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Enhanced plasma factor VIII activity in mice via cysteine mutation using dual vectors

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Hemophilia A is caused by a genetic mutation in coagulation factor VIII (FVIII) gene and gene therapy is considered to be a promising strategy for its treatment. We recently demonstrated that co-delivery of two vectors expressing M662C mutated heavy and D1828C mutated light chain genes of B-domain-deleted coagulation factor VIII (BDD-FVIII) leads to inter-chain disulfide cross-linking and improved heavy chain secretion *in vitro*. In this study, co-injection of both M662C and D1828C mutated BDD-FVIII gene expression vectors into mice resulted in increased heavy chain secretion and coagulation activity in plasma *in vivo*. Approximately (239±56) ng mL^{-1} above endogenous levels of transgenic FVIII heavy chain was found in mouse plasma using a chain-specific ELISA. For FVIII coagulation activity, approximately (1.09±0.25) IU mL^{-1} above endogenous levels were detected in co-injected transgenic mouse plasma using a chromogenic assay. These data demonstrate that inter-chain disulfide bonds likely increase heavy chain secretion and coagulation activity in the plasma of transgenic mice with an improved efficacy of a dual-vector delivery of BDD-FVIII gene. These findings support our ongoing efforts to develop a gene therapy for hemophilia A treatment using dual-AAV vectors.

coagulation factor VIII, dual-vector gene delivery, inter-chain disulfide bonding, heavy chain secretion, coagulation activity

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The current treatment for hemophilia A is protein replacement by using plasma concentrates and recombinant coagulation factor VIII (FVIII), but this approach is limited because of the high cost and other drawbacks such as the risk of transmission of blood-borne infectious diseases. Since hemophilia A is a disease caused by a single gene deficiency, gene therapy is considered to be a particularly suitable and promising treatment [1]. Adeno-associated virus (AAV) vectors are ideal gene delivery vehicles for the stable expression of transgenes, and their non-pathogenic, replication-defective characteristics, but proved difficult for the transfer of the FVIII gene because of their capacity constraint [2]. The separate delivery of FVIII heavy chain (HC) and light chain (LC) genes by a dual-vector is one strategy to circumvent the packaging limit of AAV vectors, but transgenic efficacy is adversely affected because of the inefficient secretion of the HC [3,4]. In addition, it was previously demonstrated that von Willebrand factor (vWF) is needed for the assembly of HC and LC to form the stable functional FVIII heterodimer, and helpful for its secretion in Chinese hamster ovary cells [5].

We previously reported that the FVIII LC promotes HC secretion *in cis*, and HC L303E/F309S mutations reduce its affinity to the endoplasmic reticulum (ER) protein molecular chaperone, BiP. This in turn enhances the release of spliced FVIII protein and secretion using protein *trans*-splicing technology based dual vectors to transfer the FVIII gene [6]. Studies performed by Gale *et al*. [7] have shown

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that the M662C mutation in the HC and the D1828C mutation in the LC can form a disulfide bond, and upon activation by thrombin hydrolysis, the activity of the active FVIII (FVIIIa) product was unchanged with a prolonged half-life and improved affinity for vWF. Our recent work using a dual vector system to transduce cultured cells with a M662C/D1828C mutant B-domain-deleted FVIII (BDD-FVIII) gene showed an inter-chain disulfide cross-linking between intracellularlly co-expressed HC and LC that significantly improved HC secretion [8]. In this study, a dual vector strategy was used to deliver a M662C/D1828C mutant BDD-FVIII gene into mice and observed increased plasma levels of HC secretion and coagulation activity. These studies provide an experimental basis for the ongoing study of hemophilia A gene therapy.

1 Materials and methods

1.1 Materials

Human BDD-FVIII expression vector, pCMV-F8, and individual *Ssp* DnaB intein-fused HC and LC expression vectors, pCMV-HCIntN and pCMV-IntCLC, respectively, were constructed and described in our previous study [9]. C57BL/6 mice were obtained from Vital River Laboratories (VRL, Beijing, China).

High fidelity DNA polymerase *Pfu* was purchased from Stratagene (Santa Clara, CA, USA). The DNA ligase kit was purchased from New England Biolabs (Ipswich, MA, USA). The Gel Extraction kit, PCR purification kit, Spin Miniprep kit, and HiSpeed Plasmid Midi Kit were obtained from Qiagen (Dusseldorf, Germany). Recombinant human reference factor VIII was purchased from BioChain (Hayward, CA, USA). The FVIII HC-specific monoclonal antibody (ESH5) and LC-specific monoclonal antibody (ESH8) were obtained from American Diagnostica (Greenwich, CT, USA). The horseradish peroxidase (HRP)-conjugated rabbit anti-human FVIII polyclonal antibody was purchased from Novus (Littleton, CO, USA). Normal pooled human reference plasma and FVIII-deficient plasma were obtained from George King Biomedical (Overland Park, KA, USA). The COATEST SP FVIII functional analysis kit was purchased from Chromogenix (Milan, Italy).

1.2 Vector construction

To delete the intein coding sequences fused to BDD-FVIII HC and LC in plasmids pCMV-HCIntN and pCMV-IntCLC, a *Pfu* Turbo DNA polymerase catalyzed inverse polymerase chain reaction (PCR) was performed with primers 5′-CCGTTTAAACCCGCTGATCAG-3′ (forward) and 5′-CTGAAGAGTAGTACGAGTTA TTTC-3′ (reverse) using pCMV-HCIntN as a template, and primers 5′-TCAGATCAAGAGGAAATTGACTATG-3′ (forward) and 5′-ACTAAAGCAGAATCGCAAAA GGC-3′ (reverse) using CMV-IntCLC as a template. Both linear PCR products were self-ligated respectively to obtain the HC expression vector, pCMV-HC, and the LC expression vector, pCMV-LC. Both plasmids were then used as templates for inverse PCR to introduce M662C point mutation in pCMV-HC using 5′-TGTGTCTATGAAGACACACTCACC-3′ (forward) and 5′-TTTGTGTTTGAAGGTATATCCAG-3′ (reverse), and D1828C point mutation in pCMV-LC with primer pairs 5′-TGTGAGTTTGACTGCAAAGCCTG-3′ (forward) and 5′-TTTAGTGGGTGCCATATGATG-3′ (reverse). PCR products were self-ligated to yield the circular M662C mutated HC expression vector, pCMV-C662HC, and D1828C mutated LC expression vector, pCMV-C1828LC.

1.3 Animal experiments

C57BL/6 mice were injected with 200 μg of column-purified pCMV-C662HC and pCMV-C1828LC plasmids in 2 mL saline via the portal vein, as described by Burton *et al*. [3]. At 48 h after injection of vectors, blood samples were collected by tail clipping in an anticoagulant at a final concentration of 0.38% (w/v) sodium citrate. Blood samples were centrifuged at 2000×*g* for 10 min at 4°C in a microcentrifuge and plasma was collected and analyzed for HC and LC antigen and coagulation activity.

1.4 Quantitative analysis of FVIII antigen in plasma

Chain-specific enzyme-linked immunosorbent assays (ELISAs) were performed to determine BDD-FVIII HC and LC concentration in plasma, as previously described [10]. Human recombinant FVIII protein was used as a standard and diluted in plasma blocking buffer in the range of 10–320 ng mL^{-1}. For ELISA, 96-well plates were coated with 2 μg mL^{-1} human FVIII HC-specific monoclonal antibody, ESH5, or LC-specific monoclonal antibody, ESH8, overnight at 4°C followed by washing and then blocked with blocking buffer for 2 h at 37°C. Plates were washed and diluted standard or plasma samples were applied and incubated at 37°C for 1 h. After washing, HRP-conjugated FVIII polyclonal antibody was added for continuing incubation for 1 h at 37°C. Plates were washed and the o-phenylenediamine dihydrochloride (OPD) substrate was added and incubated for 30 min at 37°C. The reaction was stopped by the addition of 2 mol L^{-1} H_2SO_4 and the plates were read at a wavelength of 490 nm. A standard curve was plotted according to the absorbance value versus concentration of the diluted standards, and transgenic human HC and LC concentrations in plasma samples were deduced from the standard curve.

1.5 Analysis of FVIII coagulation activity

A Coatest chromogenic assay was used to detect transgene-produced human FVIII coagulation activity in

mouse plasma, as described by Burton *et al*. [3], with some modifications. Total FVIII activity detected in transgenic mouse plasma comprised both mouse and human-derived activity. Normal pooled human plasma was used as a standard and one unit activity was defined as 200 ng FVIII in 1 mL of this plasma. The standard was serially diluted in FVIII-deficient human plasma ranging from 1 (undiluted) to 0.0313 U mL^{-1}. For the assays, 10 μL of each diluted standard was incubated at 37°C for 15 min with or without 2 μL ESH8 antibody (1 mg mL^{-1}). Similarly, 10 μL of diluted mice plasma samples in FVIII-deficient plasma was incubated at 37°C for 15 min without ESH8, or with 2 μL ESH8 to deplete human FVIII activity. Standards with and without treatment were prepared according to the manufacturer's instructions and read at 405 nm. A standard curve was plotted according to the difference in absorbance between untreated and ESH8-treated standards versus corresponding FVIII activity. The absorbance values of plasma samples were also measured at 405 nm and FVIII activity was extrapolated using the standard curve. The difference of FVIII activity between the ESH8-untreated sample and ESH8-treated sample represented the human FVIII activity produced by the transgene.

1.6 Statistical analysis

Data are expressed as mean±standard deviations and statistical comparisons were based on student's *t*-test. A *P*-value of <0.05 was considered significant.

2 Results

2.1 Construction of BDD-FVIII heavy and light chain mutants

FVIII has a domain structure order from N- to C-terminus arranged as A1-A2-B-A3-C1-C2 and BDD-FVIII was produced by deleting most of the nucleotides in the B domain from the I761 codon to the N1639 codon. By truncating BDD-FVIII before codon S1657, the resulting HC and LC coding sequences were independently inserted into plasmid vector pcDNA3.1(+) to derive expression vectors for HC and LC, pCMV-HC and pCMV-LC. A Cys mutation of M662 in HC and D1828 in LC was made using PCR-mediated site-directed mutagenesis using pCMV-HC and pCMV-LC as templates to produce Cys-mutated HC and LC expression vectors, pCMV-C662HC and pCMV-C1828LC, respectively. All constructs are showed in Figure 1.

2.2 Plasma HC secretion in mice administered dual-vector BDD-FVIII

To examine whether inter-chain disulfide linking facilitated plasma HC secretion in mice, a chain-specific ELISA was used to measure the amount of human BDD-FVIII HC in plasma and was determined to be (239±56) ng mL^{-1}, much higher in comparison to the wild-type human HC secretion in control mice ((84±25) ng mL^{-1}) (Figure 2). In the plasma of pCMV-C662HC or pCMV-HC alone injected mice, both showed extremely low HC secretion ((18±9) and (12±7) ng mL^{-1}, respectively). The plasma secretion of HC in mice injected with the BDD-FVIII transgene was shown to be (416±52) ng mL^{-1}. These data demonstrated that wild-type HC is inefficiently secreted, but in the presence of LC, HC secretion is significantly increased regardless of disulfide bond formation, indicating that LC could improve HC secretion both in *cis* and *trans*.

As shown in Figure 3, the secretion of LC in plasma was determined by chain-specific ELISA and showed similar plasma levels for mice co-injected with pCMV-C662HC and pCMV-C1828LC or injected with pCMV-C1828LC alone ((809±72) vs. (875±107) ng mL^{-1}). Mice co-injected with pCMV-HC and pCMV-LC or injected with pCMV-LC also displayed similar LC secretion ((779±125) vs. (835±

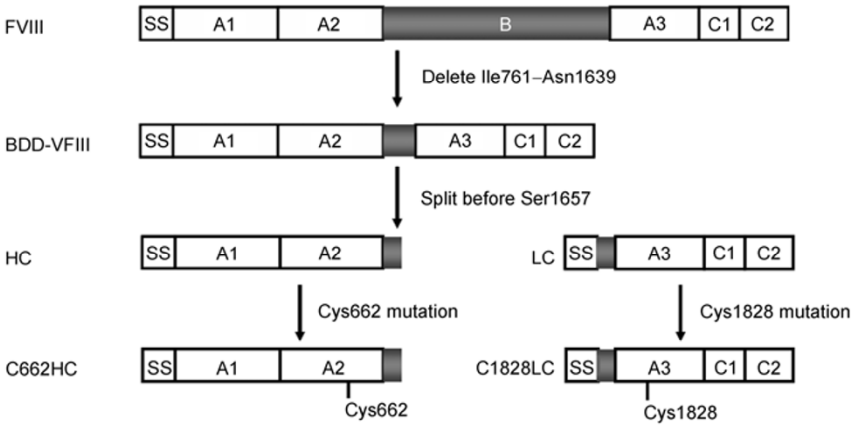

Figure 1 Schematic representation of factor VIII and its derivatives. The FVIII has a domain organization of A1-A2-B-A3-C1-C2. BDD-F VIII is derived from FVIII by deleting the B-domain from Ile761 to Asn1639. HC and LC represent the heavy and light chains by cutting BDD-FVIII before Ser1657. C662HC and C1828LC are Cys mutants of HC at M662 and LC at D1828, respectively. SS, signal sequence of FVIII.

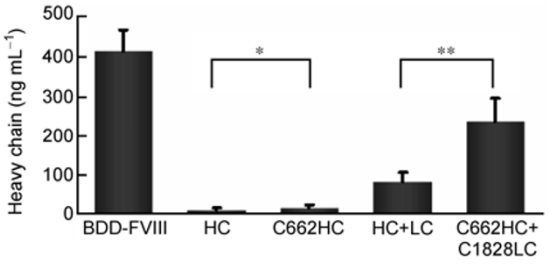

Figure 2 Inter-chain disulfide cross-linking enhances plasma secretion of heavy chain. *, P>0.05; **, P<0.01.

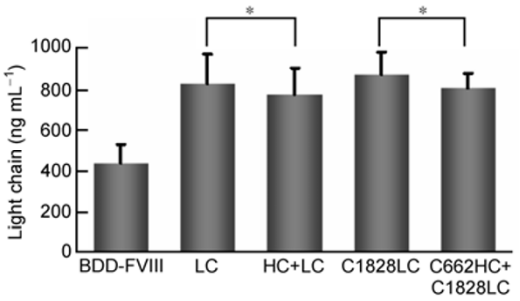

Figure 3 Inter-chain disulfide cross-linking has no effect on plasma secretion of light chain. *, P>0.05.

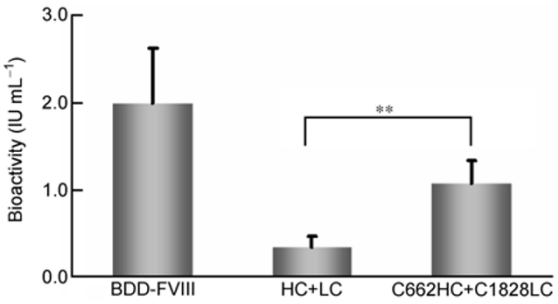

Figure 4 Inter-chain disulfide cross-linking improves plasma bioactivity. **, P<0.01.

139) ng mL^{-1}). These findings suggested that LC secretion is highly efficient compared with HC. The amount of LC secreted in mice receiving the BDD-FVIII gene is tightly related to HC because both chains have a 1∶1 stoichiometric relationship in secreted BDD-FVIII. The ratio of HC to LC in the plasma of mice co-injected with Cys-mutated HC and LC that is capable of forming inter-chain disulfide bonds was 1∶3.4 but the ratio was 1∶9.3 in plasma without engineered inter-chain disulfide bonds, indicating that the inter-chain disulfide bonds could partially relieve the chain imbalance by promoting HC secretion.

2.3 Inter-chain bonding increases plasma coagulation activity

Plasma collected from mice injected with the dual-vector were analyzed for FVIII coagulation activity using Coatest method. Coagulation activity found in plasma from mice co-administered Cys-mutated HC and LC genes ((1.09±0.25) IU mL^{-1}) was considerably higher than for control mice ((0.36±0.12) IU mL^{-1}) but less than for those that received the BDD-FVIII gene ((2.01±0.63) IU mL^{-1}). This demonstrated that mutated Cys residues in HC and LC can improve plasma coagulation activity in mice administered the dual-vector through inter-chain disulfide bonding. Assuming that 100% of HC secreted in the plasma from BDD-FVIII gene injected mice was functionally active, mice receiving Cys mutated HC and LC vectors separately had 91% of HC contributed to coagulation activity, while being 85% in control mice. These findings suggest that inter-chain disulfide bonds improve the assembly of functional heterodimer BDD-FVIII from separately expressed HC and LC proteins.

3 Discussion

Previous studies have demonstrated the ability of AAV vectors to express sustained levels of coagulation factor IX either by liver- or skeletal muscle-targeted gene delivery approaches [11,12]. Thus, AAV vectors provide a promising gene delivering system for the treatment of hemophilia A by gene therapy. However, the major obstacle for the use of AAV vectors for hemophilia A gene therapy lies with the large FVIII gene that exceeds the packaging capacity. It has been revealed that the majority of the FVIII protein expressed from FVIII gene transfected cells is hydrolyzed to heavy and light chains in the Golgi apparatus during post-translational processing and then secreted as a functional FVIII heterodimer [13]. Based on this understanding, a dual-vector strategy for FVIII gene split transfer was developed for the study of hemophilia A gene therapy [3,4].

Although FVIII and another coagulation factor, factor V (FV), share 40% sequence homology in the A and C domains, the full-length FVIII protein shows less efficient secretion than FV [14]. The molecular mechanism responsible for FVIII inefficient secretion, is mainly caused by the association of the A1 domain in the HC with several protein chaperones, including the immunoglobin-binding protein (BiP) in the lumen of the endoplasmic reticulum (ER) during intracellular processing, and thus the release of FVIII from BiP is dependent on a high concentration of intracellular ATP [15]. In addition, *N*-linked oligosaccharides in the B-domain of the FVIII heavy chain can also facilitate FVIII secretion by participating in the folding interactions within the ER as well as potentially helping facilitate ER-Golgi transport [16]. Our previous work has shown that dual-vector BDD-FVIII gene transfected cells have similar expression levels of intracellular HC and LC [10]. Therefore, dual-vector mediated FVIII gene transfer can overcome the capacity constraints of AAV vectors, but the inefficient secretion of the HC leads to its excessive intracellu-

lar deposition, which not only reduces the efficacy of gene therapy, but may also destabilize host cells and possibly induce apoptosis [17]. Moreover, the excessively secreted non-functional LC may also cause host immune responses.

With introduction of Cys mutations in both heavy and light chains that are capable of forming inter-chain disulfide bonds, the secretion of the FVIII HC was significantly improved by a dual-vector containing the BDD-FVIII gene in transfected cells [8]. Using an *in vivo* dual-vector mediated liver-targeting gene delivery in mice, the present study showed that Cys mutations promoted the formation of an inter-chain covalent disulfide bond between HC and LC, and improved the secretion of the HC in mouse plasma. In this case, increased HC secretion levels lead to the antigen ratio of HC to LC being approximately 1∶3.4, while this ratio was 1∶9.3 in the plasma of wild type BDD-FVIII gene dual-vector injected mice showing an obvious lower HC secretion. It suggested that the chain imbalance could be partially relieved in the presence of Cys mutations. With the increase in the amount of HC secretion, BDD-FVIII heterodimer was formed more efficiently from matched HC and LC, which not only improves the clotting activity of the plasma, but also reduces the amount of non-functional light chain. Meanwhile, decreased cellular deposition of heavy chain may help reduce cellular stress response in transduced cells.

The analysis of LC secretion in the plasma revealed that the LC was highly secreted and not affected in LC gene administered alone, co-administered with the HC gene, or in the presence of inter-chain disulfide. However, the secretion of the HC was inefficient but improved significantly when LC was present, much higher especially when inter-chain disulfide bond formed. Studies by Chen *et al.* [18] have shown that the secretion of LC facilitates HC secretion through its N-terminal acidic region 3 (ar3), where a mutant HC with an additional ar3 sequences at the C-terminus exhibits three- to five-fold higher secretion *in vitro*. In addition, ar3 has a role in mediating interaction of FVIII with vWF [19], but unlikely to be related to this function. When an inter-chain disulfide bond is formed, the secretion of HC increased likely driven by covalently bonded LC. In our recent studies using protein *trans*-splicing mediated dual-vector FVIII gene co-transfection in cultured cells, a *cis*-promoting effect of LC covalently linked to the HC by peptide bond on the HC secretion was found [6,10].

The secreted levels of heterodimer FVIII determines plasma clotting activity. Although *in vitro* studies have shown that a heterodimer FVIII can be formed from isolated heavy and light chains at high concentrations and in the presence of Mn^{2+} or Ca^{2+} ions [20], the heterodimerization of separately expressed heavy and light chains may only occur intracellularly. In addition, vWF can act to promote the stable assembly of FVIII subunits at the site of secretion, but physiological vWF is mainly produced in vascular endothelial cells and megakaryocyte cells, but not in hepatocytes [21]. As functional heterodimeric FVIII is produced only from cells with coexpressed HC and LC, therefore, the efficacy of the dual-vector strategy depends on the efficiency of the co-transfection of both vectors. The proportion of active HC in total plasma HC may reflect the efficiency of forming functional heterodimer in dual-vector strategy. In comparison to 85% of plasma HC being functionally active in the absence of inter-chain disulfide bonds, 91% HC was contributed to the functional heterodimer generating a higher plasma clotting activity in the presence of disulfide. These findings are consistent with an observation by Gale *et al.* [7] that also showed that Cys mutation does not affect the activity of the heterodimer. Recently, Sabatino *et al.* [22] reported that the amount of FVIII activity in plasma of dual-vector administered hemophilia A dog model correlated closely with the amount of HC in the plasma, suggesting a higher co-transfection efficiency.

In summary, we have demonstrated the feasibility of the introduction of Cys mutations that promote inter-chain disulfide cross-linking for improving the dual-vector strategy of FVIII gene delivery. This approach was proven to facilitate the assembly of the heavy and light chains into functional heterodimeric FVIII leading to an enhancing secretion of plasma HC and coagulation activity *in vivo*. The present study provides an experimental basis for ongoing investigation of the use of dual-AAV vectors for the treatment of hemophilia A in animal models.

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