Rapid Communication

Human Monocytes Support Factor X Activation by Factor VIIa, Independent of Tissue Factor: Implications for the Therapeutic Mechanism of High-Dose Factor VIIa in Hemophilia

By Maureen Hoffman, Dougald M. Monroe, and Harold R. Roberts

High doses of recombinant factor VIIa are useful in managing bleeding in hemophiliacs with inhibitors. Whether this therapeutic effect of factor VIIa is dependent on tissue factor (TF) is a matter of debate. We examined the ability of freshly isolated human monocytes (which lack TF) to support the activation of coagulation-factor X by factor VIIa. The rate of factor-X activation by factor VIIa was accelerated in the presence of monocytes compared with the rate of X activation in solution. This activation of factor X on monocytes was saturable with a $K_m$ of about 400 to 600 pmol/L factor VIIa. The rate of activation was not inhibited by an excess of inhibitory anti-TF antibody or a Gla-containing fragment of prothrombin. In contrast to monocytes, an endothelial cell line did not support activation of factor X by factor VIIa. Our findings suggest that at least one cell type can accelerate activation for factor X by factor VIIa in the absence of TF. This activity requires higher concentrations of factor VIIa than does the TF mechanism. The concentrations of VIIa required are of a similar order of magnitude to those required for a therapeutic effect of VIIa in bleeding hemophilia with inhibitors. © 1994 by The American Society of Hematology.

Materials.

Dulbecco’s modified Eagle medium (DMEM) and Eagle’s minimum essential medium (EMEM) were purchased from Gibco. Fetal bovine serum (FBS) and horse serum were supplied by Biological Industries. Endotoxin-free phosphate-buffered saline (PBS) was from Beckman. HLA-DR, HLA-DQ, and HLA-DR in combination were purchased from Immunotech. Human hybridomas producing anti-TF antibody were obtained from Dr. Kenneth Manley, National Institutes of Health, Bethesda, MD.

Methods.

Endothelial cells (EA.hy 936 line) were grown in medium 199 supplemented with 10% FBS. Mononuclear phagocytes were isolated from normal donors by a second density gradient with Ficoll. Cells were cultured in AIM V supplemented with 10% FBS. Cell viability was determined by trypan blue dye exclusion.

Results.

Factor VIIa and factor IXa activated factor X more effectively in the presence of monocytes than in the absence of monocytes. The rate of factor-X activation by factor VIIa was increased by the presence of monocytes but not by the presence of factor X. The rate of factor-X activation by factor VIIa was increased by the presence of monocytes but not by the presence of factor X. The rate of factor-X activation by factor VIIa was increased by the presence of monocytes but not by the presence of factor X.
were incubated for 4 hours in 20 mmol/L HEPES, pH 7.4) containing 2.2 mmol/L EDTA, 1 mmol/L Spectrozyme ma, and 170 mmol/L NaCl, pH 7.4 (HBS) containing 3.5 mmol/L CaCl₂, 170 mmol/L purified factor X (plasma concentration) and different concentrations of added factor VIIa. Activation of factor X was verified to be linear for incubation periods with the monocytes of up to 90 minutes. At the end of the incubation period, a 100 μL aliquot of the supernatant was removed and added to an equal volume of HBS containing 2.2 mmol/L EDTA, 1 mmol/L Spectrozyme FXa, and 1 mg/mL albumin. The absorbance at 405 nm was monitored continuously in a plate reading spectrophotometer (Vmax, Molecular Devices Corp, Menlo Park, CA). Any baseline-Xa activity (cleavage of the chromogenic substrate in the absence of added factor VIIa) was subtracted from the values measured for all of the other wells.

In some assays, TFPI was added to the monocyte monolayer. TFPI binds to factor Xa, then the bimolecular complex inhibits TF/VIIa. When TFPI concentrations near that in plasma (100 ng/mL) were added to LPS-treated or fresh monocytes, no significant factor-Xa activity could be measured. We selected the lowest concentration of TFPI (10 ng/mL) that gave significant inhibition of factor-X activation by LPS-treated monocytes. This concentration of TFPI only minimally inhibited the amount of factor Xa activity in wells containing fresh monocytes. To verify that the chromogenic substrate cleavage was not actually caused by a small amount of thrombin being generated, we assayed the factor-X–activating activity of fresh monocytes in the presence of the thrombin inhibitor hirudin. At a concentration of 2 U/mL, hirudin had no effect on chromogenic substrate cleave in assays of fresh monocytes.

The inhibitory activity of the anti-TF antibody against human monocyte TF was verified as follows. Monocyte monolayers were cultured for 4 hours with 1 μg/mL LPS to induce TF expression, then assayed for activation of factor X in the presence of 200 pmol/L factor VIIa and varying concentrations of the anti-TF antibody. An antibody concentration of 10 μg/mL was found to completely

**Fig 1.** Inhibition of factor X activation on LPS-treated monocytes by anti-TF antibody. Monocytes were plated and cultured for 18 hours in the presence of 500 ng/mL LPS and assayed for factor-X activation in the presence of 500 pmol/L factor VIIa as described in Materials and Methods. The indicated amount of antibody was added 5 minutes before the incubation of monocyte monolayers with factors VIIa and X. After the incubation period, aliquots of the monocyte supernatants were transferred to wells containing the chromogenic substrate solution, and the rate of substrate cleavage monitored for the succeeding 20 minutes. The data points represent the results of three separate experiments.

**Activity assays.** Activation of factor X was measured using a specific chromogenic substrate. TF-dependent and independent factor-X activation was assayed as follows. Monocyte monolayers were incubated for 1 hour in 20 mmol/L HEPES, 150 mmol/L NBS.
inhibit the factor-VIIa-dependent activation of factor X (Fig 1). This concentration of anti-TF antibody was used to eliminate any contribution of TF in our studies of factor-X activation on fresh monocytes. TF-dependent Xase activity was defined as factor-VIIa-dependent factor-X activation that could be inhibited by the anti-TF antibody. Non-TF-dependent activity was factor-X activation at the same time. Results shown are for a typical experiment assayed in triplicate. Factor X activation was assayed as described in Materials and Methods. The inset shows an expansion of the curve for fresh monocytes and represents the values from nine separate experiments performed on separate days.

RESULTS

The rate of factor-X activation in the presence of LPS-treated and fresh monocytes as a function of increasing factor-VIIa concentration is shown in Fig 2. The rate of factor-X activation on monocytes was at least 10-fold greater after 18 hours in culture with LPS. The titration curve with respect to factor-VIIa concentration was saturable, implying that a limited number of sites on the monocyte surface were responsible for factor-X activation. The concentration of added factor-VIIa required for half maximal factor-X activation ($K_{1/2}$) was $145 \pm 50$ pmol/L VIIa (with the factor-X concentration held constant at 170 nmol/L) for LPS-treated monocytes.

Data analysis. The data were fitted to a right hyperbola defined by the equation: $v = V_{max} \times [VIIa]/(K_{1/2} + [VIIa])$ using the curve-fitting function of DeltaGraph Professional (Delta Point, Inc, Monterey, CA).

Fig 3. The effect of an anti-TF antibody and TFPI on the amount of factor X activation by fresh and LPS-treated monocytes. Monocytes were isolated and cultured as for Fig 2. The anti-TF antibody (10 mg/mL) and TFPI (10 ng/mL) were added 5 minutes before the addition of factor VIIa to the factor-X activation assays. After the incubation period, aliquots were removed and added to separate wells containing the chromatographic substrate solution. The rate of chromogenic substrate cleavage was monitored over the succeeding 20 minutes. The figure represents the results of four separate experiments. The amount of factor X activated by LPS Monos was significantly reduced by anti-TF antibodies and by TFPI ($P < 0.05$, Student’s paired t-test). The amount of factor X activated by fresh monocytes was also slightly lower in the presence of TFPI, but the difference was not statistically significant.
mediated binding to anionic phospholipid plays a role in factor X activation by high concentrations of factor VIIa. We hypothesized that TF-independent factor-X activation by high concentrations of factor VIIa might be related to Mac-1–dependent factor-X binding and activation on monocytes.11 Therefore, we examined the effect of an anti-Mac-1 antibody on factor X activation on monocytes. Not all anti-Mac-1 antibodies inhibit factor X binding and activation.16,17 We purchased a MoAb that inhibited Mac-1–dependent binding of factor X to monocytes by 98% (M 1/70). We verified, by indirect immunofluorescence and flow cytometry, that the antibody had bound to freshly isolated monocytes (data not shown). However, this antibody had no effect on factor-X activation on fresh monocytes in the presence of 2000 pmol/L factor VIIa (data not shown).

DISCUSSION

Fresh monocytes, but not an endothelial cell line, supported TF-independent activation of factor X by factor VIIa. The rate of factor Xa generation was less than 10% of the rate achieved after induction of TF expression on the monocytes. The TF-independent factor X activation was not simply caused by the provision of anionic phospholipid by monocyte membranes, nor was it caused by a Mac-1–dependent mechanism. This factor-X–activating activity was detected at concentrations of factor VIIa greater than those required for TF-mediated activity. Thus, this TF-independent factor-X–activating activity could play a role in the ability of high doses of factor VIIa to overcome bleeding in hemophilia patients.

In this report, we only tested two cell types. The ability to support factor X activation by factor VIIa may not be unique to monocytes. However, the fact that endothelial cells do not have this property is consistent with the finding that administration of high doses of factor VIIa rarely (if ever) leads to the development of DIC.5 Monocytes comprise less than 5% of the total circulating leukocytes. Because the rate of factor Xa generation on monocytes is quite low in the absence of TF, significant amounts of factor Xa might only accumulate at sites of injury where leukocytes and platelets adhere. At sites of injury tissue macrophages or stromal cells might also be able to support factor Xa production in the presence of high doses of factor VIIa. TF-independent Xa generation might be an important source of factor Xa in a hemophilic patient, because it would not be inhibited by TFPI. Such an activity could continue to provide factor Xa after the more potent TF-dependent activity has been inhibited.

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REFERENCES


9. Reuning U, Preissner KT, Muller BG: Two independent binding sites on monolayers of human endothelial cells are responsible for interaction with coagulation factor VIII and factor Vlla. Thromb Haemost 69:197, 1993


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Human monocytes support factor X activation by factor VIIa, independent of tissue factor: implications for the therapeutic mechanism of high-dose factor VIIa in hemophilia [see comments]

M Hoffman, DM Monroe and HR Roberts