Gene Expression Profile of Antithrombotic Protein C Defines New Mechanisms Modulating Inflammation and Apoptosis*

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Human protein C is a natural anticoagulant factor, and a recombinant activated form of the molecule (rhAPC) is completing clinical evaluation for treatment of severe sepsis. Because of the pathophysiologic role of endothelial dysfunction in severe inflammatory disease and sepsis, we explored the possibility that rhAPC might directly modulate endothelial function, independent of its anticoagulant activity. Using broad transcriptional profiling, we show that rhAPC directly modulates patterns of endothelial cell gene expression clustering into anti-inflammatory and cell survival pathways. rhAPC directly suppressed expression of p50 and p52 NFκB subunits, resulting in a functional decrease in NFκB binding at target sites. Further, rhAPC blocked expression of downstream NFκB regulated genes following tumor necrosis factor α induction, including dose-dependent suppression of cell adhesion expression and functional binding of intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin. Further, rhAPC modulated several genes in the endothelial apoptosis pathway, including the Bcl-2 homologue protein and inhibitor of apoptosis protein. These pathway changes resulted in the ability of rhAPC to inhibit the induction of apoptosis by the potent inducer, staurosporine. This new mechanistic understanding of endothelial regulation and the modulation of tumor necrosis factor-induced endothelial dysfunction creates a novel link between coagulation, inflammation, and cell death and provides insight into the molecular basis for the efficacy of APC in systemic inflammation and sepsis.

Endothelial dysfunction plays a critical role in uncontrolled inflammatory conditions such as sepsis and multiorgan dysfunction syndrome. The elucidation of cytokines following insult, particularly tumor necrosis factor α (TNF),1 initiates cell surface activation affecting a number of pathways (e.g. oxidative, adhesion, cytokine release, apoptosis, and nitric oxide production), as well as releasing a tissue factor that further contributes to inflammation through thrombin-induced activation of endothelium, platelets, and vascular smooth muscle (1). The overall result of this cellular activation is a dysregulation of endothelial function, leading to microvascular thrombosis, end organ damage, multiple organ dysfunction, and often death (2, 3). In the last several years, blocking disseminated intravascular coagulation, and the microthrombi that may promote end-organ dysfunction, has been proposed as a new target for clinical treatment in sepsis (4).

Human protein C is a plasma serine protease that plays a key role in maintaining normal hemostasis (5, 6). The thrombin-activated form of protein C (APC) acts as a feedback inhibitor of the coagulation cascade and has demonstrated antithrombotic activity in numerous model systems (7). APC has shown efficacy in models of lethal endotoxemia and has been reported to prevent microvascular coagulation in patients with meningococcal sepsis (8–12). A recombinant version of human activated protein C (rhAPC) is completing clinical evaluation in patients with severe sepsis, targeting the effects of microvascular coagulation. However, there has been considerable speculation about the simultaneous monitoring of gene expression for a significant portion of a genome, has emerged as a powerful tool in genetics and biology, because it allows for the analysis of many of the signal transduction pathways and other biological systems identified from the sequencing of the human genome (14). This profiling has been used to reveal novel targets and mechanisms of action using yeast as a model system (15–17). Using this novel approach, we demonstrate that rhAPC directly modulates cell signaling and alters gene expression in two major pathways of inflammation and apoptosis. rhAPC suppressed NFκB-regulated genes by directly reducing NFκB expression and functional activity. Further, rhAPC inhibited cytokine signaling, including TNF-induction of cell surface adhesion molecules (e.g. VCAM, ICAM, E-selectin, and fractalkine). rhAPC also modulated apoptosis pathways, including up-regulation of the endothelial Bcl-2 homolog (A1), eNOS, and the inhibitor of apoptosis (IAP), and suppression of the apoptosis-associated genes calreticulin and TRMP-2. Moreover, treatment of cells with rhAPC blocked the induction of apoptosis. These data

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1 The abbreviations used are: TNF, tumor necrosis factor; APC, activated protein C; rhAPC, recombinant human activated protein C; NFκB, nuclear transcription factor κB; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; A1, Bcl-2 homologue protein; IAP, inhibitor of apoptosis protein; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cell; EMSA, electrophoretic mobility shift assay; PCNA, proliferating cell nuclear antigen; FBS, fetal bovine serum; PCR, polymerase chain reaction; TBE, Tris-buffered EDTA; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate.
provide a novel view of how inflammation is modulated at the endothelial cell level and defines new relationships among hemostasis, inflammation, and cell death/apoptosis. Moreover, our results demonstrate the value of gene profiling in defining novel mechanisms of drug action and specifically in providing possible mechanistic answers to rhAPC efficacy in sepsis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human umbilical vein endothelial cells (HUVEC) and pools HUVEC PO96, F146, or F150 were grown in endothelial growth medium-2 (FBS; Clonetics, San Diego, CA). Cells were either untreated or treated with rhAPC (El Lilly and Co., Indianapolis, IN) and/or TFα (R & D Systems, Minneapolis, MN). For the Affymetrix® experiment, cells were treated with APC for 16 h and TfN for the last 7 h. For the four conditions (untreated; vehicle of 0.1% bovine serum albumin (BSA); 100 ng/ml thrombin at subnanomolar concentrations. During the course of the experiments, samples were tested for amyloid activity in the presence and absence of hirudin, and no significant thrombin activity could be detected (<25 pmol/kit). Total RNA was isolated by TRIzol® (Life Technologies, Inc.) following the manufacturer’s recommendations. RNA was stored at −80 °C in diethyl pyrocarbonate-treated deionized water. Detailed methods for labeling the samples and subsequent hybridization to the arrays are available from Affymetrix (Sangamo BioSciences). 1.5 μg of poly(A) RNA was converted to double-stranded cDNA (Superscript; Life Technologies, Inc.) priming the first-strand synthesis with a T7-(dT)24 primer containing a T7 DNA binding site was used for specificity controls. Quantification of gel shift to a consensus NFκB 5′-GAGTTTGAGGGACTCCAGG-3′ site was performed by a scanning phosphorimager. Flow Cytometry—Primary antibody at 1–2 μg/ml in 100 μl of FACS buffer (PBS, 5% albumin, 0.02% sodium azide) was applied at 4 °C for 30 min. The secondary antibody, anti-mouse IgG-FITC, at 1 μg/ml in 100 μl of FACS buffer was applied at 4 °C for 30 min. FACS analysis was done with a Coulter® flow cytometer (Coulter). Primary antibodies were to adhesion markers ICAM-L, E-selectin, VCAM-L, and fractalkine (R & D Systems, Minneapolis, MN). Apoptosis Assay—Cells were seeded at 3 × 104 cells per well in a 96-well plate and treated with 1 μg/ml staurosporine (Sigma) for 1 h or with staurosporine and APC (pretreatment for 16 h). Cells were prepared and stained according to APOPercentage™ apoptosis assay per the manufacturer’s instructions (Bioceil Ltd., Belfast, Northern Ireland).

**RESULTS**

**Transcript Profile of rhAPC Alone and in TNF-treated Endothelial Cells**—The effect of rhAPC on TNF-activated or non-activated human endothelial cells was assessed using transcript profiling with Affymetrix® microarrays. Treatments were performed under conditions where no thrombin or other protease activity could be detected, and no detectable thrombin was being generated during the cell culture experiments as described under “Experimental Procedures.” After treatment, cells were lysed and mRNA was isolated, followed by analysis on Affymetrix® arrays, and results were confirmed by semiquantitative RT-PCR. We found that rhAPC and TNF treatments of HUVECs resulted in 10 genes that were regulated by rhAPC alone and 31 genes regulated by TNF alone. Shown in Table I are the genes whose expression was altered by rhAPC treatment alone and 13 genes of the 31 TNF-regulated genes that we found to be comodulated by rhAPC. In general, the genes clustered with respect to cellular function into those involved in inflammation/immune modulation and cell survival/apoptosis. We also observed that the TNF-activated genes, which primarily fell into the proinflammatory and apoptotic pathways, were counter modulated by the rhAPC treatment. There were several TNF-activated genes, associated with feedback pathways for controlling TNF responses, (19) that were further enhanced by rhAPC treatment. Notably, the TNF-activated A20 gene, which was enhanced by rhAPC, has recently been shown in knockout experiments to be critical in regulating TNF-induced effects and cell death responses (20). Overall, the pattern of gene expression modulation by rhAPC was consistent with an induction of anti-inflammatory and antiapoptotic pathways.

**Effect of rhAPC on the NFκB Pathway**—A very notable change observed on the transcript profile following rhAPC treatment was a suppression of p52 (NFκB2 subunit) on the array. Further analysis of the array results indicated that expression of this NFκB2 subunit was suppressed both by rhAPC alone and in combination with TNF (Fig. 1A). The average difference from the 20 perfect and mismatch signals on the Affymetrix array demonstrated a reduction in NFκB2 mRNA by rhAPC, with or without TNF cotreatment. Semiquantitative RT-PCR confirmed that the NFκB2 mRNA was suppressed by rhAPC alone and that rhAPC attenuated its induction by TNF (data not shown). To further examine the functional effect of the suppression at the mRNA level, an electrophoretic mobility shift assay (EMSA) for NFκB was used to determine the amount of NFκB DNA binding in nuclear extracts from treated HUVECs using a 32P-labeled oligonucleotide consensus probe to NFκB, (Fig. 1B). In repeated experiments, rhAPC alone suppressed NFκB nuclear extract DNA binding by ∼60% (n = 4), consistent with the reduced mRNA expression (lanes 1 and 2). Moreover, in repeated experiments,
APC Modulates Inflammatory and Apoptotic Pathways

Fig. 1. APC down-regulates NFκB gene expression in HUVECs. A, effect of rhAPC on mRNA levels by Affymetrix analysis and semiquantitative RT-PCR. Levels of NFκB2 expression were compared with untreated control cells. Data represents the average difference from 20 perfect and mismatch signals on the Affymetrix chip (duplicate chips and confirmed by RT-PCR at two cycle times with actin and transferrin receptor controls). B, functional analysis of NFκB activity using a DNA shift assay (mean ± S.D.; n = 5). Gel shows functional suppression of NFκB DNA consensus sequence binding when excess radiolabeled (32P) consensus sequence is present. Lane 1, nuclear extract from untreated cells; lane 2, with rhAPC alone (320 nM, 24-h pretreatment); lane 3, nuclear extract from untreated cells; lane 4, cells treated with TNF alone (1 ng/ml for 4 h); and lane 5, rhAPC pretreatment followed by TNF.

rhAPC also attenuated the amount of binding following TNF induction of NFκB2 (lanes 3–5). In addition to these results using HUVECs, we obtained the same results using the Eahy926 (21) human endothelial line (data not shown). The specificity of the EMSA binding assays was demonstrated with antibody supershifts to p65, p50 (NFκB1), and p52 (NFκB2), as well as competition with cold consensus sequence (data not shown). This showed that the effect was specific and that rhAPC can directly inhibit the functional NFκB pathway.

Table I

| Genes modulated by rhAPC | APC effect       | Functional category | Accession number |
|--------------------------|------------------|---------------------|-----------------|
| NFκB2                    | DEC              | Inflammatory        | C               | N               | S76638 |
| PCNA                     | INC              | Cell cycle          | C               | N               | J05614 |
| Human autoAg calreticulin| DEC              | Immune              | C               |                | M84739 |
| Human A1 Bcl-2 homologue | INC              | Antiapoptosis       | C               | N               | U29680 |
| Human Gu helicase        | INC              | Antiapoptosis       | C               |                | U41387 |
| Human IAP                | INC              | Antiapoptosis       | N               |                | U45878 |
| eNOS                     | INC              | Nitric oxide/apoptosis | C            | N               | M93718 |
| Orphan G protein-coupled receptor | DEC | Orphan GPCR | C | M67784 |
| TRMP-2                   | DEC              | Proapoptotic        | M63379          |
| B61 (Ephrin-A1)          | DEC              | Chemotactot         | M57730          |
| Genes activated by TNF that are suppressed by APC | | | |
| NFκB2                    | Inflammatory     | C                   | N               | S76638          |
| ICAM-1                   | Adhesion         | C                   | N               | M24823          |
| E-selectin               | Adhesion         | C                   | N               | M24736          |
| Fractalkine precursor (CX3C) | Adhesion     | C                   | N               | U34457          |
| VCAM-1                   | Adhesion         | C                   | N               | M30257          |
| Manganese superoxide dismutase | Oxidation    | C                   | N               | X65986          |
| Stromelysin-2            | Matrix metallo   | C                   | N               | X07820          |
| Genes activated by TNF and further enhanced by APC | | | |
| Human A1 Bcl-2 homologue | Antiapoptosis   | C                   | N               | U29680          |
| Human Gu helicase        | Antiapoptosis    | C                   | N               | U41387          |
| Human IAP homologue B    | Antiapoptosis    | C                   | N               | U45878          |
| TNF α-induced A20        | Antiapoptosis    | C                   | N               | M59465          |
| Homo sapiens MAD-3 (IκB) | Antiapoptosis    | C                   | N               | U68019          |
| Genes suppressed by TNF and activated by APC | | | |
| PCNA                     | Cell survival    | C                   | N               | J05614          |

Genes suppressed by TNF and activated by rhAPC (both NFκB1 and NFκB2) in endothelial cells.

Suppression of Cell Adhesion Molecules by rhAPC—As shown in Table I, a number of TNF-modulated genes were countermodulated by rhAPC, and the induction of many of these genes has previously been shown to be mediated through NFκB (see Table I). We focused additional studies on the adhesion molecules as being of particular importance with regard to anti-inflammatory effects. Semiquantitative RT-PCR confirmed that rhAPC mediated suppression of several of the adhesion molecule mRNAs (induced by TNF) including CX3C (fractalkine) (22), ICAM-1, E-selectin, and VCAM-1 (23). By flow cytometry, we confirmed that ICAM-1 surface expression induced by TNF could be suppressed by rhAPC in endothelial cells (Fig. 2A). The effect of rhAPC on the reduction in ICAM-1 expression was concentration-dependent, as shown in Fig. 2B. Similarly, we have shown that rhAPC could inhibit the expression both E-selectin and VCAM-1, two other important leukocyte adhesion molecules. As shown in Fig. 2C, rhAPC reduced cell surface E-selectin expression, measured by flow cytometry, in a concentration-dependent manner. Further, using a direct cell binding assay, dependent on the interaction of endothelial VCAM-1 and very late antigen-4 on a target cell (U937), we demonstrate that rhAPC directly inhibits the cell-cell interaction up-regulated by TNF (Fig. 2D). Additional studies with VCAM-1 and fractalkine showed comparable dose-dependent inhibition by rhAPC.

APC-dependent Modulation of Apoptosis—In addition to the described effect of rhAPC on NFκB and its downstream mediation of inflammation, our profiling results also suggested a second clustering around pathways promoting antiapoptosis and cell survival. As shown in Fig. 3A, rhAPC suppressed two proapoptotic genes, calreticulin, an endoplasmic reticulum luminal protein that when suppressed has been shown to decrease cell apoptosis (24), and TRMP-2, a marker of cell apoptosis (25). In contrast, rhAPC increased genes shown to be antiapoptotic or markers of cell survival such as A1 (19), IAP (26), cell cycle-related human Gu helicase (27), and proliferat-
Fig. 2. rhAPC suppresses NFκB-activated surface adhesion proteins ICAM-1 and E-selectin. A, analysis of ICAM-1 surface expression by flow cytometry in human endothelial cells. Cells were treated with rhAPC, TNF, or both combined, and the levels of mean fluorescence intensity versus events was determined using an FITC-labeled secondary antibody as described below. B, dose response for the effect of rhAPC on TNF-induced ICAM surface expression (mean ± S.D.; n = 3). C, dose response for the effect of rhAPC on TNF-induced E-selectin surface expression similarly determined by flow cytometry. In both B and C the levels were made relative to untreated control as 100%. D, effect of rhAPC on VCAM-dependent cell-cell interaction (mean ± S.D.; n = 5). Very late antigen-4-expressing U937 mononuclear cells were used in endothelial cell adhesion experiments using procedures described previously (42).

Fig. 3. rhAPC modulates genes associated with cell survival and apoptosis. A, selected gene changes affected by rhAPC related to cell cycle and apoptosis. The results are depicted as -fold differences determined from the average difference change from Affymetrix arrays (see “Experimental Procedures”) and made relative to actin controls (data from duplicate array experiments each with 20 perfect and mismatch signals on the array). B, protective effect of rhAPC against induction of apoptosis. Effect of rhAPC treatment (8 μM) on staurosporine (SS; 1 μM)-induced apoptosis in human endothelial cells and kidney 293 cells is shown. C, quantification of staurosporine-induced apoptosis in Eahy926, kidney 293 cells, and HUVECs with and without rhAPC (mean ± S.D.; n = 4).

DISCUSSION

We have utilized a novel approach to define new molecular pathways for the subcellular action of the anticoagulant human protein C. Moreover, our data demonstrate the power of transcriptional profiling in defining drug mechanism and implications for disease therapy. In Fig. 4, we diagram the expanded paradigm of the physiological role of APC. We have found that beyond its indirect effect on inflammation, via inhibition of thrombin generation, APC has novel direct anti-inflammatory effects via suppression of the NFκB pathway and an apparent ability to prevent apoptosis and modulate cell survival. This effect of rhAPC on inhibiting apoptosis appears to be separate and antithetical to most of the TNF/NFκB pathway effects, yet consistent with an effect that would be beneficial in antithrombotic/anti-inflammatory situations. In fact, there is growing evidence for the role of apoptosis in systemic inflammatory response and sepsis (30–32). The emerging data suggest that organs-specific cell death involving both parenchymal and microvascular endothelium underlies organ dysfunction, with increased apoptotic rates occurring in organ dysfunction. The concept of suppression of proinflammatory pathways and the...
switch to cellular survival mechanisms at the endothelial interface suggests a complex adaptive response at the vessel wall directly connected at the subcellular level to the action of APC. The protein C pathway in inflammatory states could protect from ischemic renal injury (34). Recently, diminished ICAM-1 that is resistant to induction of sepsis (33) and pro- 

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fig. 4. model for the expanded role for protein c pathway in endothelial cell. in addition to its classic role as a feedback inhibitor of thrombin generation, apc suppresses tnf-mediated effects though the down-regulation of nf-kb subunits and subsequent inhibition of inflammatory cell adhesion. moreover, apc modulates pathways associated with an anti-inflammatory phenotype.
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