

Mutagenesis in sequence encoding of human factor VII for gene therapy of hemophilia

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ABSTRACT

Background: Current treatment of hemophilia which is one of the most common bleeding disorders, involves replacement therapy using concentrates of FVIII and FIX. However, these concentrates have been associated with viral infections and thromboembolic complications and development of antibodies.

The use of recombinant human factor VII (rhFVII) is effective for the treatment of patients with hemophilia A or B, who develop antibodies (referred as inhibitors) against replacement therapy, because it induces coagulation independent of FVIII and FIX. However, its short half-life and high cost have limited its use. One potential solution to this problem may be the use of FVIIa gene transfer, which would attain continuing therapeutic levels of expression from a single injection. The aim of this study was to engineer a novel hFVII (human FVII) gene containing a cleavage site for the intracellular protease and furin, by PCR mutagenesis.

Methods: The sequence encoding light and heavy chains of hFVII, were amplified by using hFVII/pTZ57R and specific primers, separately. The PCR products were cloned in pTZ57R vector.

Results and discussion: Cloning was confirmed by restriction analysis or PCR amplification using specific primers and plasmid universal primers. Mutagenesis of sequence encoding light and heavy chain was confirmed by restriction enzyme.

Conclusion: In the present study, it was provided recombinant plasmids based on mutant form of DNA encoding light and heavy chains. Joining mutant form of DNA encoding light chain with mutant heavy chain led to a new variant of hFVII. This variant can be activated by furin and an increase in the proportion of activated form of FVII. This mutant form of hFVII may be used for gene therapy of hemophilia.

Keywords: Hemophilia, Recombinant human factor VII, Furin, Mutagenesis

INTRODUCTION

Hemophilia is one of the most common inherited disorders of blood coagulation which is caused by the lack of the activity of factor VIII (hemophilia A) or factor IX (hemophilia B) (1). Current treatment for hemophilia involves replacement therapy using blood-derived or concentrates of FVIII and FIX, which may be administered as it is required or on a prophylactic schedule. However, continuous injections of blood factors have been associated with viral infections and thromboembolic problems (2). The most complication of replacement therapy, is development of inhibitors against the infused protein, which occurs in about 3-5% and about 30-40% of patients with severe hemophilia B and A respectively (3,4).

Recently, recombinant activated human factor VII (rhFVII) (Novo Seven; Novo Nordisk, Bagsvaerd, Denmark) has been suggested as a safe and

effective option in the treatment and prophylaxis of bleeding complications in patients with antibodies against FVIII or FIX, because all hemophiliacs have immunological tolerance to it and the risk of serious side effects, including thrombosis or DIC, have been very low (5,6). Also, at higher concentrations, rFVIIa is able to activate FX to FXa (despite impaired FVII and FIX-dependent amplification of FX activation) independent of TF through to the binding activated platelet surface (7). The cost of rhFVII is estimated \$1 US/μg and its short half-life and rapid clearance necessitates frequent infusions and gives rise to high treatment costs (8).

One potential solution to this problem may be the use of FVIIa gene transfer, which results in long-lasting therapeutic levels of expression from a single injection. **Gene transfer of FVIIa circumvents the risk of inhibitor formation and eliminates problems**

arising from a short half-life, since it confers long-term expression (9).

In order to transfer a gene it was required to engineer a hFVII variant that would be secreted directly as hFVIIa. Insertion of a recognition sequence for PACE-furin (an intracellular protease) has been considered for activation of hFVII. This approach has the potential of homeostasis in all hemophilia patients-with or without inhibitors-against coagulation factors.

MATERIALS AND METHODS

PCR mutagenesis

In order to insert a short amino acid sequence (sequence encoding of 2RKR) at position Arg152-Ile 153, the sequence encoding light and heavy chains of hFVII, was amplified using hFVII/pTZ 57R construct as a template and specific primers, separately.

Also in order to introduce 18nt between light and heavy chains which codes a cleavage site for furin, 9 nt and 14 nt were added to the 5' end of reverse primer of the first fragment and forward primer of the second fragment of the gene, respectively. These modifications also generate restriction sites for *DraI* at the C terminal of the light chain and *BstBI* of the N terminal of the heavy chain which facilitate subsequent joining of these two fragments. There are *BamHI* restriction site at the 5' end of forward primer of the first fragment and *EcoRI* restriction site at the 3' end of reverse primer of the second fragment to clone the final sequence to express plasmid.

The PCR reaction mixture for amplification of DNA encoding light and heavy chains contained 1µg of hFVII/pTZ 57R construct, 0.17 mM dNTPs, 1.5mM MgCl₂, 1.25 units of *Taq* DNA polymerase and 20 pmol of each of the forward and reverse primers (hFVII F: 5'-GGA TCC ATG GTC TCC CAG GCC CTC AG-3' and a_m hFVII R: 5'-AAA TTT TCT TCG GCC TTG GGG TTT GCT GGC ATT-3') for the light chain and (b_m hFVII F: 5'-TTC GAA GGA AGA GGA TTG TGG GGG GCA AGG TGT G - 3' and hFVII R: 5'-GAA TTC CTA GGG AAA TGG GGC GGC ATT-3') for the heavy chain in 50µl of final volume, separately. PCR reaction were carried out within 30 cycles; denaturation at 94 °C for 30 seconds, annealation at 55 °C for 40 seconds for amplification of DNA encoding of light chain and 62 °C for 1 min for heavy chain, and elongation at 72°C for 45 seconds. The PCR products were applied to electrophoresis using 1.5% agarose gel, stained by ethidium bromide and visualized under a UV transilluminator.

Cloning

Following PCR reaction, two fragments of gene including light and heavy chains containing extra nucleotides at 3' and 5' ends were electrophoresed

on 1.5% agarose gel, separately and the bands were sliced under UV long wave. DNAs were first extracted using the DNA purification kit (Fermentas, Lithuania), then were introduced in to pTZ57R as T vector via the T/A cloning method(10). The ligation was performed by T4 DNA ligase and incubated at 22°C for 2 hrs.

Transformation

All constructs were transformed into *E.coli* JM107 strain (11). The cell was grown in agar gel plates containing ampicilin to assure that the cell contains plasmid compared to the control plate lacking ampicilin. Recombinant plasmids were screened as white colonies using agar plates containing X-gal and IPTG. The positive colonies containing recombinant plasmids were mass cultured in Luria Bertani (LB) medium. Plasmid was extracted (12) and confirmed by restriction analysis or PCR amplification using specific primers and plasmid universal primers. For mutagenesis confirmation, recombinant plasmids were digested using *DraI* and *BstBI* restriction enzymes.

RESULTS

PCR amplification

The PCR products were electrophoresed on 1.5% agarose gel in parallel with 100bp DNA ladder marker. Figures 1 and 2 show the PCR product of light and heavy chains of hFVII which were amplified from hFVII/pTZ57R as template.

Verification of cloning

Figures 3 and 4 show PCR products of recombinant plasmid by plasmid universal primers, which differentiate plasmid and recombinant plasmid because PCR product of the recombinant plasmid contains cloned gene and plasmid MCS but PCR product of plasmid contains only plasmid MCS. Figures 5 and 6 show digested plasmids with *BglII* and *NcoI* restriction enzymes for sequence encoding of light and heavy chains of hFVII, respectively, because there are no restriction sites for *BglII* and *NcoI* on pTZ57R plasmid.

Verification of mutagenesis

Figures 7 and 8 show restriction analyses of recombinant plasmids by *DraI* and *BstBI* restriction enzymes. There is no restriction sites for *BstBI* on pTZ57R plasmid but related recombinant plasmid (b_m hFVII / pTZ 57R) was lineared by this enzyme. There are three restriction sites for *DraI* on pTZ57R plasmid and one site for inserted gene (a_m hFVII), therefore digested recombinant plasmid (a_m hFVII/ pTZ 57R) produce four fragments. Because of short length of the one of fragments, only three fragments are observed after electrophoresis on agarose gel.

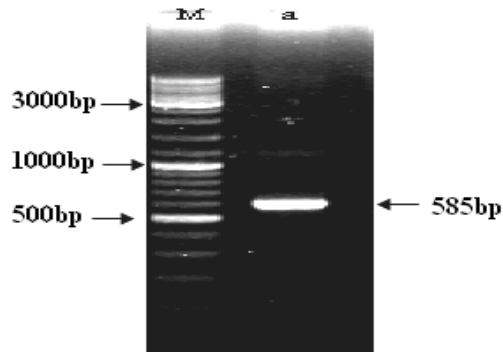


Figure 1. 1.5% agarose gel electrophoresis; lane a: PCR product of gene encoding of light chain of FVII, lane M: 100 bp DNA ladder marker.

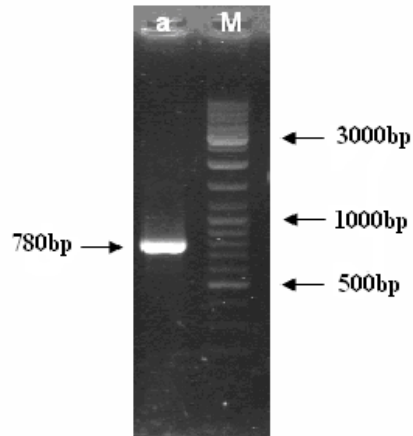


Figure 2. 1.5% agarose gel electrophoresis; lane a: PCR product of gene encoding of heavy chain of FVII, lane M: 100 bp DNA ladder marker

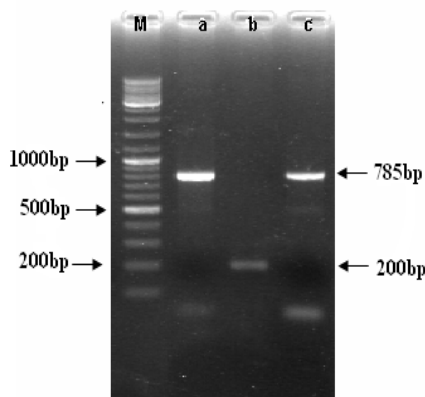


Figure 3. 1.5% agarose gel electrophoresis; lane a,c: PCR product of gene encoding of light chain of FVII with T3/T7 primer for pTZ57R; lane b: PCR product of pTZ57R with T3/T7 primer as control, lane M: 100 bp DNA ladder marker

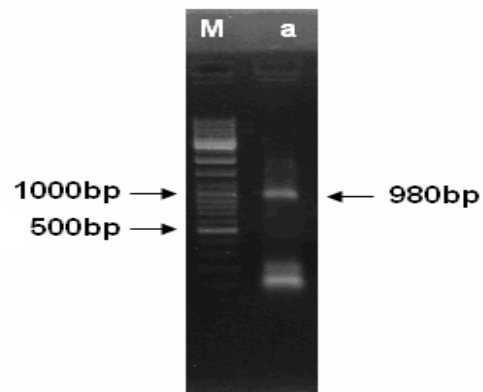


Figure 4. 1.5% agarose gel electrophoresis; lane a: PCR product of gene encoding of heavy chain of FVII with T3/T7 primer for pTZ57R, lane M: 100 bp DNA ladder marker

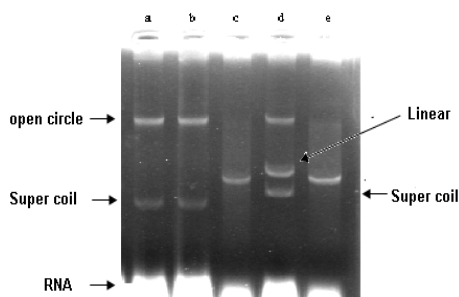


Figure 5. 1% agarose gel electrophoresis; lane a: Digested plasmid pTZ57R by *Bgl*II, lane b: Intact plasmid pTZ57R, lane c: Digested recombinant plasmid (a_m FVII/pTZ57 R) by *Bgl*II, lane d: Intact recombinant plasmid (a_m FVII/pTZ57 R)

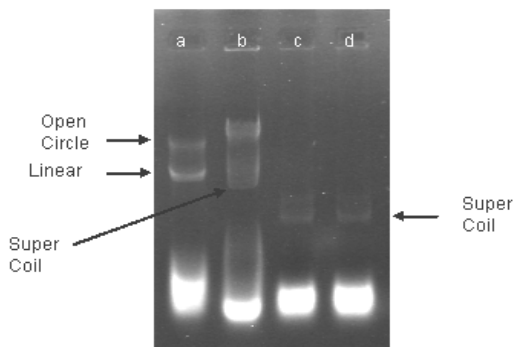


Figure 6. 1% agarose gel electrophoresis; lane a: Digested plasmid b_m FVII/pTZ57R by *Nco*I; lane b: Intact plasmid b_m FVII/pTZ57R, lane c: Digested plasmid (pTZ57 R) by *Nco*I, lane d: Intact plasmid (pTZ57 R)

DISCUSSION

Hemophilia is one of the most common genetic disorders. **Hemophilia A and B occur 1 in 5000 male and 1 in 30000 male, respectively**(13). The common treatments for hemophilia are based on replacement therapy with missing coagulation factors, but these treatments have been associated with viral infections, allergic reactions, high cost because of the required repeated infusion (2, 14).

Inhibitor formation has been recognized as one the most serious problem of hemophilia. The management of these patients requires a lot of economic and human resources (2). **Hedner and colleagues** have established rFVIIa as an ideal by-passing agent (15). The effects of inhibitors of FVIII and FIX can be overcome by rFVIIa, which can activate FX directly in the absence of a functional tenase complex, and in the presence of an excess of antibodies against FVIII or FIX. More investigations have shown that rFVIIa is a suitable option for treatment of hemophiliacs, because it can restore homeostasis, independent of

FVIII & FIX (16, 17) .

Gene transfer of hFVII (9), can circumvent limitation arising from previous hemophilia treatment, including risk of inhibitor formation and repeated infusion of coagulation concentrates (18-20) .

CONCLUSION

With the purpose of activation of hFVII after secretion, several nucleotides were inserted to the ends of sequence encoding light and heavy chains of hFVII by using specific primers for these fragments, separately. Joining these mutant forms of light and heavy chains together leads to the new variant of FVII. This variant may increase proportion of activated form of FVII after activation by furin.

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