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HUMAN FACTOR VII ASSOCIATED WITH ENDOTOXIN STIMULATED MONOCYTES IN WHOLE BLOOD

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Summary: Evidence is provided for a calcium dependent binding of factor VII to stimulated monocytes in whole blood. The binding of factor VII to the monocytes in this study was directly related to the exposure of tissue thromboplastin on the surface of the endotoxin stimulated monocytes. In such monocytes synthesis of tissue thromboplastin is well known to be enhanced. Antibodies against the apoprotein of tissue thromboplastin prevented the appearance of factor VII activity in monocyte suspensions isolated from blood incubated with endotoxin. This could be explained by the failure of factor α-VIIa generation in blood containing thromboplastin antibodies. Factor α-VIIa is probably the form of factor VII being bound to the monocytes.

A rapid disappearance of factor VII has been observed in the circulation of patients with fulminant meningococcemia (1). A similar reduction of factor VII concentration has also been found in patients with meningococcal infection in our hospital (unpublished data). Thus, in one case, the factor VII level fell from 0.90 U/ml to 0.12 U/ml in the first 1½ hours after admission to the hospital. There are several ways factor VII might be removed from the circulation although no exact mechanism is known at the moment. It is well established that factor VII is converted to an active form α-VIIa by factor Xa in the presence of phospholipids, or by factor IXa and factor XIIa (2-6). However, in vivo studies showed that the active form of factor VII persisted in the circulation, and had a half life of 144 min (7) compared to 290-355 min for native factor VII (8). This long half life may be explained by the failure of antithrom-

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bin III to neutralize the active form of factor VII (9). This contrasts with the neutralization of other activated serine protease clotting factors by antithrombin III. Based on these facts, it may be reasonable to assume the existence of an unknown mechanism for the removal of factor VII in patients with meningococcal infection.

Monocytes incubated with endotoxin are known to accelerate their tissue thromboplastin synthesis (10,11,12). This has also been shown in vivo after injecting endotoxin intravenously (13). Recently, we demonstrated that about 70% of the tissue thromboplastin synthesized in the monocytes of blood incubated with endotoxin, became available on the surface of the monocytes (14). This finding prompted us to investigate the extent of factor VII binding to endotoxin stimulated monocytes. An extensive decrease in leucocyte count is quite often associated with the meningococcal infection, and if factor VII is strongly bound to the cells it would be removed too, a possible mechanism for the fast disappearance of factor VII from the circulation of these patients.

MATERIALS AND METHODS

MATERIALS

Endotoxin was E.coli 0.26:B6 obtained from Difco Laboratories, Detroit, Mich., USA. A stock solution of 4 mg/ml endotoxin was prepared by dissolving it in 0.15 M NaCl and kept at 4°C. Blood was drawn from normal donors into heparin anticoagulant (25 U/ml blood) or 0.10 M sodium citrate-citric acid (15) in plastic tubes.

Isolation of monocytes. Mononuclear cells were isolated on Lymphoprep gradient (Nyegaard, Oslo, Norway) by the method of Bøyum (16). The cells were further washed by resuspending the band containing mononuclear cells in 0.15 M NaCl containing 3 mM $CaCl_2$. The cells were then collected by centrifugation at 1500 x g for 10 min. The cell sediment from 2 ml blood was resuspended in 0.2 ml 0.15M NaCl, and tested for factor VII activity.

The whole blood recalcification time. Two hundred µl whole blood was pipetted into 0.9 cm x 5.5 cm polycarbonate

tubes at 37°C. Then 100 µl 0.035 mM $CaCl_2$ was added and the clotting time measured. Further details of this method were as previously described (14).

Factor VII activity measurements. Factor VII clotting activity was determined in a one stage assay which measures the ability of a test substance to shorten the clotting time of a hereditary factor VII deficiency substrate plasma in the presence of thromboplastin (17). A coupled amidolytic assay for factor VII was carried out as described earlier (20).

Factor VII and tissue thromboplastin antibodies. Antibodies to factor VII were purchased from Behring Werke AG, Marburg, West Germany. The IgG fraction was isolated and used as the factor VII antibody reagent. This antibody had no effect on factors IX and X or prothrombin. Tissue thromboplastin antibodies were raised by injecting purified tissue thromboplastin apoprotein into rabbits (18). The IgG fraction of the rabbit antiserum was used as the antibody reagent. As control materials, an IgG fraction from normal rabbit serum was used.

RESULTS AND DISCUSSION

Factor VII binding to stimulated monocytes.

Fig. 1 shows an increase in the binding of factor VII activity to monocytes isolated from heparinized blood incubated with 5 µg/ml endotoxin over time (open circles), which parallels the appearance of newly synthesized tissue thromboplastin (solid circles). The extent of the exposure of the latter on the surface of the monocytes in blood incubated with endotoxin, was recently quantitated and found to be about 70% of the total tissue thromboplastin . The test used was the ability of tissue thromboplastin antibodies to abolish the activity when added to whole blood (14).

As seen from the lower curve of Fig. 1, marked with triangles, the factor VII activity measured in monocyte suspensions isolated from blood incubated without endotoxin was quite insignificant. When using citrated blood for incubation with endotoxin, no factor VII activity was found in the isolated monocytes despite significant exposure of tissue thromboplastin on the surface of the monocytes. The factor VII binding is therefore calcium dependent.

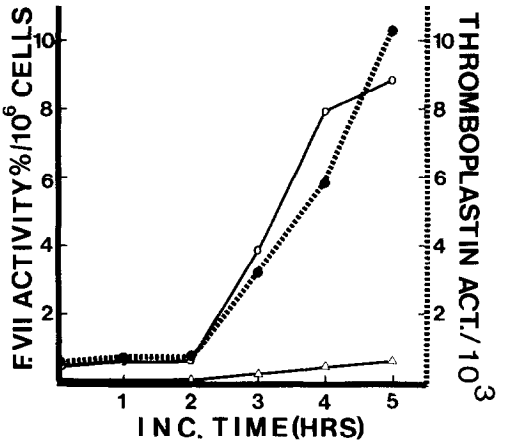

Fig. 1 Factor VII activity in monocyte suspensions isolated from blood incubated with and without endotoxin. Heparinized blood was incubated with endotoxin (5 μg/ml, ——O——) or without endotoxin (——Δ——), and at the intervals shown, 2 ml blood aliquots were removed. Then the mononuclear cells were isolated, counted and immediately tested for factor VII activity. The factor VII activity relates to normal plasma designated as 100%. The curve ---●--- represents the tissue thromboplastin activity exposed in whole blood incubated with 5 μg/ml endotoxin. The thromboplastin activity measurement has been described elsewhere (14). and the activity refers to a crude human brain tissue thromboplastin reagent (undiluted reagent designated as 100% activity). The undiluted reagent clotted normal plasma in 18 sec using the Quick time system. The counts of monocytes were carried out as described (22).

That the increased factor VII activity measured in the endotoxin stimulated monocytes really represented factor VII activity, was shown by adding the factor VII antibodies to the monocyte suspensions 5 min before assaying them in the factor VII assay. The antibodies neutralized all the activity.

The factor VII bound to the monocytes in endotoxin stimulated heparinized blood could be either native factor VII or activated factor VII (α-VIIa), since the clotting assay used does not discriminate between the two forms. Of these forms the latter has been demonstrated to have a much higher binding affinity for tissue thromboplastin than the former (19), and its formation could by itself explain the enhanced binding of factor VII to the tissue thromboplastin producing monocytes. To test this possibility, a coupled amidolytic assay for factor VII, was carried out as described earlier (20) in addition to the

clotting assay. In a typical experiment the activities found were 8.9%/10^6 cells and only 0.3%/10^6 cells in the clotting and amidolytic assays respectively. Such a discrepancy between the values obtained in the two assays has been shown to be caused by the presence of factor α-VIIa (20), and accordingly this is probably the predominant form of factor VII bound to the monocytes. Thus, as expected heparin has apparently not prevented the generation of trace amounts of factor Xa initiated by the newly synthesized tissue thromboplastin, factor Xa being known in turn to generate factor α-VIIa. The latter by virtue of its high affinity remains bound to the cells.

The effect of tissue thromboplastin antibodies on the binding of factor VII to monocytes.

When tissue thromboplastin antibodies were added to whole blood and incubated together with endotoxin, the isolated monocytes possessed no factor VII activity. In a typical experiment, the factor VII clotting activity measured in monocyte suspensions isolated from blood incubated for 5 hours with endotoxin only, was found to be 8.4%/10^6 cells. In contrast, when the endotoxin incubation was carried out in the presence of thromboplastin antibodies, the factor VII activity was 0.8%/10^6 cells in the isolated monocyte suspension. A clotting activity of 0.6%/10^6 cells was obtained for a monocyte suspension isolated from blood incubated without endotoxin. Clearly, the antibodies are capable of blocking the binding of factor VII on the surface of the stimulated monocytes. This effect is probably indirect by preventing the formation of factor α-VIIa. When the appearance of the high affinity form of factor VII is abolished, only small amounts of the factor can be bound. To what extent the antibodies prevent the binding of native factor VII or already formed factor α-VIIa cannot be deduced from our studies.

The binding of factor VII to monocytes in blood incubated with various concentrations of endotoxin.

Blood samples with various concentrations of endotoxin were incubated for 4 hours at 37°C. The factor VII activity measured in the various monocyte suspensions isolated from blood incubated with 5 µg, 1 µg, 0.5 µg, 0.05 µg endotoxin or buffer was found to be 7.6%/10^6 cells, 4.1%/10^6 cells, 2.3%/10^6 cells, 1.3%/10^6 cells and 0.4%/10^6 cells respectively. (Average of three experiments). This increasing efficiency of factor VII binding to monocytes correlates nicely with the amount of tissue thromboplastin synthesized and exposed in whole blood incubated with various concentrations of endotoxin as recently reported from our laboratory (14). The result is in accordance with our finding that factor α-VIIa, which would be generated in proportion to the amount of tissue thromboplastin present, is by far the predominant bound form.

Our results offer a plausible explanation for the fast disappearance of factor VII from the circulation of meningococcal infected patients. The generation of tissue thromboplastin in the monocytes may result in an even more pronounced generation of factor α-VIIa in this *in vivo* situation than in our whole blood system with heparin present from the beginning of the incubation. The factor α-VIIa remains bound to the tissue thromboplastin by virtue of its high affinity. Any large decrease in leukocyte count, which is often associated with the disease (21), would by this mechanism imply a rapid removal of factor VII too.

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