Acta Medica et Biologica
Vol. 36, No. 1, 1-5, 1988

STUDIES ON THE STABILITY OF FACTOR VIII MODIFIED BY POLYETHYLENE GLYCOL

NOBUO SAKURAGAWA, KANAME KONDO, MASAHIRO NIWA, KAORU TAKAHASHI AND KIMIHIRO SHIMIZU

Central Clinical Laboratory, Toyama Medical and Pharmaceutical University, Toyama, Japan

(Received December 4, 1988)

INTRODUCTION

Factor VIII conentrate preparation is infused for the stoppage of bleeding in cases with hemophilia A. It is absolutely necessary to infuse it frequently to keep Factor VIII level on the therapeutic level because of its short half-life in circulation.[1] The main cause of its short half-life are the inactivation of Factor VIII by such serine proteases as plasmin, thrombin and protein Ca.[2,3,4,5,]

In our previous studies on the effects of polyethylene glycol (PEG)-modification on urokinase, it was observed that this modification made the half-life of urokinase longer because of the avoidance of inactivation of urokinase by inhibitors.[6]

It is surmised from the results that PEG-modification of Factor VIII would also show the distinct effects to keep the Factor VIII biological activity stable.[7,8]

In this paper, the effect of PEG-modification on Factor VIII was investigated from the viewpoint of the stability of Factor VIII against inactivation by the proteases.

MATERIALS AND METHODS

1. Reagents: Factor VIII concentrate preparation (Koäte, Cutter Co. Ltd, 500 units/vial) was dissolved in 20 ml of 100 mM phosphate buffer (pH 7.0). Plasmin (Green-Cross Co., 12.5 CTA units/ml) was diluted in 50 mM Tris-buffer to make 0.4 and 0.04 CTA units/ml. Thrombin (Mochida Co. Ltd., 500 units/ml) was diluted in 50 mM Tris-HCl-100 mM-NaCl-0.1% BSA buffer to make 100 units/ml (native Factor VIII).

Antithrombin III (Green Cross Co. Ltd., 500 units/ml) was diluted to 100 units/ml; heparin (Kodama Co. Ltd, 1000 units/ml) was diluted to 10 units/ml; and protamin sulfate (Shimizu Co. Ltd., 5% solution) was diluted to 0.1% with 50 mM Tris-HCl-100 mM-NaCl-0.1% BAS (pH 8.0) buffer, respectively. Crude protein C was prepared by Sala's method.[9] By the addition of 0.7 ml of 100 units/ml of thrombin solution to 0.2 ml of the eluate of citrate solution from adsorbed $BaSO_4$, the reaction mixture was kept at 37°C for 60 min to activate protein C. The crude protein Ca solution was obtained from the reaction mixture after a further addition of 0.04 ml of antithrombin III (100 units/ml) and 0.04 ml of heparin (10 units/ml) to inactivate the residual thrombin at 37°C for 30 min. Finally 0.04 ml of 5.1% protamin sulfate solution was mixed with this reaction mixture to neutralize the added heparin.

2. Assay methods: Enzymatic activity of Factor VIII (Factor VIII: C) was assayed by the standard one stage method.[10] Thrombin, plasmin and protein C were assayed by a chromogenic substrates.

3. Preparation of PEG-Factor VIII: The following reaction were carried out in a cold room. Native-Factor VIII of 25 units/ml in various concentrations of activated PEG was kept at 37°C for 60 min to bind the lysine residues of Factor VIII to PEG, and the PEG-Factor VIII preparation was obtained.[11]

The ratio of the modification of Factor VIII by various concentrations of PEG made various levels of Factor VIII activity. The activity of Factor VIII decrease depending on the amount of PEG added because of the binding of PEG to Factor VIII lysine residues. We decided to use 4.2 mM of PEG in this reaction mixture to obtain 75% of Factor VIII for the convenience of the research.

4. Stability of PEG-Factor VIII against the serine proteases: Such serine proteases as thrombin, plasmin and protein Ca were used as follows: 2 units/ ml of both native and PEG factor VIII were added with equal volumes of 0.4 or 0.04 CTA units/ml of plasmin, 2×10^{-2}or 2×10^{-3} units/ml of thrombin, and the above-mentioned protein Ca solution at 37°C, respectively. The control studies were carried out by using the protein Ca preparation without the addition of thrombin.

RESULTS

1. Changes of Factor VIII by addition of plasmin:

By using 0.2 CTA units/ml of plasmin, native-Factor VIII was inactivated in 10 min,

but PEG-Factor VIII was sustained the Factor VIII biological activity for 2 hours.

By using 0.02 CTA units/ml of plasmin, native-Factor VIII was inactivated to 25 % in 2 hours, but, as shown in Figure 1, PEG-Factor VIII was kept stable.

2. Changes of Factor VIII by addition of protein Ca:

Native-Factor VIII was inactivated for 6 hours, but, as shown in Figure 2, PEG-factor VIII was stable for 9 hours.

3. Changes of Factor VIII by addition of thrombin:

By addition of 1×10^{-3} units/ml of thrombin, both native and PEG-Factor VIII activities were elevated to about 300 % in 5 min, and by 1 x 10^{-3} units/ml, these were elevated to about 200 %. But finally, they were inactivated gradually.

DISCUSSION

In the previous studies of PEG-modification on urokinase, we obtained the results that PEG modified urokinase showed a longer half-life due to the avoidance of inactivation by the inhibitors.[1)]

In this paper we applied this PEG-modification on Factor VIII to prolong the half-life

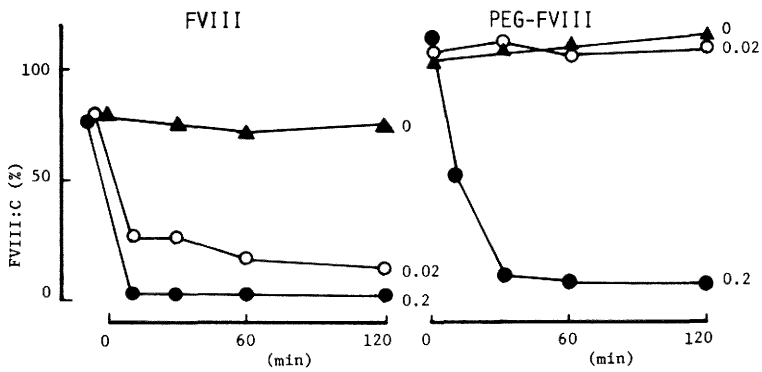

Fig. 1. Stability of native- and PEG-factor VIII against plasmin.

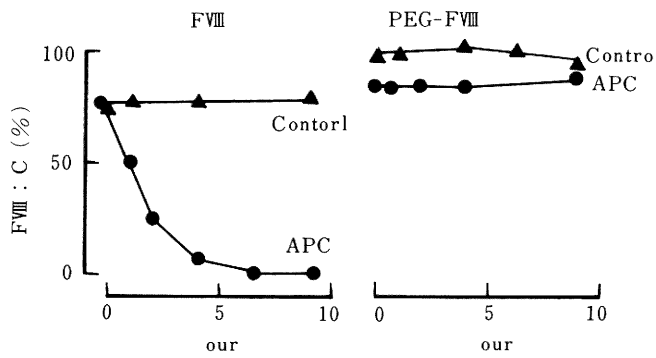

Fig. 2. Stability of native- and PEG-factor VIII against activated protein C.

of Factor VIII in blood circulation.

Factor VIII C:Ag is composed of both heavy chain with Mr 92,000 and light chain with Mr 79,000-80,000. It is clear by SDS-PAGE that with protein Ca and plasmin the heavy chain of Factor VIII C: Ag was partially proteolysed to yield a small fragment of Mr 45,000 to reduce its activity. The partial proteolysis of thrombin on heavy and light chains elevates their biological activities. After these reactions, the proteolysis on light chian occurred , and then yielded a fragment of Mr 71,000-72,000 to reduce its activity.[12] The proteolysis of thrombin works on the N-terminal portion of Factor VIII C: Ag, and that of plasmin or protein Ca works on the middle portion.

It is presumed that PEG combined with the lysine residue of Factor VIII makes a conformation change on Factor VIII and protects the proteolysis by the serine proreases.

From our results, PEG-Factor VIII was stable agaist protein Ca and plasmin to keep activity stable. But with thrombin, Factor VIII was activated to 200-300% because of the less influence of PEG on Factor VIII protein. Therefore, it is proposed that PEG-modification is useful as a therapeutic for keeping Factor VIII stable in circulation as a protection against inactivation by serine proteases to maintained a longer half-life. In addition, it will also helpful to avoid generation of antibody against Factor VIII.

The future question to solve is whether or not PEG is safe on the human body.

SUMMARY

The stability studies of Factor VIII modified by PEG against serine proteases; thrombin, plasmin, and protein Ca, wrere carried out.

PEG-Factor VIII was stable against plasmin and protein Ca, but was not stable against thrombin.

These differences may be due to the different characteristics of each of the serine proteases.

The PEG modification will be useful for therapeutic use by maintaining the level of Factor VIII much longer.

REFERENCES

1) Abildgaard, C. F.: Current concepts in the management of hemophilia. *Semin. Hematol.* 12: 223-232, 1975.
2) Guisasola, J. A., Cockburn, C. G. and Hardisty, R. M.: Plasmin digestion of factor VIII : Characterization of the breakdown products with respect to antigenicity and von Willebrand activity. *Thromb. Haemostas.* 40: 302-315, 11978.
3) Hoyer, L. W. and Trabold, N. C.: The effect of thrombin on human factor VIII. J. Lab. Clin Med. 97: 50-64, 1981.
4) Fay, P. J. et al.: Purification and characterization of a highly purified human factor VIII consisting of a single of polypeptide chain. *Proc. Natl. Acad. Sci. (USA).* 79: 7200-7204, 1982.
5) Fulcher, C. A., Roberts, J. R., and Zimmerman, T. S.: Thrombin proteolysis of purified factor VIII procoagulant protein : Correlation of activation with generation of a specific polypeptide. *Blood* 61: 807-811, 1983.

6) Sakuragawa, N. et al.: Studies on the effect of PEG-modified urokinase on coagulation-fibrinolysis using beagles. *Thromb. Res.* **41**: 627-635, 1986.
7) Abuchowski, A. et al.: Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.* **252**: 3582-3586, 1977.
8) Shimizu, K. et al.: Changes of characteristics of urokinase by chemical modification. *Seikagaku* **54**: 824 (in Japanese). 1982.
9) Sala, N., Owen, W. G., and Collen.: A functional assay of protein C in human plasma. *Blood* **63**: 671-675, 1984.
10) Hardisty, R. M. and Macpherson, J. C.: A one-stage factor VIII (antihaemophilic globulin) assay and its use on venouse and capillary plasma. *Thromb Diath. Haemorrh.* **7**: 215-229, 19662.
11) Chen, R. H. L. et al.: Properties of two urate oxidases modifid by the covalent attachment of polyethylene glycol. *Biochi. Biophys. Acta,* **660**: 293-298, 1981.
12) Fulcher, C. A. et al.: Proteolytic inactivation of human factor VIII procoagulant protein by activated human protein C and its analogy with factor V. *Blood* **63**: 486-489, 1984.