Effector Cell Protease Receptor-1 Is a Vascular Receptor for Coagulation Factor Xa*

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The binding and assembly of the coagulation proteases on the endothelial cell surface are important steps not only in the generation of thrombin and thrombogenesis, but also in vascular cell signaling. Effector cell protease receptor (EPR-1) was identified as a novel leukocyte cell surface receptor recognizing the coagulation serine protease Factor Xa but not the precursor Factor X. We now demonstrate that EPR-1 is expressed on vascular endothelial cells and smooth muscle cells. Northern blots of endothelial and smooth muscle cells demonstrated three abundant mRNA bands of 3.0, 1.8, and 1.3 kDa. 125I-Labeled Factor Xa bound to endothelial cells in a dose-dependent saturable manner, and the binding was inhibited by antibody to EPR-1. No specific binding was observed with a recombinant mutant Factor X in which the activation site was substituted by GlntopreventtheproteolyticconversiontoXa.

EPR-1 was identified immunohistochemically on microvascular endothelial and smooth muscle cells. Functionally, exposure of smooth muscle cells or endothelial cells to Factor Xa induced a 3-fold and a 2-fold increase in [3H]thymidine uptake, respectively. However, receptor occupancy alone is insufficient for mitogenic signaling because the active site of the enzyme is required for mitogenesis. Thus, EPR-1 represents a site of specific protease-receptor complex assembly, which during local initiation of the coagulation cascade could mediate cellular signaling and responses of the vessel wall.

The ordered assembly of proteins of the coagulation cascade on cell surfaces results in greatly enhanced kinetic efficiency of protease function and protease generation as well as protection of proteases from their extracellular inhibitors. Endothelial cells, which constitute the cellular barrier between blood and the smooth muscle cells of the vessel wall, may initiate and assemble functional thrombin-generating cascades following vessel injury. In addition to thrombus generation, proteins in the cascade can directly initiate receptor-coupled cellular signaling and functional responses. In addition to its role in clotting and in the anticoagulation pathways mediated by specific proteolytic conversion of fibrinogen to fibrin and thrombomodu-
conjugated goat anti-mouse IgG was from Tago Immunologisch (Burlingame, CA). 5-fluorodeoxyuridine (FUDR) was purchased from Calbiochem (La Jolla, CA). Anti-annexin II (12, 13) was provided by Dr. J. T. Martin, University of Virginia (Charlottesville, VA). A biotinylated rabbit anti-human IgG was from Zymed (South San Francisco, CA). A fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse IgG was from Tago Immunologisch (Burlingame, CA). 3-Amino-9-ethylcarbazole (AEC) chromagen (Dako) was obtained from Kimoto (Dako). Hematoyxlin was used as a counterstain.

**RESULTS**

**Characteristics of Factor Xa Binding to Vascular Cells—**Radiolabeled Factor Xa was recombinantly expressed in a baculovirus expression system (13). The purity of the recombinant Factor Xa was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (14). The purity of the recombinant Factor Xa was determined to be >95% by mass spectrometry. The purity of the recombinant Factor Xa was determined by mass spectrometry to be >95%. The purity of the recombinant Factor Xa was determined to be >95%. The purity of the recombinant Factor Xa was determined to be >95%.

**Fluorescence-activated Cell Sorter—**Flow Cytometry—Human endothelial cells were plated in 96-well plates at a density of 3 × 10^4 cells/well and cultured for 24 h to allow for maximum adherence. The cells were then incubated with various concentrations of recombinant Factor Xa for 24 h. The cell monolayers were then washed twice in PBS, 0.02% BSA, and resuspended in 1% NIH-15 medium. The cell monolayers were then washed twice in PBS, 0.02% BSA, and resuspended in 1% NIH-15 medium. The cell monolayers were then washed twice in PBS, 0.02% BSA, and resuspended in 1% NIH-15 medium. The cell monolayers were then washed twice in PBS, 0.02% BSA, and resuspended in 1% NIH-15 medium.

**Statistical Analyses—**The data were analyzed by ANOVA. Differences in the mean values were considered significant if p < 0.05.
binding sites/cell (not shown). A Factor X mutant (Arg\textsuperscript{196}→Gln), which is not converted to the active serine protease Factor Xa by TF\textsuperscript{VIIa}, did not exhibit significant binding to endothelial or smooth muscle cells (Fig. 1), thereby providing supplemental data to support the specificity for Factor Xa.

Receptor Identity—In order to identify the endothelial cell receptor for Factor Xa, monoclonal antibodies recognizing the Factor Xa receptor previously identified on leukocytes, namely EPR-1 (1), were tested for their ability to inhibit binding of \textsuperscript{125}I-Factor Xa to endothelial cell monolayers (Fig. 2). Anti-EPR-1 monoclonal antibody B6 diminished specific binding to endothelial cells by approximately 60%, whereas characterized antibodies to annexin II (12, 13), TF (14, 15), and PECAM (17) failed to block binding of Factor Xa (not shown). In addition, no significant inhibition of Xa binding to endothelial cells was observed with monoclonal antibody OKM1 to Mac-1, which blocks binding of Factor X to leukocyte surface Mac-1 (16) (not shown). To further substantiate endothelial cell surface expression, we demonstrated mAb B6 reactivity with the endothelial cell surface by flow cytometry (Fig. 3).

To determine whether vascular cells can synthesize and express EPR-1 \textit{in vivo}, immunohistochemical analysis for EPR-1 was carried out (Fig. 6). Anti-EPR antibody strongly reacted with blood vessels in frozen sections of human tonsil (Fig. 6A). Both vascular smooth muscle cells and endothelial cells were immunoreactive. In contrast control mouse IgG did not react (data not shown).

To explore the potential functional impact of docking of Factor Xa with vascular cell EPR-1, \textit{in vitro} mitogenesis experiments were performed. These experiments were designed to
exclude proliferative responses that might result from local
generation of thrombin. In Fig. 7, we show a representative
experiment that demonstrates the ability of Factor Xa at 50 nM,
slightly in excess of the 32 nM $K_d$, to induce 3-fold increased
proliferation of arterial smooth muscle cells relative to cells
grown in medium alone in an 18-h assay. Factor Xa-induced
proliferation was inhibited by a monoclonal antibody (B6)
 against EPR-1. Notably, addition of the thrombin inhibitor,
hirudin, did not diminish Factor Xa-induced mitogenesis,
thereby excluding cellular signaling by thrombin via the
thrombin receptor. Also, neither very highly repurified Factor
X, with Factor VII contamination at $<1:50,000$, nor a nonacti-
vable mutant Factor X, neither of which bind or react with
EPR-1, influenced these cell proliferation assays.

To determine if the active site of Factor Xa was required for
Factor Xa-induced smooth muscle cell mitogenesis, we per-
formed proliferation assays in the presence of recombinant
TAP, which blocks Xa conversion of prothrombin to thrombin,
or with Factor Xa, which was inactivated at the active site by
reacting with the covalent inhibitor glutamyl-glycyl-arginyl-
chloromethylketone. In Fig. 8 we show that Factor Xa (50 nM)
increased smooth muscle cell mitogenesis by more than 4-fold
relative to untreated cells. However, Factor Xa in the presence
of TAP (50 µg/ml) or glutamyl-glycyl-arginyl-chloromethyl-
ketone (50 nM) did not significantly increase smooth muscle cell
mitogenesis, implying that the intact catalytic active site of
Factor Xa is required for EPR-1/Xa vascular cell signaling.

**DISCUSSION**

The macromolecular assembly of the proteins of the coagu-
lation cascade on cell surfaces can result in a diverse set of
effects including conversion of zymogens to highly specific pro-
teases, generation of proteolytic fragments of various substrate
proteins, platelet and endothelial cell activation via the throm-
bin receptor, and formation of the fibrin gel. Assembly of these
proteases on cell surface receptors results in receptor-protease
complexes with greatly enhanced function such as for assembly
of the TF-VIIa initiation complex (10), the thrombin-thrombo-
modulin complex (26), the prothrombinase complex Factor Xa-
Factor Va (27), and the intrinsic complex factor Ixa-Factor
VIIIa (28). Less well appreciated has been the docking of pro-
teases on cognate cell surface receptors, a molecular mecha-
nism well recognized for the adhesive proteins and their recep-
tors but also evidenced by the docking of Factor X on the
integrin Mac-1 (16), the assembly of protein S and homologous
proteins on Tyro 3 (29), and the identification and cloning of
EPR-1, a novel high affinity receptor for Factor Xa (1, 9). These
receptors as well as the assembly of Factor VIIa on tissue factor
not only mediate functional molecular assemblies on the cell
surface but possess the capability of cellular signaling via their

**FIG. 5. Western analysis of EPR-1 in human EC and smooth
muscle cells.** Endothelial and smooth muscle cells were washed, sol-
ubilized in homogenization (lysis) buffer, and centrifuged. Superna-
tants (220 µg of protein/lane) were analyzed on 7% SDS-PAGE gels.
Following transfer to Immobilon-P, the membrane was reacted with
anti-EPR-1 monoclonal antibody B6 at 4 µg/ml. Samples were visual-
ized using the ECL detection kit.

**FIG. 6. Immunohistochemical iden-
tification of EPR-1 in the microvas-
culature.** Frozen sections of human ton-
sil were incubated with monoclonal
antibody to EPR-1 (1:1000) (A). Adjacent
sections of tonsil were incubated with
monoclonal anti-human Factor VIII
(1:500) (B). Reactions were developed
with second antibody in an 3-amino-9-
ethylcarbazole chromagen substrate sys-
tem (magnification, 400×).
sent the mean of triplicate wells. Radioactivity was quantified by scintillation counting. The data demonstrated that an antibody to EPR-1 reacted with a specific binding (Fig. 2). Western immunoblotting experiments (1) demonstrated EPR-1 immunostaining of vessels within human tissue, introducing the potential for EPR-1 to participate in local activation of coagulation cascades, which might therefore elicit smooth muscle cell mitogenesis in vivo. Recent data suggest that in addition to participating in protease generating molecular pathways, some of these proteins may directly initiate receptor-coupled intracellular signaling and cell activation. Thrombin, in addition to cleaving fibrinogen to fibrin, also proteolytically activates the cell surface thrombin receptor resulting in intracellular signaling via a G-protein-coupled mechanism (32, 33). Protein S, an homologous γ-carboxylated protein, but not a protease, binds to the receptor Tyro 3 resulting in intracellular signals (29), at least under some experimental conditions (34). Similarly, TF initiates a cytosolic Ca²⁺ signal following binding of the coagulation protease factor Vila (30). In addition, the product of the gas6 gene, which is related to protein S, a negative co-regulator of the coagulation cascade, potentiates smooth muscle cell proliferation mediated by Ca²⁺ mobilizing receptors (35).

EPR-1 was originally identified on monocytes and various leukocyte subsets as a binding protein for the protease Factor Xa (1, 2). On these cell types, the assembled EPR-1-Xa complex can facilitate activation of prothrombin to thrombin, though weakly (1, 36). In addition, occupancy of EPR-1 by Factor Xa or by certain surrogate mAb ligands, increases cytosolic free [Ca²⁺], in single adherent T cells and co-stimulates lymphocyte proliferation initiated by suboptimal stimulation through the CD3/T cell receptor complex (2). Whether this is a direct result of receptor ligation or signaling is initiated by other means (i.e. patching the receptor) has not been established. Because mitogenesis has been implicated as one of the responses triggered by Factor Xa binding to vascular cells (6, 37), we initially hypothesized that a molecular mechanism for the action of Factor Xa might involve direct receptor-ligand assembly of Factor Xa and EPR-1. Factor Xa induced a 3-fold increase in thymidine incorporation, which was inhibited by an antibody to EPR-1. Importantly, very highly purified Factor X and mutant recombinant Factor X, which cannot be proteolytically cleaved to Factor Xa, did not stimulate arterial smooth muscle cell proliferation. This supports our concept that Factor X must be cleaved to Factor Xa before binding to EPR-1 occurs, and the cellular response is evoked by EPR-1-Xa formation. Although Gasic et al. (37) demonstrated that Factor X was mitogenic for smooth muscle cells, our results suggest that proteolytic activation of Factor X to Factor Xa is necessary to elicit the cellular...
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response. We have found functionally significant Factor VII contamination of conventional highly purified Factor X. These experiments were conducted with additionally purified Factor X, which has significantly reduced Factor VII levels (<1.50,000). We attribute differences in experimental results of prior studies to the quality of protein preparations. This interpretation is further supported by the lack of mitogenic effect of the mutant recombinant Factor X where the activation of the sessile bond is was mutated by the replacement of Arg<sup>196</sup> with Gin.

The mitogenicity observed in our studies was directly mediated by Factor Xa but not via thrombin generation. This was confirmed by the inability of the thrombin inhibitor, hirudin, to decrease Factor Xa-induced response-coupled mitogenic activity (Fig. 7). However, the intact catalytic active site of Factor Xa is required for EPR-1-Xa smooth muscle cell mitogenic signaling because glutamyl-glycyl-arginyl-chloromethylketone or Factor Xa in the presence of TAP did not induce smooth muscle cell mitogenesis (Fig. 8). Binding of Xa to EPR-1 did not require the active site because both Factor Xa and Factor Xa in the presence of TAP bind equivalently to vascular smooth muscle cells (data not shown). Thus, post-receptor binding signaling events but not EPR-1-Xa binding require the catalytic active site of Xa to be functional. It will now be necessary to define the proteolytic events leading to this cellular response. Factor Xa was also mitogenic for endothelial cells, inducing a 2-fold increase in [<sup>3</sup>H]thymidine incorporation (data not shown). However, the mitogenic response was not blocked by a purified monoclonal antibody directed against EPR-1. Therefore, the mechanism(s) of mitogenic signaling by factor Xa in endothelial cells is not clear and is the subject of further investigation.

Previous reports by Stern and others have documented the ability of factor Xa to bind to vascular cells (6, 38, 39). Although endothelial cells synthesize Factor V (40), it is unclear whether they express Va on their surface to support the association of factor Xa. Colman and his colleagues were unable to detect Factor V on the surface of adherent monolayers of human endothelial cells; however, they found that injury induced expression of Factor V (41). Endothelial cells grown in human serum, devoid of factor V coagulant activity, expressed approximately 15,000 molecules/cell as measured by a monoclonal antibody directed against factor VVa (42). The source of this Va, i.e. endothelial cell or serum derived, was unclear; however, the reported number of Factor Va molecules per cell are entirely inadequate to account for the ~220,000 sites for Factor Xa binding found in the present studies. Others have shown that although prothrombin activation by endothelial cells is inhibited by anti-Factor V antibodies, the binding of Factor Xa does not appear to be affected (39). The binding of Factor Xa to HepG2 and J82 tumor cells has also been shown to be Factor Va-independent (43).

The specific role played by EPR-1 on endothelial cell prothrombinase complex assembly and prothrombin formation is under investigation. Vascular mechanisms of prothrombin activation have been extensively characterized in the platelet model, in which membrane assembly of Factor V/Va functions as a nonenzymatic co-factor to increase the catalytic activity of factor Xa by 300,000-fold (27). Although the platelet is probably the primary site of coagulation complex assembly in vivo, the endothelium, in vitro, is also capable of forming a prothrombinase complex (44). The catalytic efficiency of prothrombin activation by the endothelium (in cell culture) is similar to platelets (44). Synthetic EPR-1 peptides inhibit prothrombinase activity on endothelial cells (45) and monoclonal anti-EPR-1 antibodies inhibit prothrombinase activity on platelets (46) in a dose-dependent manner (each in the absence of exogenous V/Va). However, the relationship of EPR-1 and factor Va and the relative importance of each to prothrombin formation on the endothelial cell surface in vivo remains to be determined. More importantly, the contribution of the vascular endothelium (relative to the platelet) to prothrombinase complex assembly and coagulation in vivo is also unclear, but the platelet is likely to play a more important role in this regard.

In summary, our data support the hypothesis that EPR-1, a receptor on endothelial cells and vascular smooth muscle cells, can specifically mediate a Factor Xa interaction with a concomitant cellular response. Further experiments are needed to determine the signaling events, which are initiated by the binding of Factor Xa to EPR-1, but because the active site of Xa is required for mitogenic signaling, it is likely that the enzymatic activity of the protease and not receptor occupancy per se is involved in the signaling cascade. In addition, it is recognized that the coagulation and related protease activity are generated in vivo during inflammatory and hemostatic responses. Binding of locally generated Factor Xa to EPR-1 on vascular cells may generate an “independent accessory signal” to increase arterial smooth muscle cell proliferation and contribute to the early molecular events associated with vascular cell activation that accompanies vascular disease.

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Note Added in Proof—Mitogenesis of vascular smooth muscle cells in response to Factor Xa (but not Factor X) was also reported by Ko et al. (Ko, F., Yang, Y. C., Huang, S. C., and Ou, J. T. (1996) J. Clin. Invest. 98, 1493–1501). Consistent with our results, they attribute this mitogenic effect to the serine protease activity of Factor Xa with the subsequent release of PDGF by these cells.

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