

## Review

# New insights into the protein C pathway: potential implications for the biological activities of drotrecogin alfa (activated)

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## Abstract

It has been hypothesized that the protein C pathway is a pivotal link between the inflammation and coagulation cascades. The demonstration that a survival benefit is associated with administration of drotrecogin alfa (activated) (recombinant human activated protein C [APC]) in severe sepsis patients has provided new insights into the protein C pathway. APC was originally identified based on its antithrombotic properties, which result from the inhibition of activated Factors V and VIII. In the early 1990s, any potential anti-inflammatory properties of APC were thought to relate primarily to its inhibition of thrombin generation. However, the mid-1990s saw the identification of the endothelial protein C receptor (EPCR), which has subsequently been shown to be neither endothelial specific nor protein C specific, but has a primary function as a cofactor for enhancing the generation of APC or behaving as an APC receptor. Thus, the potential biologic activities of APC can be classed into two categories related either to the limiting of thrombin generation or to cellular effects initiated by binding to the EPCR. Intracellular signaling initiated by binding of APC to its receptor appears to be mediated by interaction with an adjacent protease-activated receptor (PAR), or by indirect activation of the sphingosine 1-phosphate pathway. Based mostly on *in vitro* studies, binding of APC to its receptor on endothelial cells leads to a decrease in thrombin-induced endothelial permeability injury, while such binding on blood cells, epithelial cells, and neurons has been shown to inhibit chemotaxis, be anti-apoptotic, and be neuroprotective, respectively. In the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study, drotrecogin alfa (activated) was associated with improved cardiovascular function, respiratory function, and a prevention of hematologic dysfunction. This article discusses the way in which the interactions of APC may alter the microcirculation.

## Introduction

Activation of the innate immune system is the first phase of the human response to invading microorganisms [1,2]. In

most instances, this results in a localized inflammatory and procoagulant response that is beneficial in limiting spread of the infection, clearing pathogens, and aiding tissue healing [3,4]. However, in a significant number of sepsis patients, activation of the immune system is poorly regulated, resulting in a systemic inflammatory and procoagulant response that is frequently fatal [5,6]. Severe sepsis and septic shock represent the more severe complications of an uncontrolled immune response to infection.

Activated protein C (APC), an endogenous vitamin K dependent serine protease with multiple biological activities, is an important modulator of the host systemic response to severe infection [7]. APC exhibits antithrombotic properties via inhibition of activated Factors V and VIII [8,9], and profibrinolytic properties via inhibition of plasminogen activator-inhibitor 1 [10,11]. Inhibition of thrombin production results in indirect anti-inflammatory properties [12]. Additionally, APC may exhibit direct anti-inflammatory and anti-apoptotic [13] properties via interaction with its receptor (endothelial protein C receptor [EPCR]) on the endothelium [14], neutrophil [15], monocytes [15], eosinophil [16], and airway epithelial cells [17]. Profound species specificity has been widely shown for the anticoagulant/antithrombotic activity of APC [18-22]. Little is known about the species specificity of its nonanticoagulant activities. Many published *in vitro* and *in vivo* pharmacology studies exploring its nonanticoagulant activities have been conducted using concentrations of APC much higher than median steady-state plasma levels (45 ng/ml or 0.8 nM in patients with severe sepsis) [23] in humans given 96 hours drotrecogin alfa (activated) (recombinant human APC) therapy at 24 µg/kg per hour (Table 1), the dose for the treatment of severe sepsis at high risk of death. Since 2003,

APC = activated protein C; EPCR = endothelial protein C receptor; IL = interleukin; PAI-1 = plasminogen activator inhibitor-1; PAR = protease-activated receptor; PROWESS study = Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis Study; S-1-P = sphingosine-1-phosphate; S1P<sub>1</sub> = sphingosine-1-phosphate receptor (Edg-1); TAFI = thrombin activatable fibrinolysis inhibitor; TNF-α = tumor necrosis factor-α.

**Table 1**

| <b>Concentrations of protein C, APC, or rhAPC in humans</b>                      |                        |
|--|------------------------|
|  | Concentrations (ng/ml) |
| Normal endogenous protein C levels   | 4,000                  |
| Normal endogenous APC levels   | 1                      |
| rhAPC in PROWESS patients<br>(median steady-state levels)                        | 45                     |
| Levels of APC/rhAPC used in many<br>non-clinical <i>in vitro/in vivo</i> studies | 1,000–20,000           |

APC, activated protein C; PROWESS, Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis; rhAPC, recombinant human activated protein C (drotrecogin alfa [activated]).

more *in vitro* and *in vivo* studies have been conducted with APC concentrations or infusion rates that approximate those achieved or given in patients with severe sepsis. These studies suggest that some of the nonanticoagulant activities of APC may be less species-specific than the anticoagulant activity. These nonanticoagulant activities include reducing leukocyte interactions with activated endothelium, and reducing chemotaxis of leukocytes in response to chemokines [15,16,24].

This review summarizes the most recent insights into the protein C pathway, emphasizing results from clinical studies as well as potentially more clinically relevant preclinical studies (i.e. those that have incorporated concentrations or dosing regimens of APC that are approximately equal to the therapeutic dose of APC approved for the treatment of severe sepsis).

## Major components of the protein C pathway in severe sepsis

### Protein C and APC

Protein C is converted to APC when thrombin complexes with thrombomodulin, an endothelial surface glycoprotein [25]. The activation of protein C is facilitated by the EPCR, which appears to be primarily located on major blood vessels [12,14]. In healthy individuals, circulating levels of protein C and APC are 3,000–7,000 ng/ml and 1–3 ng/ml, respectively. Under normal conditions, circulating levels of APC are dependent on the concentrations of protein C and thrombin [26]. Infusing low concentrations of thrombin in healthy baboons results in concentrations of APC exceeding 200 ng/ml [27]. Activation of the protein C pathway in patients undergoing thrombolysis for acute myocardial infarction results, on average, in APC concentrations of 69 ng/ml, possibly related to the release of thrombin from lysing thrombus [28]. Consequently, in the setting of a normal endothelium, activation of the protein C pathway would be expected to result in an increase in circulating levels of APC. In severe sepsis, however, the host response leads to a generalized systemic dysfunction of the endothelium [4].

In studies of drotrecogin alfa (activated) in adult patients with severe sepsis, endogenous protein C and APC concentrations were measured in placebo-treated patients at variable time points during the first 4 days of study participation. In a Phase II study, 80% of placebo-treated patients had no detectable levels of APC (lower limit of detection = 5 ng/ml) [29,30]. The remaining patients had transiently detectable levels that displayed no discernible pattern, and no patient had a level exceeding 20 ng/ml. In a Phase III study, only 11 of 333 placebo-treated patients had measurable levels of APC (lower limit of detection = 10 ng/ml) [23]. In these 11 patients, only 13 of the 36 total samples collected had measurable concentrations of APC, and only two samples contained concentrations exceeding 20 ng/ml. Data from studies with a small number of severe sepsis patients confirm that levels of endogenous APC are much lower than the therapeutic levels (45 ng/ml) achieved with drotrecogin alfa (activated) treatment, and are not sustained [31,32].

### Acquired protein C deficiency in sepsis in humans and in animal models

In both Phase II and III studies with drotrecogin alfa (activated), over 85% of patients presented with protein C levels below the lower limit of normal, consistent with previous reports demonstrating protein C deficiency in severe sepsis [33,34]. Potential explanations for this acquired protein C deficiency include degradation by neutrophil elastases [35], conversion to APC, decreased synthesis by the liver [36,37], and increased trapping by the soluble form of EPCR in sepsis patients [38,39]. Neutrophils are key to sepsis-induced inflammation, and it has been demonstrated that mediators released from neutrophils, such as elastase, can significantly degrade protein C stores [36]. Since protein C is synthesized almost exclusively by the liver, it is difficult to examine this parameter in patients with severe sepsis, but animal models of sepsis can offer unique insights. Heuer and colleagues demonstrated that protein C mRNA levels in the liver are significantly reduced 20 hours after cecal ligation and puncture in the rat [37]. The effect on protein C mRNA levels in this model of sepsis appears to be selectively reduced compared with other proteins produced by the liver, such as antithrombin [40].

### Thrombomodulin and EPCR

In severe sepsis, the host response also leads to a generalized systemic dysfunction of the endothelium [4,41]. Thrombomodulin is required for activation of protein C, and *in vitro* studies have shown that endotoxin and inflammatory cytokines can downregulate endothelial-surface thrombomodulin [42,43]. Thrombomodulin can also be cleaved by neutrophil elastases and released into the systemic circulation. In a study of pediatric patients with severe sepsis from meningococcal infection, thrombomodulin and EPCR were reduced in skin biopsy specimens, which can contribute to low levels of APC [44].

EPCR, a type I transmembrane protein with homology to CD1d/major histocompatibility complex class I proteins [45] involved in antigen presentation, facilitates the conversion of protein C to APC. A recent *in vivo* study reported that EPCR mRNA expression was upregulated in the liver, kidney, and lung 24 hours after cecal ligation and puncture in protein C heterozygous mice [40]. Gu and colleagues demonstrated that intravenous injection of lipopolysaccharide increased EPCR mRNA levels in the lung and heart, and increased (by approximately fourfold at 6 hours, the peak of expression) the soluble EPCR serum level in rodents. However, the cell-surface EPCR levels in the lung and heart changed little in response to endotoxin challenge, suggesting that the increase of mRNA may compensate for the increased shedding of the receptor from the endothelium [46].

In severe sepsis patients, deficiencies in the protein C pathway can contribute significantly to the decrease in APC generation. In summary, low concentrations of circulating APC can be explained by low protein C concentrations, downregulation or shedding of thrombomodulin and EPCR, and/or APC trapping by soluble EPCR. The low levels of protein C and APC provide a scientific rationale for giving exogenous APC to patients with sepsis-induced coagulopathy and inflammation.

### Targeting the host response to infection

The generally accepted concept that limiting or suppressing the host response to infection would be beneficial in mitigating organ dysfunction in severe sepsis has been the focus of sepsis research for more than 20 years. Most of the early focus was on blocking the excessive inflammatory response, but most recent studies have begun to investigate targeting of the coagulation cascade. As the anticoagulant activity of the APC pathway displays species specificity [18-22], there were few preclinical studies investigating the efficacy of APC for severe sepsis prior to the approval of drotrecogin alfa (activated). Taylor and colleagues demonstrated that infusion of high-dose, plasma-derived human APC in baboons in a bacteremic model prevented the coagulopathic, hepatotoxic, and lethal effects of an otherwise lethal dose of *Escherichia coli* [47]. More interestingly, blocking endogenous activation of protein C in the same model using an antibody to protein C resulted in a more severe response to a lethal dose of *E. coli*, and a sublethal dose was made lethal. The blockade of EPCR during infusion of 10% of a lethal dose of *E. coli* in baboons greatly increased interleukin (IL)-8 concentrations and leukocyte infiltration into the tissues [48]. The disruption of the binding of APC to its receptor may suggest a role for EPCR in the regulation of leukocyte trafficking in the host response to bacterial infection. In EPCR transgenic mice, EPCR was overexpressed in both large vessels and capillaries, resulting in a survival advantage to endotoxin challenge [49]. This study reported higher levels of endogenous APC in these transgenic EPCR mice on endotoxin challenge compared

with wild-type mice. These recent data suggest that EPCR may be a key modulator of both endogenous anticoagulation and the interaction between leukocytes and the endothelium in health and in disease (Fig. 1).

Taken together, these data are consistent with the hypothesis that, in patients with severe sepsis, acquired protein C deficiency and diffuse endothelial injury may result in the inability to convert protein C to APC. Consequently, providing APC, rather than protein C concentrate, ensures administration of a biologically active therapeutic capable of providing a survival benefit.

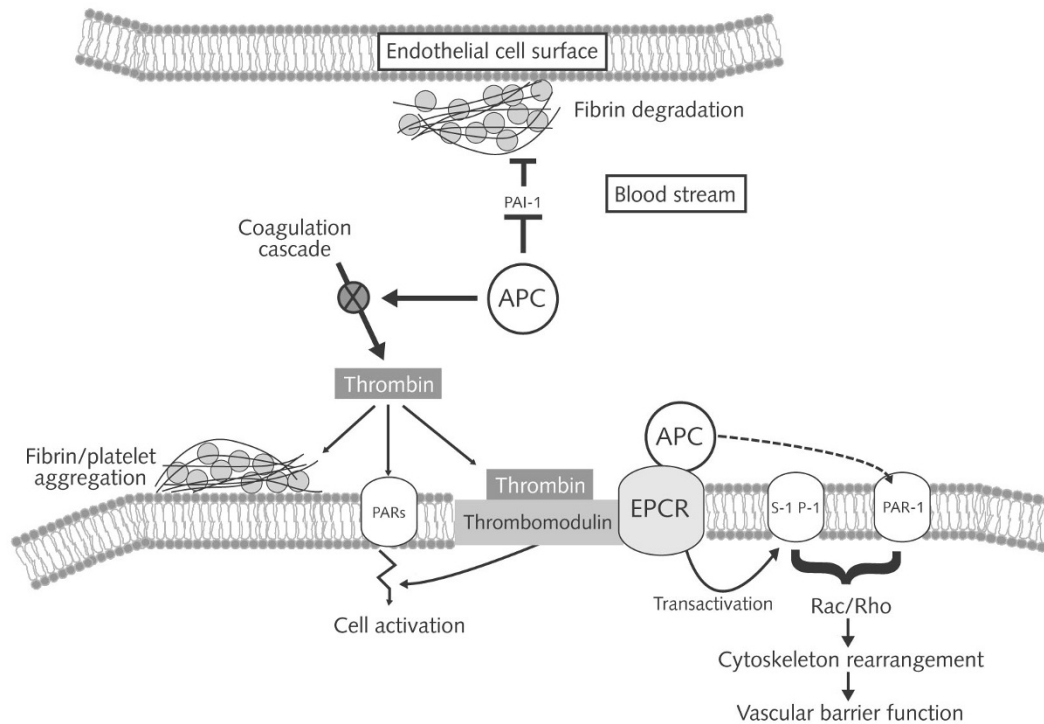
### The multipotent protein C pathway

The first known activity of the protein C pathway was anticoagulation, with this property of APC first reported by Seegers and colleagues in 1960 [50]. Similar to thrombin, APC is a serine protease and appears to have multiple biological activities, both alone and via EPCR. The species specificity of the anticoagulant activity of the protein C pathway influenced experiments exploring other nonanticoagulant activities of this pathway during the 1980s and 1990s. In examining the anticoagulant/antithrombotic activity of human APC in nonprimates, much higher doses of human APC were used to overcome the cross-species barrier effect. For example, the dose of human APC that produced an antithrombotic effect in a guinea pig was about 2 mg/kg per hour compared with a dose of about 0.015 mg/kg per hour in rhesus monkey [51,52]. Given the antithrombotic effects of APC, it also serves as an indirect inhibitor of the inflammatory activities of thrombin. There has been a growing interest in the potential direct anti-inflammatory activities of APC [53-57]. Preclinical experiments done in the 1980s and 1990s almost inevitably used supratherapeutic exposure of APC. As such, some of the reported activities of APC from these studies may not be clinically relevant, and recent data also suggest that APC at high concentrations appears to have opposing effects to lower concentrations [58-60].

### The anticoagulant/antithrombotic activity of APC

The antithrombotic activity of APC has been well established in various thrombotic models and in multiple animal species [9,52,61-65]. The antithrombotic activity of drotrecogin alfa (activated) was demonstrated in patients with severe sepsis by the reduction in levels of D-dimers and markers of thrombin generation (F1.2, thrombin-antithrombin complex) compared with placebo-treated patients [66]. Surprisingly, unlike several other anticoagulants [67-72], drotrecogin alfa (activated) does not significantly reduce markers of thrombin generation in a human model of low-dose endotoxemia [73,74]. The unexpected differences in pharmacodynamic effects of drotrecogin alfa (activated) observed between patients with severe sepsis and the human endotoxemia model will be important in future studies and should prompt caution in extrapolating data from human endotoxemia models to actual patients with severe sepsis.

Figure 1



The protein C pathway: modulation of thrombin generation and cell signaling. APC, activated protein C; EPCR, endothelial protein C receptor; PAI-1, plasminogen activator inhibitor 1; PAR, protease-activated receptor; Rac, ras-related protein; Rho, ras-homolog; S-1 P-1; sphingosine 1 phosphate 1-receptor.

### The profibrinolytic activity of APC

Preclinical studies suggest that APC may enhance the endogenous fibrinolytic pathway by inhibiting tissue plasminogen activator with plasminogen activator inhibitor-1 (PAI-1), and by limiting the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) by thrombin [75,76]. However, the PAI-1 concentration in plasma is several orders of magnitude lower than the other four known plasma serine protease inhibitors ( $\alpha_1$ -antitrypsin,  $\alpha_2$ -macroglobulin,  $\alpha_2$ -antiplasmin, and protein C inhibitor) for APC. Thus, in the actual milieu of the circulation, the effect of APC on PAI-1 may be minimal. This may explain why, in patients with severe sepsis, drotrecogin alfa (activated) treatment does not significantly lower PAI-1 levels compared with placebo patients [66]. Even in human endotoxin models studied with drotrecogin alfa (activated), there was no significant decrease in the levels of plasma PAI-1 compared with placebo [73,74]. In a human model of local inflammation with pulmonary low-dose endotoxin [77], drotrecogin alfa (activated) given systemically blunted the rise in PAI-1 levels in the bronchoalveolar lavage fluid as compared with the placebo group, but did not appear to influence the endogenous fibrinolytic potential [78].

TAFI is now known to be an acute phase reactant [66,79] and, thus, would not be an appropriate biomarker to study the

profibrinolytic activity of the protein C pathway in sepsis. In summary, the profibrinolytic properties of drotrecogin alfa (activated) may be a minor mechanism of action.

### The anti-inflammatory activity of APC

There have been many preclinical *in vitro* and *in vivo* studies, almost all using suprapharmacological concentrations of APC, suggesting that APC has direct anti-inflammatory activity by downregulating the expression of inflammatory cytokines such as IL-1 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [80-84]. However, to date, no such effects have been observed in any clinical studies of drotrecogin alfa (activated). A study of patients with severe sepsis showed that there were no significant differences between drotrecogin alfa (activated) and placebo groups in the levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-8, and IL-10, but that there was a faster reduction in IL-6 levels in the drotrecogin alfa (activated) group [66]. Two independent, placebo-controlled, blinded studies [73,74] were conducted with drotrecogin alfa (activated) in a human endotoxemia model. In both studies, compared with placebo, drotrecogin alfa (activated) did not significantly decrease levels of multiple cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and IL-10) and leukocyte cell-surface adhesion molecules. In addition, in a placebo-controlled human pulmonary endotoxin model [77], drotrecogin alfa

(activated) was given intravenously at 24 µg/kg per hour for 16 hours, starting 2 hours prior to the endotoxin challenge. Drotrecogin alfa (activated) treatment did not have a significant effect on the levels of inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10, and monocyte chemoattractant protein-1) in the bronchoalveolar lavage fluid compared with placebo.

### The effect of APC on leukocyte–endothelial cell interactions

More recently, preclinical studies have explored the nonanticoagulant activities of APC using therapeutic levels of APC. These recent studies suggest that the anti-inflammatory properties of the protein C pathway may not involve lowering inflammatory cytokine levels, but rather may involve lowering the chemotactic response of leukocytes and modulating the interaction of leukocytes with the activated endothelium. Intriguingly, the effect of APC on leukocytes appears to be limited to chemotaxis, as other leukocyte functions, such as phagocytic and oxidative burst, are unaffected [15,16,85].

Using intravital microscopy of the dorsal skin fold of a hamster endotoxemia model, Hoffmann and colleagues [24] demonstrated that intravenous administration of human plasma-derived APC at 24 µg/kg per hour significantly reduced endotoxin-induced leukocyte rolling and adhesion in both arterioles and venules. At this infusion rate, there is minimal anticoagulant activity of human APC in the hamster due to species specificity [52]. The study by Hoffmann and colleagues [24] strongly suggests that these anti-inflammatory properties of APC are independent of its anticoagulant activity. *In vitro* studies [15,16] using therapeutic concentrations of both plasma-derived human APC and drotrecogin alfa (activated) suggest that the effects observed by Hoffmann and colleagues may occur via the lowering of the chemotactic response of leukocytes to chemokines. The effect of APC on leukocyte chemotaxis is mediated by EPCR, which is present both on endothelial cells and on neutrophils. This may explain the significant decrease of leukocytes in the bronchoalveolar lavage fluid observed in a human pulmonary endotoxin model [77] for individuals treated with drotrecogin alfa (activated) compared with placebo.

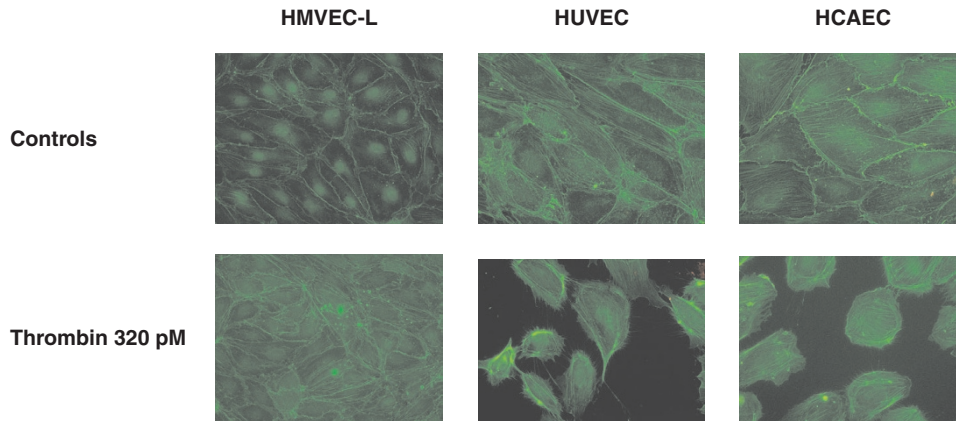
Transendothelial migration of leukocytes from the circulation also involves concerted endothelial cell–cell and cell–matrix interactions [86]. Several *in vitro* studies have examined the effects of drotrecogin alfa (activated) or plasma-derived human APC on the barrier function of primary human endothelial cells [59,60,87]. These studies, each using primary human endothelial cells derived from different vascular beds, showed that APC was able to protect the endothelial barrier from thrombin-induced disruption. Thrombin-induced transient endothelial barrier disruption (maximum around 30 min and recovered by 2–3 hours) occurs by activating protease-activated receptor (PAR)-1, one of four PARs on the endothelium [88,89]. The data from

these studies suggest that the protective effects of APC involve interaction with EPCR and PAR-1. These studies also suggest that the mechanism of action of APC is linked to the sphingosine-1-phosphate (S-1-P) pathway and the Rho-kinase pathway (Fig. 1). In extending these intriguing *in vitro* observations to future studies, it is important to note the significant complexity of these signaling pathways. It is known that there is a wide variation in the tissue distribution of the receptors implicated in these *in vitro* studies of APC. For example, Edg-1 (also known as S1P<sub>1</sub>), the receptor for S-1-P, has been shown to be abundant in the brain and lung, but virtually absent in the kidney vasculature [90]. One *in vitro* study offers an important insight [60] into the opposing effects of thrombin in endothelial barrier function above and below the half-maximal thrombin concentration for activating PAR-1 (about 40–50 pM [91]). At thrombin concentrations below 40–50 pM, thrombin strengthens endothelial barrier function, while at higher concentrations thrombin disrupts the barrier. Primary human endothelial cells derived from different vascular beds, however, appear to have different sensitivities to thrombin-induced barrier disruption. Human endothelial cells derived from the lung microvascular bed are more resistant to thrombin-induced barrier disruption than cells derived from the coronary arterial or umbilical venous bed (Fig. 2). The thrombin concentration used in this experiment (320 pM) is an estimate of the levels of thrombin generated in patients with severe sepsis from the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study (Table 2) [33]. We speculate from these recent studies that the multiple biological activities of APC may differ from tissue to tissue, governed by the tissue distribution of the various receptors, intracellular signaling pathways, and sensitivity of the cells to various inflammatory stimuli.

### Conclusion

More than four decades since the discovery of the anticoagulant activity of APC, we are continuing to learn about the diverse biological activities of this molecule. Drotrecogin alfa (activated) treatment has been shown to reduce mortality in patients with severe sepsis and has been approved for the treatment of severe sepsis patients at significant risk of death in more than 50 countries. An improvement in respiratory function and more rapid resolution of cardiovascular dysfunction were demonstrated in the pivotal Phase III PROWESS study. The exact mechanisms by which drotrecogin alfa (activated) exerts its beneficial effects on organ function and survival are yet to be fully understood. However, it is likely that the multiple biologic activities of this agent were critical to its success in PROWESS. Most of these activities appear to involve the modulation of endothelial function, modulation of leukocyte activity, and improvement in microvascular perfusion in severe sepsis, thus improving organ function. New and current noninvasive technologies may allow researchers to study the effect of drotrecogin alfa (activated) treatment in the microvascular beds of patients with severe sepsis. Further insights into the

Figure 2



Effect of thrombin on monolayer and cytoskeletal rearrangement of human primary endothelial cells derived from three different vascular beds. At early passages, cultured cells were plated in 8-well fibronectin-coated CultureSlides (Becton Dickinson, Bedford, MA, USA), 35,000 cells/well. After 24–48 hours, confluent monolayer cells were stimulated with 320 pM human thrombin (Sigma, St Louis, MO, USA) for 30 min at 37°C. Cells were fixed with 4% formaldehyde and stained for f-actin using Fluorescein isothiocyanate-conjugated phalloidin (Sigma, catalog number P5282). HCAEC, human coronary arterial endothelial cells; HMVEC-L, human lung microvascular endothelial cells; HUVEC, human umbilical venous endothelial cells. All cells were obtained from Cambrex (Walkersville, MD, USA). All images are shown at  $\times 40$  magnification.

Table 2

#### Concentrations of thrombin used in experiments

|   | Concentration (nM) |
|---|--------------------|
| Most historical experiments   | 20–500             |
| EC <sub>50</sub> for PAR-1 activation   | 0.05               |
| Baseline levels of markers of thrombin generation in PROWESS patients (severe sepsis) | ~0.1–1             |
| More recent studies of APC's effects on thrombin/PAR-1/endothelial cells              | 0.02–1             |

APC, activated protein C; EC<sub>50</sub>, Concentration inducing half-maximum activation of PAR-1; PAR-1, protease-activated receptor 1; PROWESS, Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis.

potential mechanisms of action of drotrecogin alfa (activated) will require the translation of preclinical study results to clinical research, and finally to the bedside.

#### Competing interests

All authors are employees and stockholders of Eli Lilly and Company. Drotrecogin alfa (activated) (Xigris®) is a product of Eli Lilly and Company. Ownership and all rights of issued patents are signed over from employees to Eli Lilly and Company.

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