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THE INTERACTION OF HUMAN BLOOD COAGULATION FACTOR VII AND TISSUE FACTOR: THE EFFECT OF ANTI FACTOR VII, ANTI TISSUE FACTOR AND DIISOPROPYLFLUOROPHOSPHATE

Bjarne Østerud*, Eirik Bjørklid and Stephen F. Brown

Department of Medicine, University of California, San Diego, U.S.A. and Institute for Medical Biology, University of Tromsø, Tromsø, Norway

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SUMMARY: The effect of Factor VII antibody and an antibody to the apoprotein of tissue factor has been tested on the product formed between Factor VII, tissue factor and calcium ions. The antibody to the apoprotein of tissue factor neutralized tissue factor but had no effect on the extrinsic Factor X activator activity when Factor VII had been allowed to react with tissue factor before the addition of the antibody. The Factor VII antibody neutralized Factor VII and it also blocked the Factor X activator activity when Factor VII had been incubated with tissue factor and calcium ions prior to the addition of Factor VII antibody.

Diisopropylfluorophosphate (DFP) was found to neutralize native purified Factor VII and Factor VII in human plasma. This inhibition of Factor VII was very slow and required high concentrations of DFP. However, when the Factor VII had been preincubated with tissue factor and calcium ions, the neutralization of Factor VII by DFP occurred rapidly, and at much lower concentration of DFP.

INTRODUCTION: Factor VII is interacting with tissue factor and calcium ions to form the extrinsic activator of Factor IX (1) and Factor X (2-4). In a bovine system it has been shown that Factor VII forms a complex with tissue factor (5,6) and this complex is thought to be the extrinsic activator of the coagulation system. In a human system it has been reported that the interaction of crude Factor VII with tissue factor and calcium ions led to the formation of Factor VIIa, that no longer needed tissue factor for its activation of Factor X (7). The formed Factor VIIa was neutralized by phospholipase C indicating that the activated form of Factor VII had essential phospholipids attached to the Factor VII molecule.

*Present address: Department of Clinical Chemistry, University Hospital, 9012 Tromsø, Norway.

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Factor VII in bovine plasma is completely inactivated by diisopropylfluorophosphate (DFP) (8). Thus the bovine Factor VII in its native form has an available active serine site with esterase activity (9). DFP has not been found to inactivate native human Factor VII (10). However, when human Factor VII first was allowed to react with tissue factor and calcium ions, then the Factor VII was neutralized by addition of DFP (10).

The experiments reported herein were undertaken to study the interaction between purified human Factor VII and tissue factor utilizing specific antibodies to Factor VII and the apoprotein of tissue factor. Evidence is presented that the reaction between Factor VII and tissue factor results in a Factor X activator product that no longer can be neutralized by antibodies to the apoprotein of tissue factor, but still is completely inactivated by the antibody ot Factor VII. In addition, Factor VII is neutralized both in purified form and in plasma by DFP, and it is shown that this inactivation occurs more rapidly when Factor VII has interacted with tissue factor.

MATERIALS AND METHODS: Tissue factor was isolated from human brain as described (11).

Antibody to apoprotein of tissue factor. Monospecific antibody to the apoprotein of tissue factor was produced by injecting purified apoprotein in rabbit as has been reported earlier (11, 12).

Factor X. Factor X was purified to homogeneity by utilizing Ba-citrate precipitation of plasma, DEAE-cellulose chromatography in batch, preparative polyacrylamide gel electrophoresis, and heparin agarose affinity chromatography as described in detail elsewhere (13-14). Monospecific antibody to Factor X was raised in goat. The antiserum was tested against normal human plasma, and it was found that 1 part antiserum neutralized 320 parts of normal plasma. The isolated immunoglobulin fraction was generally used to abolish the Factor X activity.

Factor VII. Factor VII was partially purified by using the three first steps in the purification of Factor X. The Factor VII preparations were then subjected to heparin-agarose affinity chromatography and finally chromatographed through a column of anti-prothrombin-agarose. The final preparations of Factor VII labelled with ^{125}I gave two bands on SDS polyacrylamide gel electrophoresis in the presence or absence of reducing agent. By testing for Factor VII activity utilizing a clotting assay (15) and a coupled amidolytic assay (16) the same value was obtained. Earlier studies have shown that only native Factor VII gives the same value in the clotting assay and the coupled amidolytic assay (16).

Factor VII antibody. The Factor VII antibody used in this study was a gift from Behringwerke, Germany. It did not neutralize the coagulation activity of any other proteins than Factor VII.

Coupled amidolytic assay for Factor VII. This assay has been described in detail elsewhere (16). In the present study the assay was performed as follows. Ten µl of the test material that had been incubated with DFP or isopropyl alcohol alone as control was added to 500 µl trisbuffered saline containing 1 mg/ml albumin. From these diluted samples, 50 µl aliquotes were pipetted off and added to 50 µl Factor X (1.9 U/ml), 10 µl tissue factor and 5 µl 50 mM $CaCl_2$ and incubated for 3 min at 37°. The reaction was stopped by adding 10 µl 0.3 M Na_2EDTA. One hundred µl from each incubation mixture was tested for Factor Xa activity by adding it to 600 µl tris-buffered saline buffer and 100 µl S-2222, (Kabi, Sweden), 1 mg/ml, and the increase in optical density at 405 nm was recorded using a Gilford spectrophotometer.

RESULTS: Interaction between Factor VII and tissue factor. Factor VII was incubated with dilute tissue factor and calcium chloride and tested over time for its ability to shorten the clotting time of Factor VIII deficiency plasma in the presence of added phospholipids and calcium-chloride. Although the Factor VII preparation did not contain measureable Factor X activity, a trace amount of an antibody to Factor X was incubated with Factor VII prior to the addition of tissue factor and calcium chloride to avoid a possible trace formation of Factor Xa in the incubation system. As seen in Fig. 1, a time consuming reaction, between Factor VII, tissue factor and calcium ions could be demonstrated since subsamples from the incubation mixtures with Factor VII, tissue factor and calcium chloride progressively shortened the clotting time of Factor VIII deficiency plasma from 56 sec at zero time to 32 sec at 10 min.

Effect of antibody to Factor VII on the reaction product of Factor VII and tissue factor. A monospecific antibody to Factor VII was utilized to determine whether the activity generated by Factor VII, tissue factor and calcium chloride was dependent on the Factor VII moiety. In preliminary experiments Factor VII and the antibody were incubated together for 150 sec at 37°, then tissue factor and calcium chloride were added and subsamples were removed over time and tested for their ability to clot Factor VIII deficiency plasma. As seen in Fig. 1, the antibody neutralized the procoagulant activity of Factor VII (93 sec clotting time at zero and 113 sec at 4 min incubation time).

When the antibody of Factor VII was added to the 10 min incubation mixture of Factor VII, tissue factor and calcium chloride as shown in Fig. 1, the clotting time of the samples

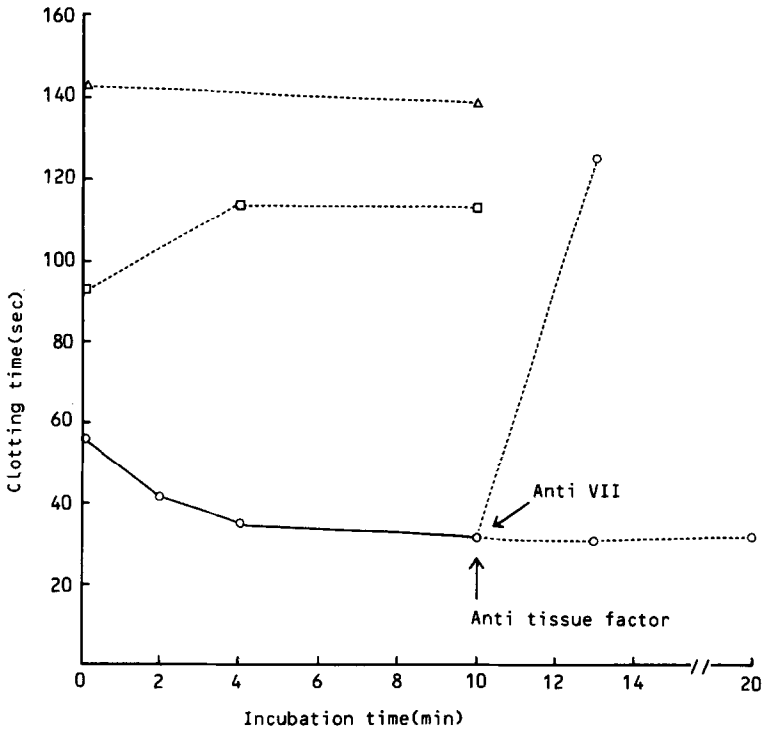

Fig. 1.
Interaction of Factor VII and tissue factor and the effect of anti tissue factor and anti Factor VII. Two hundred µl Factor VII (0.2 U/ml) was added 200 µl TBS/BSA and 1 µl anti-Factor X and incubated for 150 sec at 37°. Then 20 µl tissue factor and 50 µl 50 mM $CaCl_2$ were added and the whole mixture incubated at 37°. Subsamples (50 µl) from the mixture were removed over time and tested for its potency to clot VIII deficiency plasma as described in "Methods and Materials". At 10 min incubation time, either 10 µl anti-apoprotein of tissue factor or 10 µl anti-Factor VII was added, and the mixtures were further incubated for 3 min when new subsamples were removed and tested. The upper curve -ΔΔ- shows the clottingtimes obtained with Factor VIII deficiency plasma as substrate when 10 µl of anti-apoprotein of tissue factor together with 100 µl TBS/BSA were incubated for 120 sec at 27° followed by addition of 100 µl Factor VII (0.2 U/ml) and 25 µl mM $CaCl_2$. Fifty µl subsamples were tested in the clotting assay. The line -□-□- indicates the results obtained when 100 µl Factor VII (0.2 U/ml), 100 µl TBS/BSA, 10 µl anti-Factor VII, 1 µl anti-Factor X were incubated for 150 sec at 37° followed by adding 10 µl tissue factor and 25 µl 50 mM $CaCl_2$. Subsamples of 50 µl of this mixture were then tested over time in the clotting assay.

increased from 32 sec to 125 sec indicating that Factor VII even after incubation with tissue factor and calcium chloride still was required for the activation of Factor X.

Effect of antibody to the apoprotein of tissue factor on the reaction-product of Factor VII and tissue factor. When tissue factor and the antibody to the apoprotein of tissue factor were incubated for 2 min at 37°, followed by the addition of Factor VII and calcium chloride, the subsamples of this final mixture gave a very prolonged clotting time when tested with VIII deficiency plasma, at zero time 143 sec as compared to 56 sec without antibody and at 10 min incubation time 138 sec as compared to 32 sec without antibody. However, when the antibody to the tissue factor apoprotein was added to a mixture of Factor VII, tissue factor and calcium chloride that had been allowed to react for 10 min, the antibody had no effect as shown by the clotting time of 32 sec on a subsample from the mixture just prior to the addition of anti tissue factor and the clotting time of 31 sec on a subsample from the same mixture 3 min after the addition of the antibody (see Fig. 1). Thus, when Factor VII, tissue factor and calcium chloride have been interacting the antibody of tissue factor had no neutralizing effect upon the formed activator of Factor X.

All experiments described so far were carried out in the presence of a very diluted Factor X antibody to neutralize possible trace amounts of Factor X in the incubationmixtures. To rule out a possible antibody interaction in the Factor X activation, the experiments were also performed in the absence of the weak Factor X antibody. Absence of the Factor X antibody in the incubationmixtures did not alter the results, as a Factor VII antibody still neutralized the formed Factor X activator whereas the antibody to the apoprotein of tissue factor had no effect when Factor VII had interacted with tissue factor.

Effect of DFP on Factor VII in purified system. Two different incubation systems were used for this experiment. In one system the native Factor VII was first incubated with tissue factor and calcium chloride for 10 min before the DFP was added.

After 10 min incubation of varying concentrations of DFP with the test materials at 37° followed by 4 hours incubation at 4°, the subsamples were diluted and tested for Factor VII activity in a coupled amidolytic assay. As seen in Fig. 2 the DFP had a much higher potency to neutralize Factor VII that had been preincubated with tissue factor and calcium chloride than the native Factor VII molecule. At a final concentration of

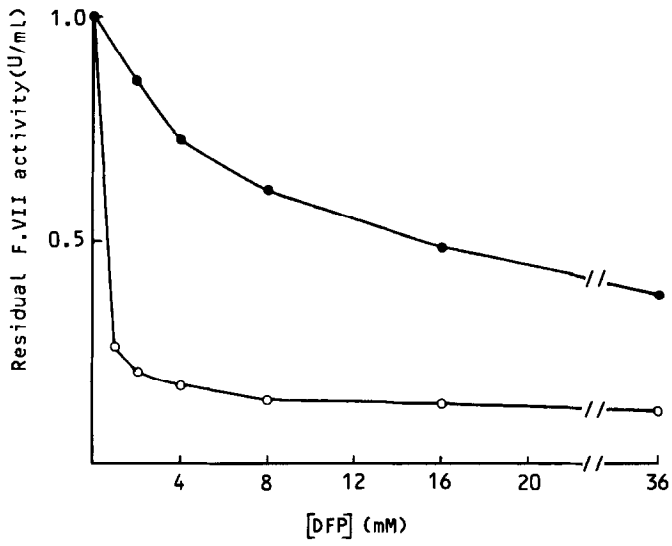

Fig. 2.
The effect of DFP on purified Factor VII and Factor VII plus tissue factor. A serie of 1.4 ml conical polypropylene centrifuge tubes containing 50 µl Factor VII (4.5 U/ml) and 200 µl TBS/BSA were set up. To another serie of tubes containing 50 µl Factor VII (4.5 U/ml), 160 µl TBS/BSA, 20 µl of tissue factor and 20 µl 50 mM $CaCl_2$ were added and the mixtures preincubated for 10 min at 37°. Then 0.25 µl, 0.5 µl, 1.0 µl, 2.0 µl, 4.0 µl and 9 µl 1 M DFP in isopropylalcohol were added to each serie of tubes. The samples were incubated for 10 min at 37° and 4 hours at 4°, diluted and tested for Factor VII as describes in detail under "Material and Methods". —●—●— native factor VII + DFP; —o—o— Factor VII interacted with tissue factor + DFP.

2 mM DFP, the preincubated Factor VII had lost 80 % of its activity compared with only 14 % loss of Factor VII activity in the native Factor VII mixture. Even a concentration of 14-15 mM DFP neutralized only 50 % of the Factor VII activity in the mixtures which had not been preincubated with tissue factor and calcium chloride.

Effect of DFP on Factor VII in plasma. It had been demonstrated earlier that Factor VII in bovine plasma is neutralized by DFP, but so far attempts at neutralizing Factor VII activity in human plasma have been negative. With the new coupled amidolytic assay system for factor VII we hoped to be able to determine whether plasma Factor VII would react with DFP or not. As seen in Fig. 3 a partial inhibition of Factor VII activity was obtained with DFP when the incubation time was the same as used in the experiments with purified Factor VII. Only 45 % of the Factor

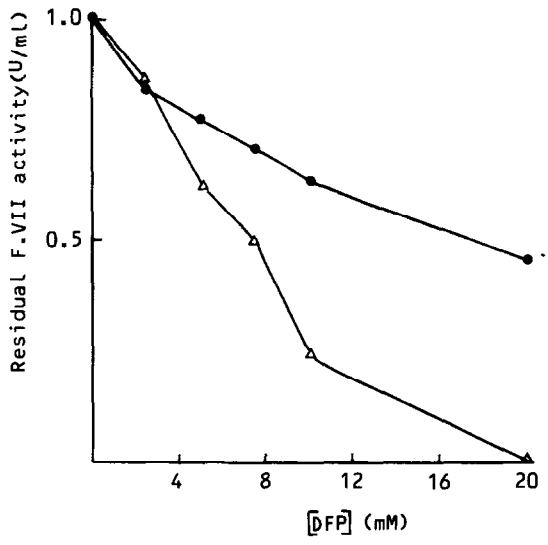

Fig. 3.
The effect of DFP on plasma Factor VII. Conical polypropylene tubes (1.4 ml) containing 200 µl of normal plasma were added 0.5 µl, 1.0 µl, 1.5 µl, 2.0 µl and 4 µl 1 M DFP in isopropyl alcohol. The samples were incubated at 37° for 10 min and 4 hours at 4°, diluted and tested for Factor VII as described in "Materials and Methods". Residual Factor VII activity is indicated by line —●—●—. The undiluted samples were further incubated for 21 hours at 4° and again tested for Factor VII as indicated by line —△—△—.

VII activity was neutralized by DFP at a final concentration of 20 mM. By increasing the incubation time at 4° from 4 hours to 24 hours the effect of DFP was more effective as shown by 77 % inhibition of Factor VII activity at 10 mM DFP. In control experiments without DFP it was shown that the long incubation of plasma with the solvent for DFP, isopropyl alcohol, did not result in any significant decrease in Factor VII activity.

DISCUSSION: It is established that both Factor VII and tissue factor are needed to form an extrinsic activator of Factor X, and as more recently reported, also an extrinsic activator of Factor IX (1).

In an earlier study we obtained some evidence for the formation of an activated Factor VII in incubationmixtures of tissue Factor, Factor VII and calcium ions (7). The product of Factor VII formed did not any longer require tissue factor for its activation of Factor X. The separation of this Factor X activator from the tissue factor was accomplished by filtration through a

millipore filter. Although the Factor VIIa activity was quite weak (clotted Factor VIII deficiency plasma in 36 sec), it possessed properties different from all other activated coagulation factors as the isolated factor VIIa was not neutralized by antithrombin III (17).

The neutralization of the Factor X activator activity in this study by the addition of Factor VII antibody to an incubation mixture of Factor VII, tissue factor and calcium ions (see Fig.1) demonstrates that Factor VII is an essential part of the Factor X activator. The failure of tissue factor antibody to block the activity of the product formed between Factor VII and tissue factor indicated that Factor VII may have been modified to an active Factor X activator no longer dependent on tissue factor apoprotein in this reaction. However, one cannot exclude the possibility that sites in the tissue factor apoprotein molecule which are pertinent for procoagulant activity after the interaction with Factor VII are inaccessible for the antibodies.

In a previous study by Nemerson and Pitlick (3) it was found that also the bovine Factor X activator, once formed, may be dependent on Factor VII antigens but not on tissue factor antigens.

Additional evidence for an alteration of the Factor VII molecule in the reaction between tissue factor and Factor VII stems from the studies with DFP. As shown in Fig. 2, DFP is more reactive with the Factor VII that has reacted with tissue factor than the native Factor VII. These results confirm the finding of Wijngaards and Immerzeel (10) who showed that the Factor VII in a mixture with tissue factor and calcium ions could be neutralized by DFP. However, they were not able to demonstrate an inhibition of native human Factor VII. This contradicts our results (see Fig. 2 and Fig. 3) since we were also able to block the Factor VII activity of purified native Factor VII and Factor VII in plasma with DFP.

It is interesting that the first experiments with bovine Factor VII and DFP initially also resulted in no effect of DFP on native Factor VII, but that when Factor VII was incubated with tissue factor, the Factor VII activity was neutralized (6). This indicates that bovine Factor VII and human Factor VII behave quite similar in regard to DFP and that the reaction of both bovine Factor VII and human Factor VII with tissue factor results

in a product of Factor VII that has a more DFP sensitive site than native Factor VII. Our results with human Factor VII indicate that Factor VII may be altered to an activator of Factor X, possibly of molecular size much smaller than native tissue factor, that afterwards can function without tissue factor. Further studies on the caracteristics of the activator may reveal important information about the mechanism of interaction between tissue factor and Factor VII.

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