complex was subjected to gel filtration (Sephacryl S-300; Amersham). The shift of rFVIII^m peak was confirmed by measuring the FVIII cofactor activity.

Results

Production of baculoviruses expressing rFVIII^m

Insect cells cotransfected with recombinant transfer vectors carrying the FVIII-B^m (pAcSG2-FVIII-B^m) and *Bsu*I-digested linearized baculovirus DNA (pBacPAK6) were cultured for 72 h, and viruses released into the culture media were harvested by ultracentrifugation (100,000 g, 1 h) and saved for the plaque assay. The plaque assay was performed after serial dilution of viral stock solution and viral titer of 3.1×10^7 pfu/ml was determined. The presence of FVIII-B^m sequence was confirmed by PCR amplification of the entire sequence of FVIII-B (4.9 kbp) present in the viral plaque.

Identification and quantitative determination of rFVIII^m

Figure 2. Western blot immunodetection of pFVIII (A panel) and rFVIII^m (B panel). rFVIII^m released from insect cells (Sf9) transfected with baculoviruses harboring FVIII-B^m cDNA and pFVIII in normal human blood were partially purified by immunoaffinity column chromatography. pFVIII and rFVIII^m were then subjected to SDS polyacrylamide gel (10%) electrophoresis (left panels of A and B). Proteins in each gel were electrotransferred onto nitrocellulose membrane, blotted and immunodetected by ECL kit using anti-FVIII:C monoclonal antibody as a primary antibody and horse radish peroxidase-conjugated anti-mouse IgG as a secondary antibody (right panel of A and B). M: molecular weight standards; A panel (pFVIII): lane 1, 5 g pFVIII; lane 2, 20 g pFVIII; lane 3, 20 g pFVIII treated with EDTA (1 mM). B panel: lane 1 and 2, 20 g of rFVIII^m; lane 3 and 4, 20 g of rFVIII^m treated with EDTA (1 mM). sc: single chain of pFVIII or rFVIII^m; lc: light chain of pFVIII or rFVIII^m.

The culture media of insect cells (Sf9) transfected with the rFVIII^m-expressing recombinant baculoviruses were collected and mixed with FVIII buffer. rFVIII^m was partially purified by anti-FVIII:C-Ag conjugated Sepharose-4B immunoaffinity column chromatography and was identified by ECL-associated western blot immunodetection system (Figure 2B). The cofactor activity of rFVIII^m in the culture media was 8.0 IU/ml, and the specific activity of the immunoaffinity purified rFVIII^m was 2,013 IU/mg protein.

Effects of thrombin and APC on FVIII cofactor activity

Treatment of rFVIII^m or pFVIII with -thrombin increased their cofactor activities for the first 2 min and rapidly decreased thereafter (Figure 3). The patterns of activity change by the treatment of rFVIII^m and pFVIII with thrombin were similar, indicating that the processing of both peptides to their active forms required thrombin proteolysis for a short period of time (2 min). The thrombin-mediated degradation of peptides (rFVIII^m, pFVIII) was also shown by SDS-PAGE (Figure 2A).

Figure 3. Activation of rFVIII^m and pFVIII by thrombin. Five hundred units of pFVIII(●) and rFVIII^m(■) were treated with thrombin(1 unit/ml) at 37°C. Samples were taken at various time points indicated, and FVIII activities were determined.

Figure 4. Changes of FVIII activities of pFVIII and rFVIII^m by APC treatment. Each 100 units of pFVIII(●) and rFVIII^m(■) were treated with each 1ml of protein C substrate(AccucolorTM protein C) and protein C activator, followed by incubation at 37°C. FVIII activities in samples taken at various time points indicated were measured. No significant differences between pFVIII and rFVIII^m at each time point were observed($p>0.05$).

Treatment of the thrombin-activated pFVIII with APC resulted in gradual decline of its cofactor activity during 10 min of experimental period, however, there was a lag period for the first 4 min of incubation (Figure 4). Treatment of rFVIII^m with APC also slowly decreased their cofactor activities although the activity changes is little less than the pFVIII during the first 4 min of incubation (Figure 4). Whether such loss of cofactor activity of rFVIII^m is generated by cleavage of other sites by APC is not clear.

Interaction of rFVIII with vWF

Addition of vWF to rFVIII^m (50:1 molar ratio) resulted in the formation of vWF-rFVIII^m complex, evidenced by the

Figure 5. Elution profiles of gel filtration of rFVIII^m added to vWF. rFVIII^m (50ug) was applied onto Sephacryl S-300 column (1.6 x 30 cm) and eluted with FVIII buffer (20 mM Hepes, pH 7.2, 40 mM CaCl₂ and 1.0 mM 2-mercaptoethanol) at a flow rate of 2 ml/min (upper panel). vWF (2 mg) was added to rFVIII^m (50 ug) and incubated at 37°C for 20 min. The reaction mixture was applied onto the same column and eluted with the FVIII buffer at the same conditions. FVIII activity (◆) in each eluted fraction was measured. vWF (■) in each fraction was measured by ELISA method using anti-vWF monoclonal antibody as a primary antibody and horse radish peroxidase-conjugated anti-mouse IgG antibody as a secondary antibody.

Figure 6. Effect of vWF on FVIII activity of rFVIII^m treated with thrombin. vWF(5 mg/0.5 ml) was added to rFVIII^m(50 g/0.5 ml) and the mixture was incubated for 20 min at 37°C, and then treated with thrombin(1 NIH unit). Samples(50 µl each) were taken at various time points and analysed for their FVIII activity. pFVIII without vWF: ●; rFVIII^m without vWF: ◆; rFVIII^m with vWF: ■.

appearance of high molecular weight protein complex on the gel filtration profile (Figure 5). vWF was added to rFVIII^m and allowed to incubate for 20 min at 37°C formed a large molecular weight complex that migrated near to void volume of gel filtration column and contained all the FVIII activity. Association of vWF with rFVIII^m resulted in prolongation of lag period of thrombin-mediated activation of rFVIII^m. Without vWF, both rFVIII^m and pFVIII were rapidly activated by thrombin in 2~4 min,

while vWF-associated rFVIII^m generated the cofactor activity slowly after 4 min of incubation (Figure 6).

Discussion

In this study, an APC-resistant B-domain deleted recombinant FVIII protein (rFVIII^m) expressed in baculovirus-insect cell system was examined for its biological characteristics.

The rFVIII^m protein expressed in insect cells and pFVIII as its counterpart were partially purified by immunoaffinity column chromatography. Cofactor activities of rFVIII^m and pFVIII were 2,013 and 2,485 IU/mg protein, respectively, which are somewhat lower than the value of rFVIII (5,000 IU/mg) reported by Fay *et al.* (1986). The difference might have been due to the different assay method of FVIII activity, i.e. chromogenic assay vs clotting assay. The presently described result indicates that rFVIII^m produced in insect cells are equally functional although they are smaller in size than their counterparts produced in mammalian cells. Glycoproteins in insect cells contain high mannose-type small oligosaccharides in the molecules which may result in different size and charge of protein molecules (Luckow, 1991). The molecular sizes of pFVIII peptides were heterogeneous ranging between 40 and 200 kDa, and rFVIII^m had a major peptide band at about 150 kDa region, indicating that only a small fraction of rFVIII^m was degraded in the present study (Figure 2). The appearance of rFVIII^m with various sizes was thought to be due to the partial processing or degradation of the single chain polypeptide. The activation process of FVIII has been known to involve limited proteolytic cleavage of single chain FVIII by specific proteases, thrombin and FXa (Harris *et al.*, 1986). Treatment of both rFVIII^m and pFVIII with thrombin increased their cofactor activity with a similar changing pattern, suggesting that both proteins have been processed by thrombin cleavage into their active forms.

It has been known that APC inactivates the activated FVIII by cleaving at Arg³³⁶ of the heavy chain of FVIII (Eaton *et al.*, 1986; Fuss, 1991). Treatment of rFVIII^m and pFVIII with APC slowly decreased their cofactor activities and the former activity changed little less than the latter during the first 4 min of incubation (Figure 4). The prolonged activity of rFVIII^m for the first 4 min by APC treatment might have been resulted from the mutation of Arg³³⁶, the site of degradation by APC during the inactivation process of pFVIII. The resistance of rFVIII^m to APC-mediated inactivation may have a sparing effects of FVIII activity when infused into hemophilia A patients.

It has been reported that APC resistance is frequently observed in thrombophilia patients with abnormal FV accumulation, however, FVIII accumulation in these patients has not been observed (Ingerslev *et al.*, 1996).

Therefore, the application of rFVIII^m to hemophilia A patient seems to be no serious risk factor for thrombophilia.

In the present study (Figure 5), strong association of vWF with rFVIII^m to form a high molecular weight protein complex has been observed, measured by gel filtration. vWF added to rFVIII^m effectively blocked its cofactor activity and the addition of thrombin reduced the lag period of activation, as shown in Figure 6. These results suggest that vWF was bound to rFVIII^m and induced the molecules into non-activated state prior to the thrombin-mediated activation. Therefore, it is reasonable to speculate that the addition of vWF to rFVIII^m may stabilize the molecules in circulating blood.

The beneficial role of vWF in FVIII stabilization has been demonstrated earlier by Morfini *et al.* (1992). The lower clearance rate and the longer half-life of rFVIII in hemophilia A patients (14.6 h) than those in von Willebrand disease patients (4.5 h) have been observed by these investigators.

The present results demonstrate rFVIII^m to be fully functional and comparable with pFVIII in physiologically important aspects such as activation by thrombin, inactivation by APC and vWF-associated stabilization.

In summary, physiologically functional rFVIII^m with high specific activity has been produced in baculovirus-insect cell system, however, further studies on thrombophilic risk due to APC resistance and possible inhibitor formation of the product are required before its clinical application.

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