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CULTURED HUMAN ENDOTHELIAL CELLS BIND AND INTERNALIZE HIGH MOLECULAR WEIGHT KININOGEN. Freek van Iwaarden, Philip G. de Groot and Bonno N. Bouma. Dept. of Haematology, University Hospital, P.O.Box 16250, 3500 CG Utrecht, The Netherlands.

The presence of High Molecular Weight kininogen (HMWK) was demonstrated in cultured human endothelial cells (EC) by immunofluorescence techniques. Using an enzyme linked immunosorbent assay a concentration of 58 ng HMWK/10^6 cells was determined. Immunoprecipitation studies performed with lysed metabolically labelled endothelial cells and mono-specific antisera directed against HMWK suggested that HMWK is not synthesized by the endothelial cells. Endothelial cells cultured in the presence of HMWK-depleted serum did not contain HMWK. This suggests that endothelial cells can internalize HMWK. Using ^{125}I-HMWK it was demonstrated that cultured endothelial cells bind HMWK in a time-dependent, specific and saturable way. The cells were found to internalize ^{125}I-HMWK, since ^{125}I-HMWK was detected in solubilized endothelial cells after the cell bound ^{125}I-HMWK had been eluted with dextran sulphate.

The binding of ^{125}I-HMWK required the presence of zinc ions. Optimal binding of ^{125}I-HMWK was observed at 50 μM Zn^{++}. Calcium ions inhibited the Zn^{++} dependent binding of ^{125}I-HMWK to EC. In the presence of 3 mM $CaCl_2$ the total binding of ^{125}I-HMWK was significantly decreased, and a concentration of 200 μM Zn^{++} was required for the binding of ^{125}I-HMWK to the cells. Higher Ca^{++} concentrations did not further decrease the binding of ^{125}I-HMWK. Analysis of the binding data by the ligand computer program indicated 3.2 x 10^6 binding sites per cell for HMWK with a Kd of 35 nM at 50 μM $ZnCl_2$ and 1 mM $CaCl_2$. Specific binding of HMWK did also occur at physiological plasma Zn^{++} concentrations. Half maximal binding was observed at HMWK concentrations of ± 105 nM at 10 μM $ZnCl_2$ and 45 nM at 25 μM $ZnCl_2$. The HMWK binding sites were saturated at HMWK concentrations of 130 nM with 1.6 x 10^6 molecules of HMWK bound per cell and at 80 nM with 2.8 x 10^6 molecules of HMWK bound per cell at 10 and 25 μM $ZnCl_2$ respectively. These results suggest that at physiological zinc, calcium and HMWK concentrations the HMWK binding sites on the endothelial cell are saturated. The presence of HMWK on the endothelial cell surface may play a role in the initiation of the intrinsic coagulation pathway.

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INTERACTION OF THROMBIN AND FACTOR Xa WITH BOVINE VASCULAR ENDOTHELIAL CELLS, SMOOTH MUSCLE CELLS AND RAT HEPATOMA CELLS. Maciej Dryjski, M.D., Ph.D., Be-Sheng Kuo, Ph.D., and Thorir D. Bjornsson, M.D. Division of Clinical Pharmacology, Department of Medicine and Department of Surgery, Thomas Jefferson University, Philadelphia, PA, U.S.A.

The inhibition of thrombin as well as of factor Xa has been thought to occur primarily in plasma through the neutralizing action of the serine protease inhibitor antithrombin III (AT-III). However, inhibition of thrombin and Xa by this mechanism may not be sufficient for effective elimination of these clotting factors in states of increased coagulation activity. The potential role of the vascular endothelium in the inhibition of clotting factor activities has therefore received attention in recent years. The aim of the present investigation was to characterize the binding and inhibition of thrombin and factor Xa to the vascular endothelial cell (EC), smooth muscle cell (SMC) and rat hepatoma cell (RHC) in vitro, as well as to evaluate the effects of plasma constituents upon the inhibition of these factors. Purified bovine thrombin and factor Xa were used. The enzymatic activities of both factors were assayed using chromogenic substrates. The cells were exposed for 5 U/ml thrombin or 0.5 U/ml factor Xa. After 10 minutes incubation, the initial thrombin activity in the solution had decreased by about 20% in case of EC and SMC and about 11% when incubated with RHC. Thrombin activity recovered from the cell surface amounted to 0.02 U/cm^2. When the cells with the surface bound enzyme were incubated with defibrinogenated plasma or AT-III for 30 seconds, only about 10% and 25-40%, respectively, of initial activity could be found. In similar experiments with factor Xa, after 10 minutes incubation, the initial activity in the solution had decreased by 10%. Factor Xa activity recovered from the cell surface was 0.001 U/cm^2. After 30 seconds exposure to AT-III, no cell surface related factor Xa activity was recovered, whereas 10% of the cell surface activity was recovered after incubation with defibrinogenated plasma. It is concluded that thrombin and factor Xa are taken up and inhibited by EC, SMC and RHC cell surfaces in similar ratios suggesting that cell surface-mediated inactivation of activated clotting factors is not restricted to vascular wall cells. The inactivation of factor Xa was dependent on AT-III, however, the inactivation of thrombin was further promoted by an additional unidentified plasma constituent.

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VON WILLEBRAND FACTOR AS A REGULATOR OF ENDOTHELIAL CELL-DEPENDENT FACTOR Xa FORMATION. Joost A.Koedam (1), Jan J. Sixma (1), Bonno N. Bouma (1), David M. Stern (2), and Peter P. Nawroth (2). Dept. of Haematology, University Hospital Utrecht, The Netherlands (1), and Oklahoma Medical Research Foundation, OK, USA (2).

Factor Xa (FXa) formation on the endothelial cell surface involves a membrane protein which promotes assembly of the Factor IXa-VIII-X complex. Since Factor VIII (FVIII) can also interact with von Willebrand factor (VWF), which is both present in the plasma and expressed by endothelium, we examined the effect of VWF on FXa formation. When monolayers of conditioned endothelium were incubated with FIXa (2.8 nM), FVIII (0.1 unit/ml), and FX (65 nM), the rate of FXa formation could be decreased in a dose-dependent manner by addition of VWF. At 10 min of incubation, a VWF concentration of 5 μg/ml caused a 93% inhibition of FXa formation. Addition of a polyclonal antibody $(F(ab')_2)$ directed against VWF which blocks formation of the FVIII-VWF complex, increased endothelial cell-dependent Factor IXa-VIII-mediated activation of FX by 2- to 3-fold in the absence of exogenous VWF, indicating a role for endogenous VWF. Since no VWF was detectable using a sensitive radioimmunoassay in reaction mixture supernatants, endothelial cell-associated VWF was considered as a potential binding site for FVIII, thereby removing it from the reaction mixture. In addition, we found no effect of either exogenous VWF nor anti VWF-antibodies when FVIII was activated with thrombin before starting the incubation.

Radioligand binding studies were carried out with ^{125}I-FVIII and demonstrated binding to a limited number of sites on intact endothelial cell monolayers which could be partially blocked by anti-VWF $F(ab')_2$. These results suggest that VWF may regulate FXa formation on the endothelial cell surface.

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EXTRINSIC ACTIVATION OF HUMAN COAGULATION FACTORS IX AND X ON THE ENDOTHELIAL SURFACE. V.J.J. Bom (1), V.W.M. van Hinsbergh (2), J.H. Reinalda-Poot, R.W. Mohanlal (1) and R.M. Bertina (1). Haemostasis and Thrombosis Research Unit, Leiden University Hospital (1) and the Gaubius Institute, Health Research Division TNO (2), Leiden, The Netherlands.

In previous studies using purified human tissue factor (TF) apoprotein reconstituted with PS/PC membranes, no dramatic differences between the kinetic parameters for extrinsic activation of factor X (FX) and factor IX (FIX) could be observed. In vivo however, TF is an integral part of a complex biological membrane. Therefore we studied the kinetics of extrinsic FX and FIX activation on endothelial cells.
TF expression was stimulated by incubation of cultured human endothelial cells with endotoxin (4 hr). The cells were washed and incubated with FVII(a), FX and/or FIX in the presence of $CaCl_2$. FXa and FIXa formation were measured with a sensitive spectrophotometric assay (S2337) and an immunoradiometric assay (IRMA-IXa), respectively. Unstimulated cells did not induce significant activation of FX or IX. Stimulation of the cells and recombination of exposed TF with FVIIa resulted in rapid activation of both substrates. Rates of FXa formation reached a maximum after a lag of about 1 min. Upon prolonged incubation the rate of FXa formation progressively decreased, probably by inactivation of FVIIa by product Xa. At the other hand, FIXa formation was linear in time for at least 30 min. When added together, FIX was found to be a weak, competitive inhibitor of FX activation, while FIX activation was severely inhibited by FX. Both FX and FIX activation were dependent on the presence of TF (sensitive to α-TF antibodies) and FVIIa. The calculated Km of FIX (~0.09μM) and of FX (~0.07μM) for extrinsic activation at the endothelial surface were both higher than those observed in a cell free system using purified TF apoprotein and the ratio of Km-FIX/Km-FX was about 6-fold increased. The ratios of Vmax-FIX activation/Vmax-FX activation for both systems were similar (~0.3). These data indicate that the microenvironment of TF in the biological membrane does not introduce important alterations in the kinetic parameters of TF-FVIIa dependent activation reactions in favour of extrinsic FIX activation.

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