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The protein C/protein S anticoagulant pathway has been proposed to be a common link between coagulation and inflammation. Studies have suggested that a component of the anticoagulant pathway, activated protein C (APC), may play a role in the inflammatory response by modulating the effects of cytokines such as TNF and by blocking neutrophil activation. Cytokines are known to be intimately involved in the inflammatory response and to function in part to restore hemostatic balance. To begin to delineate what role APC may have in the inflammatory response, we have investigated the effect of APC on the production of the proinflammatory cytokines IL-6 and IL-8 in primary HUVEC, human microvascular endothelial cells, and human coronary artery endothelial cells. Our results have demonstrated that physiologic concentrations of APC significantly up-regulated the production of both IL-6 and IL-8. This increase, which was seen at both the RNA and protein level, was not due to either thrombin or LPS contamination of the APC preparation. Additional studies also showed that the APC-mediated up-regulation of IL-6 and IL-8 was IL-1 independent. Although neither purified protein C nor protein S alone had an effect on cytokine production, protein S, the cofactor for APC, significantly enhanced the ability of APC to up-regulate IL-6/IL-8 production. These results provide further evidence for a role for APC in the inflammatory response. The Journal of Immunology, 1998, 161: 2567–2573.

The endothelium plays a critical role in maintaining physiologic equilibrium between procoagulant and anticoagulant forces. Inherent in the maintenance of this balance is the regulation of endothelial cell surface-associated coagulation and anticoagulation proteins by proinflammatory cytokines (1, 2). Among the cytokine-regulated coagulation proteins are the procoagulant protein tissue factor, which can be up-regulated by TNF (3), and the anticoagulant proteins thrombomodulin and protein S, which are conversely down-regulated by TNF (3–5). However, the opposite can also be true, in which coagulation proteins are able to modulate cytokine production. Recent studies have reported that factor Xa has the ability to up-regulate platelet-derived growth factor (PDGF) in endothelial cells (6) while activated protein C (APC)2 could down-regulate TNF-α production in macrophages (7).

The anticoagulant protein C zymogen, when activated by the thrombin/thrombomodulin complex, becomes a serine protease in the natural anticoagulant pathway (8). Classically, APC has functioned not only to inactivate the procoagulant factors Va and VIIIa (9, 10) but to also neutralize the activity of plasminogen-activator inhibitor-1 (PAI-1) (11). Further underscoring the importance of APC as an anticoagulant protein was the recent observation that the molecular basis of resistance to activated protein C or Factor V Leiden was a mutation in an APC cleavage site of Factor V (12, 13).

The protein C/protein S anticoagulant pathway has been suggested to be a common link between coagulation and inflammation (1). Animal studies have demonstrated that an infusion of APC could block the lethal effects of disseminated intravascular coagulation in Escherichia coli-induced sepsis (14). Data from these studies indicated that APC may play a role in the inflammatory response by modulating the effects of cytokines such as TNF and by blocking neutrophil activation (7, 15, 16). These observations have been supported by more recent in vitro studies in which it was shown that not only could protein C and APC block E-selectin-mediated cell adhesion (17) but, as alluded to earlier, APC has been shown to block LPS, phorbol ester, and γ-IFN-induced TNF production (7). In further support of the protein C/protein S pathway serving as a link between inflammation and coagulation was the observation that infusion of the APC cofactor, protein S, reversed the deleterious effects of infused C4bBP in E. coli-induced sepsis (18).

The production of cytokines has an important role in the pathogenesis of many diseases, particularly those with an inflammatory component. In the inflammatory state, cytokines are part of a host’s response to maintain or restore hemostatic balance. Cytokines can function to induce the acute-phase response, initiate mononuclear cell migration to the site of injury, induce wound repair, and both augment and amplify the immune response. More recent data have implicated cytokines such as IL-8 as important physiologic participants in cross-talk between coagulation and inflammation (19). In addition to being beneficial, cytokines can also have a deleterious effect on the host. Acute and chronic inflammation as well as thrombosis are among the pathologic conditions that have a significant cytokine component (1, 20, 21).

Following vascular injury, there is an exquisite interplay between coagulation, anticoagulation proteins, cytokines, and inflammatory cells in an attempt to resolve injury. Not only is the endothelium fundamental to the resolution of injury through the elaboration of chemotactic cytokines and expression of adhesion molecules that allow the recruitment and adherence of neutrophils, monocytes, and lymphocytes, but it is also pivotal in maintaining a balance between the procoagulant and anticoagulant forces.
These multiple interrelated functions are thought to be linked, and, in support of the protein C/protein S anticoagulant pathway serving as one possible common link between inflammation and coagulation, we report that APC can up-regulate the expression of the proinflammatory cytokines IL-6 and IL-8 in endothelial cells.

Materials and Methods

Reagents

Purified protein C, protein S, activated protein C, and the chromogenic substrate 2-oxo-4-thiazolidine carboxyl-prolyl-arginy1-paranitroanilide-HCl (TCPA-pNA) for the determination of APC amidolytic activity were obtained from Celsus Laboratories (Cincinnati, OH). Other commercial sources of APC were American Diagnostica (Greenwich, CT), Alexis (San Diego, CA), and Enzyme Research (South Bend, IN). With indicated exceptions, experiments were performed with APC preparations obtained from Celsus Laboratories. Before use, lyophilized activated protein C was rehydrated in sterile distilled water and dialyzed overnight against cold PBS, pH 7.3. Frozen (−70°C) APC (Enzyme Research) was used without dialysis. DFP, Hirudin, E. coli LPS, and polymyxin B were obtained from Sigma, St. Louis, Mo. A purified murine Ab (No. 2375) specific for the beta-actin (Sigma), ribosomal L32, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), while the hCK-5 set contained Ltn (lymphotoxin), RANTES, IP-10, (interferon induced protein 10, also known as monokine induced by interferon gamma), MCP-1 (monocyte chemoattractant protein), and IL-8. Commercial kits were obtained and used for the measurement of IL-6 and IL-8 (Biosource International, Camarillo, CA) and IL-1 (IL-1Ra, IL-1β, IL-1α), IL-6, and TNF-α (tumor necrosis factor alpha) according to the sequence of the NF-IL-6 binding site (bp 3’AGG TCA ATG TAT ATT TTA TAA 3’), which is found in promoter regions of a variety of genes involved in inflammation and cell proliferation. In order to ensure that equivalent amounts of RNA were present in each lane, ribonuclease protection assay (RPA) and DNA probes in a final volume of 20 µl were hybridized overnight with 15 to 20 µg of RNA, and the reactions were subsequently digested with a mixture of RNase A and T1 for 45 min followed by proteinase K digestion for 15 min. Ribobrome-RNA complexes were isolated using a phenol-chloroform extraction step and ethanol-acetate precipitation. The RNA-ribobrome complexes were then run on an 8-M urea sequencing gel for 2 h at 55°C. After drying, the gel was exposed to x-ray film from 1 to 5 days.

ELISA

Commercial kits were obtained and used for the measurement of IL-6 (R&D Systems, Minneapolis, MN, and CytImmune Science, College Park, MD) and IL-8 (Biosource International, Camarillo, CA) according to the manufacturer’s protocols. Absorbance was determined with an EL312e Microplate Reader (Biotek, Winooski, VT) and data reduction was accomplished with the KinnetCalc software package (Biotek).

APC chromogenic assay

APC (0.04 µmol/ml) was preincubated with 5 µl hirudin at room temperature for 30 min before addition to 2.5 µmol/ml TCPA-pNA substrate in a final volume of 100 µl of 1% BSA-PBS, pH 8.2, in 96-well clusters (Costar). The change in optical density at 405 nm was measured at 1-min intervals with an EL312e kinetic plate reader (Biotek).

APC inactivation by DFP

DFP treatment of APC was conducted at room temperature for 2 h at a final concentration of 2 µM and followed by overnight dialysis against PBS, pH 7.6, at 4°C before addition to the culture media.

Ribonuclease protection assay (RPA)

Total cellular RNA was isolated using a Tri-Reagent isolation kit (Sigma) according to the manufacturer’s protocol. RPAs were performed using RiboQuant (PharMingen, San Diego, CA) and DNA probes in a final volume of 20 µl. The change in optical density at 405 nm was measured at 1-min intervals with an EL312e kinetic plate reader (Biotek). The RNA-riboprobe complexes were then run on an 8-M urea sequencing gel for 2 h at 55°C. After drying, the gel was exposed to x-ray film from 1 to 5 days.

Results

IL-6 and IL-8 up-regulation

APC up-regulation of both IL-6 and IL-8 in HUVECs occurred in a dose-dependent fashion within a range of 0 to 90 nM (Fig. 1, A and B). At the equivalent protein C physiologic concentration of ~50 nM, APC had no effect on either endothelial cell viability or proliferation. However, APC at 180 nM did elicit a change in cellular morphology and decreased the viability of endothelial cells (data not shown). As determined by ELISA, significant increases in both IL-6 and IL-8 production were detected as early as 8 h after the addition of APC, and cytokine levels continued to increase as one possible common link between inflammation and coagulation.
increase through 48 h (Fig. 1, C and D). These increases in cytokine levels, with increasing APC concentrations or exposure over time, compared with control levels (Fig. 1, A–D), were statistically significant by Student’s t test (range \( p, 0.001- < 0.05 \)). The effect of APC on endothelial cell IL-6 and IL-8 production was significantly potentiated (\( p, 0.01 \)) when the APC-purified cofactor, protein S, was added to cell cultures in combination with APC (Fig. 2, A and B). However, when used alone, neither purified protein S nor protein C had any effect on IL-6 or IL-8 production (Fig. 2, A and B). On a transcriptional level, IL-6 RNA was up-regulated by 50 nM APC at 3 h but returned to control levels by 6 h (Fig. 3A), whereas IL-8 up-regulation was observed at 3 h and 6 h and returned to near control levels by 24 h (Fig. 3B). In some experiments a modest induction of the p35 chain of IL-12 was observed, but, unlike IL-6 and IL-8, this effect was not consistent in all experiments. No induction of either IL-1\( \alpha \), IL-1\( \beta \), IL-10, IL-1Ra, γ-IFN, RANTES, lymphotixin, IP-10, MIP-1\( \alpha \), or MIP-1\( \beta \) RNA was observed (data not shown).

**Specificity of the APC effect**

The inactivation of APC by DFP demonstrated that the active site of APC was required for cytokine up-regulation as shown for IL-8 in Figure 4. APC activity was also inhibited by the serine protease inhibitor, aprotinin (1 TIU/ml), further documenting the specificity of APC (Fig. 4). To rule out possible thrombin or LPS contamination of the APC preparation as a source of cytokine up-regulation, experiments were conducted using the respective inhibitors, hirudin and polymyxin B, which alone had no effect on APC activity. Hirudin, up to concentrations of 20 U/ml, had no effect on the ability of APC to up-regulate IL-6/IL-8. (Fig. 4). It should be noted, however, that thrombin alone at \( \geq 10 \) U/ml (\( \approx 5 \times \) the amount that should be expected in the APC preparation based on a typical protein C activation with EDTA/thrombin (24)) did up-regulate IL-6 and IL-8, but the effect was completely inhibited by coinubcation with excess hirudin (data not shown). When used alone at \( \leq 5 \) U/ml, thrombin had no measurable effect on IL-6 or IL-8 production. Similarly, polymyxin B (10 μg/ml) had no effect on the action of APC (Fig. 4), thereby indicating that the effect was not due to LPS contamination. In further support that the effect was not due to LPS, endothelial cell activation by the APC preparation was heat labile and was abolished after heating the preparation at 56°C for 30 min (Fig. 4). At the concentrations employed, neither hirudin, aprotinin, or polymyxin B had any effect on cell morphology, viability, IL-6, or IL-8 levels. At concentrations ranging from 50 to 250 nM, purified protein C had no effect on the APC-mediated up-regulation of either IL-6 or IL-8 (Fig. 2 and Fig. 5). Pre-incubation of up to fivefold excess of protein C in cell culture 30 min before the addition of APC did not diminish or block APC-mediated up-regulation of either IL-6 or IL-8 (Fig. 5). Furthermore...
coincubation of APC with threefold molar excess murine monoclonal anti-thrombomodulin or an eightfold molar excess immunopurified goat anti-thrombomodulin Abs had no effect on cytokine up-regulation (data not shown).

Although APC-mediated induction of either IL-1α or IL-1β was not observed, experiments using IL-1Ra were conducted to further exclude the possibility that IL-1 may have been responsible for IL-6/IL-8 up-regulation. IL-1Ra at the concentrations of 10 ng/ml had no effect on APC induction of either IL-6 or IL-8, thereby demonstrating that the APC effect was IL-1 independent (data not shown).

To establish that the APC effect was not unique to one commercial preparation, purified APC was obtained from three other commercial sources. As illustrated in Table I, each APC preparation had the ability to significantly increase the production of IL-6/IL-8 from endothelial cells over that of basal levels. APC from Enzyme Research Laboratories had the greatest effect with approximately a 44- and 18-fold increase in IL-6 and IL-8 production, respectively. The potency of the APC preparation from Enzyme Research was further highlighted by its high amidolytic (functional) activity as compared with the other commercial sources (Table I).

The effect of the combination of IL-6/IL-8 on production of the APC cofactor, protein S

Since it has been reported that IL-6 could up-regulate the vitamin K-dependent anticoagulant protein, protein S, in the HepG-2 cells (25), experiments were conducted to determine what effect IL-8 may have on protein S production. As shown in Figure 6, while having no direct effect itself, IL-8 synergized with IL-6 to significantly increase protein S production over that of IL-6 alone in the HepG-2 cells. IL-8 had no effect on protein S production in endothelial cells (data not shown), which was not surprising, since these cells have no IL-8 receptors (26).

The effect of APC on microvascular and coronary artery endothelial cells

As shown in Figure 7, APC, in a dose-response fashion, induced IL-6 and IL-8 in both the microvascular and coronary artery endothelial cells (Fig. 7, A-D). As depicted in Figure 8, microvascular endothelial cells produced 2 to 3 times more IL-6 and IL-8 in response to 50 nM of APC than did HUVECs. Coronary artery endothelial cells produced significantly less IL-6 and IL-8 than HUVECs, in response to APC, and 3 to 4 times less IL-6 and IL-8 than did the microvascular endothelial cells (Fig. 8). Furthermore, compared with both HUVECs and microvascular endothelial cells, APC at the concentration of 50 nM induced only a modest increase in IL-6 and IL-8 production in coronary artery endothelial cell (CAEC) cultures. The experimental data sets for Figures 7 and 8 were generated with APC obtained from Enzyme Research Labs, hence the overall greater levels of IL-6/IL-8 induction compared with Figures 1, 2, 4, and 5.

Gel shift analysis

The transcriptional factor NF-IL-6 was maximally expressed at 30 min with a decrease in expression noted at 60 min following exposure to 50 nM APC (Fig. 9). Specificity of the response was determined by using unlabeled DNA as a competitive inhibitor at a 30-fold and 100-fold excess.

Table I. APC activation of endothelial cell cultures with four different APC preparations shows a parallel between increased cytokine production and the amidolytic activity of the different APC preparations

<table>
<thead>
<tr>
<th>APC (source)</th>
<th>Activity</th>
<th>IL-6</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celsus</td>
<td>0.215* (0.999)</td>
<td>6.0†</td>
<td>3.0</td>
</tr>
<tr>
<td>American</td>
<td>0.164 (0.997)</td>
<td>3.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Diagnostica</td>
<td>0.545 (0.998)</td>
<td>43.9</td>
<td>18.0</td>
</tr>
<tr>
<td>Enzyme Research</td>
<td>0.218 (0.999)</td>
<td>4.0</td>
<td>3.5</td>
</tr>
</tbody>
</table>

* Change in absorbance (slope of regression) with the APC amidolytic assay at room temperature with 5 U hirudin and under conditions of excess substrate (2.5 μmol/ml TCPA-pNA and 0.04 nmol/ml APC), i.e., zero order reaction rate in which product formation was linear with time. The number in parentheses is the coefficient of correlation of the regression.
† Fold increase, after 24 h, in endothelial cell culture cytokine concentration following activation with 50 nM APC.

FIGURE 6. The effect of IL-6 and IL-8 on protein S production in the HepG2 cells. IL-6 but not IL-8 significantly increased protein S production (* p = 0.001). However the combination of IL-6/IL-8 significantly increased the production of protein S over that of IL-6 alone at 24 and 48 h (* p = 0.01).

FIGURE 7. The up-regulation of IL-6 by APC in (A) human microvascular endothelial cells (HMVEC) and (B) human coronary artery endothelial cells (HCAEC). IL-8 up-regulation by APC in both HMVEC (C) and (D) HCAEC.
Discussion

The protein C/protein S natural anticoagulant pathway has been proposed to serve as a link between inflammation and coagulation (1, 14). This has been supported in part by past experimental evidence that has implicated inflammation in the development of a physiologic clot into a pathologic thrombus (27). Activated protein C, the pivotal protein in the natural anticoagulant pathway, not only has been suggested to have a role in cytokine production but also has been shown to block TNF induction by LPS in human monocytes/macrophages (7). The work reported here supports the concept that APC can be important in cytokine regulation. Our results demonstrate that a) APC, in a dose-dependent fashion, up-regulated the production of both IL-6 and IL-8 in umbilical vein endothelial cells; b) the increase was seen at both the transcriptional as well as at the protein level; c) the transcriptional factor NF-IL-6 was induced; d) the active site of APC was required for cytokine up-regulation; e) protein S, a cofactor for APC, while having no direct effect, significantly potentiated the APC effect; f) the induction of IL-6/IL-8 was IL-1 independent; and g) in addition to HUVECs, APC induced IL-6/IL-8 in both microvascular and coronary artery endothelial cells.

Though the anticoagulant role of APC is well recognized, little is known about its effect in the acute but localized inflammatory state that involves the microvasculature. This report provides data that suggest that APC, in addition to its anticoagulant properties, may also function to restore hemostasis via cytokine induction. The finding that APC significantly increased the production of both cytokines, particularly IL-8, in the microvascular endothelial cells, as compared with the HUVECs and the coronary artery endothelial cells, was especially interesting since leukocyte trafficking and the development of the inflammatory response occurs in the microvasculature. Moreover, the observation that endothelial cells from coronary arteries produced the least amount of IL-6/IL-8 in response to APC was not surprising since it could be argued that a robust inflammatory response in this particular vascular bed probably would not be advantageous to the host.

Our data, together with work by Johnson et al. (19), which reported an increase in IL-8 production during coagulation, suggest the following. Concomitant with limiting clot formation, APC could induce IL-8 production independent of coagulation to produce enough IL-8/IL-6 to achieve one of two opposing physiologic endpoints, with the outcome dependent on the extent of injury. One endpoint would be the maintenance of IL-8/IL-6 levels required to maintain leukocyte trafficking and adherence at the site of injury, thereby amplifying the inflammatory response. In addition, increased levels of both IL-6 and IL-8 could also contribute to the restoration of the integrity of the endothelium by formation of a fibrin clot by inducing thrombogenesis through the induction of tissue factor in monocytes (28). Conversely at the other endpoint, the inflammatory response could be attenuated by APC through alterations in the local IL-8 concentration gradient that would inhibit rather than induce neutrophil migration (29, 30), and, likewise, increased IL-6 levels would also contribute to the inhibition of neutrophil accumulation (31). In indirect support of the latter, a recent report demonstrated that APC inhibited LPS-induced pulmonary vascular injury by inhibiting the accumulation of activated neutrophils (16). Furthermore, increased levels of IL-6, mediated by APC, not only would serve to induce the acute phase response but could increase the levels of protein S, the cofactor for APC (32). In addition, the observation that IL-8 could synergize with IL-6 to up-regulate hepatic derived protein S (25) within a specific time-frame further suggests that APC may be able to control its enzymatic activity via the indirect up-regulation of protein S. Our data does in fact demonstrate that exogenously added protein S can increase IL6/IL-8 production induced by APC.

Members of the serine protease family, which includes APC, have become recognized as important mediators in leukocyte function (33). Some of the newly recognized functions of serine proteases include induction of apoptosis, chemotaxis, cell adhesion, and proliferation, as well as cytokine modulation. It is thought that these proteases initiate their cellular effects through signal transduction via protease receptors. Factor Xa, a serine protease that has been classically defined as a clotting protein responsible for catalyzing the activation of prothrombin to thrombin, has recently been shown to induce cellular proliferation. Gajdusek et al. (6) have reported that factor Xa induced endothelial cell proliferation.
through the induction of platelet-derived growth factor. These observations were extended by Gasic et al. (34), in which it was shown that smooth muscle cell proliferation was induced by factor Xa. In contrast to endothelial cells, the effect of factor Xa on smooth muscle cells was independent of any known mitogens or cytokines.

The mechanism(s) through which APC exerted its effect on endothelial cells is presently not known. However, since DFP blocked the ability of APC to up-regulate IL-6/IL-8, it was apparent that the APC active site was necessary for its biologic effect. DFP has been used by many investigators to block the active site to determine APC specificity. Grey et al. (7) used DFP to show that APC could block TNF production by human macrophages, while Hancock et al. (35) used it to demonstrate that high affinity APC receptors were present on these phagocytic cells. We have further demonstrated specificity by abolishing the APC effect through the use of the serine protease inhibitor, aprotinin. In further support of APC specificity, the APC cofactor, protein S, was shown to augment IL-6/IL-8 up-regulation when exogenously added together with APC. Contamination of the APC preparation with thrombin or LPS was ruled out since inhibitors of both thrombin and LPS failed to abolish cytokine up-regulation and furthermore neither purified protein C or protein S, when used alone, had any effect. Although thrombin was also capable of IL-6 and IL-8 up-regulation, much higher levels were required (≥10 U/ml) than would be expected if residual thrombin were present in the APC preparation.

Since APC up-regulated IL-6/IL-8 at the transcriptional level, which in part may have been due to the induction of the NF-IL-6 transcriptional factor, it could perhaps be argued that the APC effect was receptor mediated. Receptors for APC have been identified on both macrophages (36) and endothelial cells (37, 38). Recent investigations have suggested that the Ca²⁺-independent macrophage APC receptor is probably distinct from the Ca²⁺-dependent APC receptor found on endothelial cells. Although our preliminary data suggest that the APC effect is Ca²⁺ independent (our unpublished observations), we have yet to determine whether or not the effect is mediated through a specific receptor. The observed APC effect was also probably independent of the endothelial cell protein C receptor (EPCR) (37) since protein C not only was unable to induce IL-6/IL-8 but also could not block the effect of APC. The APC effect was also independent of APC-thrombomodulin interaction since anti-thrombomodulin Abs had no effect on IL-6/IL-8 induction by APC. Presently, we cannot rule out the possibility that APC may act through a nonreceptor mechanism by the formation of a complex with protein S on the phospholipid membrane of the endothelial cell or that APC may initiate an intracellular signal via the cleavage of a membrane-bound serine protease-sensitive protein.

In summary it can be argued that our results suggest that, in addition to the benefits reported by others, APC may also have a negative effect on the host through the induction of proinflammatory cytokines. Although this possibility cannot be excluded, we would like to propose that APC may contribute to the resolution of injury in part by regulating cytokine production crucial to the regulation of either the early or delayed inflammatory response, and that the magnitude of this response may be dependent on the type or location of the vascular bed. These results further extend the biologic functions of a protein that has classically been defined as an anticoagulant protein and provides further evidence for a role for APC in the inflammatory response.

Acknowledgments

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References


