Activation Mechanism of Anticoagulant Protein C in Large Blood Vessels Involving the Endothelial Cell Protein C Receptor

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Summary

Protein C is an important regulatory mechanism of blood coagulation. Protein C functions as an anticoagulant when converted to the active serine protease form on the endothelial cell surface. Thrombomodulin (TM), an endothelial cell surface receptor specific for thrombin, has been identified as an essential component for protein C activation. Although protein C can be activated directly by the thrombin–TM complex, the conversion is known as a relatively low-affinity reaction. Therefore, protein C activation has been believed to occur only in microcirculation. On the other hand, we have identified and cloned a novel endothelial cell surface receptor (EPCR) that is capable of high-affinity binding of protein C and activated protein C. In this study, we demonstrate the constitutive, endothelial cell–specific expression of EPCR in vivo. Abundant expression was particularly detected in the aorta and large arteries. In vitro cultured, arterial endothelial cells were also found to express abundant EPCR and were capable of promoting significant levels of protein C activation. EPCR was found to greatly accelerate protein C activation by examining functional activity in transfected cell lines expressing EPCR and/or TM. EPCR decreased the dissociation constant and increased the maximum velocity for protein C activation mediated by the thrombin–TM complex. By these mechanisms, EPCR appears to enable significant levels of protein C activation in large vessels. These results suggest that the protein C anticoagulation pathway is important for the regulation of blood coagulation not only in microvessels but also in large vessels.

Protein C pathway is an indispensable regulatory mechanism of blood coagulation, since deficiencies in this pathway lead to thrombosis (1, 2). Protein C is a γ-carboxyglutamic acid containing protein (3) and circulates as a zymogen form of serine protease (4). Protein C functions as an anticoagulant when converted to its active form by thrombin (5), and activation of protein C was demonstrated to be greatly enhanced on the endothelial cell surface (6). Thrombomodulin (TM) has been identified as an essential component on the endothelial cell surface (7, 8), and direct activation of protein C by the thrombin–TM complex has been demonstrated. However, the activation by the complex was observed as a relatively low-affinity reaction and the $K_d$ value was calculated as 0.7–1.0 μM (9–11), which is 15 times higher than that of the protein C concentration (65 nM) in the circulation (12). Under these conditions, protein C activation appears unlikely to occur under normal physiological conditions. One of the proposed explanations for this has been that protein C activation mediated by the thrombin–TM complex occurs only in the microcirculation because of the greater ratio of endothelial cell surface area to blood volume (13).

On the other hand, we (14) and others (15) found that protein C and activated protein C (APC) bound to cultured endothelial cells with a relatively high affinity ($K_d = 30$ nM). By expression cloning, we identified a novel endothelial cell surface receptor that is capable of protein C–APC binding in the presence of physiological concentrations of calcium (14) and magnesium (16). We designated this molecule as an endothelial cell–protein C–APC receptor (EPCR). EPCR is a type 1 transmembrane glycoprotein containing...
two domains in the extracellular region that are homologous to the α1 and α2 domains of CD1/MHC class I molecules (17). The highly conserved structural features and ligand binding function of EPCR that are found between species suggest an important physiological function for the receptor molecule (18). In vitro cultured human cells, significant levels of EPCR expression has been demonstrated only in human umbilical vein endothelial cells (14). In this study, we demonstrate in vivo expression of EPCR by immunohistochemical analysis. The abundant expression of EPCR was found on the endothelial cells of the aorta where protein C activation has been considered unlikely to occur. In addition, we found that protein C activation mediated by the thrombin-TM complex was promoted as a relatively high-affinity reaction in the presence of EPCR.

Materials and Methods

Cells. Human primary venous endothelial cells (VECs) and arterial endothelial cells (AECs) were obtained from Cell Systems (Kirkland, WA) and maintained according to the manufacturer's protocol. Human kidney 293 cells (CRL 1573; American Type Culture Collection, Rockville, MD) were maintained in DMEM containing 10% fetal bovine serum. Stable transfected cell lines of 293 cells expressing human EPCR and/or TM were established as follows: Cells were transfected with a human EPCR cDNA construct in a mammalian expression vector, pEF-BOS (19), and/or a human TM cDNA (a gift from J.F. Parkinson, Lilly Research Lab., Indianapolis, IN) constructed in a mammalian expression vector pBKH-EP (a gift from T. Fujimoto, Hiroshima University, Hiroshima, Japan) by the calcium/phosphate method as described (14). Cell lines were established by G-418 selection, followed by subcloning. T2 cells are positive for TM, ET1 cells and ET2 cells are positive for both EPCR and TM. Establishment of these cell lines was done in the Oklahoma Medical Research Foundation (Oklahoma City, OK) under the support of C.T. Esmon (Oklahoma Medical Research Foundation). Negative control N1 cells and EPCR-positive E7 cells were established as described (16).

Histology. Sections (4 μm) of human aorta and lung were fixed in cold acetone, paraffin embedded processed, and then stained with JRK-1 monoclonal antibody and anti-EPCR goat Ig (16). Constitutive, endothelial cell-specific expression of EPCR was demonstrated with these antibodies. The most abundant expression of EPCR was detected on the endothelial cells of the aorta (Fig. 1A). Moderate expression was also detected on the endothelial cells of relatively large-sized vessels (data not shown). On the other hand, little or no expression could be detected in microvessels such as the lung capillaries (Fig. 1C).

We also examined serial sections using an anti-TM monoclonal antibody. TM was expressed widely in both arterial and venous vessels as described (21), and strong expression was detected in the capillaries (data not shown). These results indicated that EPCR and TM coexpress on the endothelial cells in vessels with relatively large size. Coexpression of EPCR and TM was also demonstrated by the flow cytometer analysis using in vitro cultured endothelial cells (Fig. 2). Strong expression of EPCR was detected on cultured AECs. EPCR was also detectable in cultured VECs, however, the expression level was several times lower than that in the AECs. TM was expressed on both AECs and VECs. In contrast to EPCR, strong expression of TM was detected on VECs and a lower and heterogeneous expression was detected on AECs. Since the dissociation constant between protein C and EPCR is 30 nM, which is approximately half of the blood concentration of protein C, the majority of EPCRs expressed to the blood stream should be holding protein C under physiological conditions. These findings encouraged us to reinvestigate the mechanism of protein C activation, which has been believed to be mediated by only the
thrombin–TM complex. We compared activities of protein C activation by AECs and VECs, which express different amounts of EPCR s and TM on their surface (Fig. 2). In both cases, protein C was converted to APC only in the presence of thrombin. Interestingly, promotion of protein C activation by AECs was comparable to VEC-mediated activation (Fig. 3), despite the lower level of TM expression (Fig. 2). In addition, activation of protein C on AECs was observed as a relatively high-affinity reaction with a $K_d$ value of 125 ± 72 nM, suggesting contribution by the high-affinity protein C receptor to the reaction. The $K_d$ value for the reaction by VECs was slightly lower (162 ± 54 nM) than that of AECs. This could be due to the low level of expression of EPCR on VECs.

To analyze in detail the activation mechanism, we established transfected human kidney 293 cell lines that express receptor molecules. The 293 cells are negative for EPCR and TM. Mock-transfected N1 cells are used as a negative control, E7 cells are EPCR positive as described previously (16), T2 cells are positive for TM alone, and ET1 and ET2 are dual-positive cell lines expressing both EPCR and TM. Stable antigen expression was demonstrated by flow cytometer analysis as shown in Fig. 4. Protein C was incubated with these cell lines in the presence of thrombin and the subsequently generated APC was measured.
bin-mediated protein C activation was not detectable on the negative control N1 cells. EPCR-positive E7 also could not promote activation. On the other hand, thrombin-dependent protein C activation was detected with cells positive for TM. T2 cells promoted activation only in the presence of thrombin. The activation was completely inhibited by a functional blocking anti-TM monoclonal antibody, CTM1009 (Fig. 5A). These results indicated that activation of protein C on T2 cells was mediated by the thrombin–TM complex. However, the conversion rate was quite low when the rate per cell was calculated. In contrast, dramatic activation was demonstrated with EPCR/TM dual-positive cells (Fig. 5A). Five to six times more activation was detected in the case of dual-positive cells as compared with cells positive for TM alone.

We next compared APC generation by T2 cells and ET1 cells in the presence of increasing protein C concentration (Fig. 5B). Protein C activation by T2 cells was greatly dependent on the protein C concentration. On the other hand, dual positive ET1 cells could promote significant activation, even at the lowest concentration of protein C. The $K_d$ value of the reaction was calculated as 869 ± 396 nM for T2 cells (Fig. 5D) and 140 ± 43 nM for ET1 cells.
The low-affinity reaction by cells positive for TM alone and the high-affinity reaction by dual-positive cells were also demonstrated with the other cell lines (data not shown). The $K_d$ value obtained using T2 cells was identical with that calculated in a reconstitution experiment using solubilized TM in phospholipid membranes (11). In contrast, the $K_d$ value for ET1 cells was almost identical with that of AEC (Fig. 3). Therefore, EPCR/TM dual-positive cells, but not cells positive for TM alone, might mimic the protein C activation mechanism observed in in vitro cultured endothelial cells. TM appears to be an essential component for activation even in the presence of EPCR, because complete inhibition of protein C activation could be demonstrated again using the anti-TM monoclonal antibody (Fig. 5 A).

To analyze the function of EPCR on endothelial cells, we established several functional blocking monoclonal antibodies against EPCR (Ye X., N. Tsuneyoshi, K. Fukudome, and M. Kimoto, unpublished data). One of these, RCR-252 (rat IgG1), was found to inhibit APC binding to EPCR-expressing cells (Fig. 6 A). RCR-252 also inhibited protein C activation mediated not only by EPCR/TM dual-transfected ET1 cells (Fig. 6 B), but also by primary cultured AECs (Fig. 6 C) in the same manner. The inhibitory effect of RCR-252 was specific for EPCR function, since protein C activation mediated by TM single-positive T2 cells was not affected (Fig. 6 B). Therefore, EPCR on cultured AECs appears to function for protein C activation. Inhibition of protein C activation by RCR-252 was only partial (~60%), in sharp contrast with the complete inhibition by anti-TM antibody (Fig. 6 C). This would be par.

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EPCR appears to concentrate protein C on the endothelial cell surface. Protein C appears to change conformation when it binds to EPCR because the velocity of the catalytic reaction mediated by the thrombin–TM complex increased in the presence of EPCR. Although EPCR can bind protein C with high affinity, the binding has been demonstrated as a reversible reaction. Therefore, bound protein C should be replaced continuously in circulation. By these mechanisms, protein C activation appears to be promoted effectively on the endothelial cell surface. Whether EPCR and TM exist as a complex on the endothelial cell surface or the complex is formed after binding of protein C to EPCR remains to be investigated.

Clinical studies related to the protein C pathway have been mainly focused on thrombosis in microvessels, since until now protein C activation has been considered to be restricted to the microcirculation (Fig. 7A). However, in this study, we have demonstrated a novel mechanism for APC generation involving EPCR (Fig. 7B). The high-affinity protein C receptor is expressed in large vessels in which protein C activation has been believed unlikely to occur. EPCR appears to enable significant levels of protein C activation in large vessels under physiological conditions. Generation of APC might function as an important regulatory mechanism for blood coagulation in large vessels. In fact, some clinical reports of arterial thrombosis appear to be related to defects in the protein C pathway (22–24). In the microvessels, we could not detect EPCR. Whether the physical condition of increasing the endothelial cell surface area to blood volume make up the low-affinity reaction or another unidentified molecule is involved in the activation remains to be investigated.

The authors thank Drs. K. Miyake and Y. Kamikubo for their helpful discussion; Dr. F. Nestel for critical reading of the manuscript; and S. Chen, F. Mutho, and C. Brown for technical support.

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan to K. Fukudome and M. Kimoto, and from the Ryouichi Naitoh Foundation for Medical Research to K. Fukudome.

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Received for publication 28 October 1996 and in revised form 15 December 1997.

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