Published in final edited form as:

Thromb Haemost. 2018 April ; 118(4): 630–638. doi:10.1055/s-0038-1629902.

α_2 -Macroglobulin Is a Significant *In Vivo* Inhibitor of Activated Protein C and Low APC: α_2 M Levels Are Associated with Venous Thromboembolism

Laura Martos^{1,*}, Luis Andrés Ramón^{1,*}, Julia Oto¹, Álvaro Fernández-Pardo¹, Santiago Bonanad^{1,2}, Ana Rosa Cid^{1,2}, Andras Gruber³, John H. Griffin⁴, Francisco España¹, Silvia Navarro¹, and Pilar Medina¹

¹Grupo de Investigación en Hemostasia, Trombosis, Arteriosclerosis y Biología Vascular, Instituto de Investigación Sanitaria La Fe (IIS La Fe), Hospital Universitario y Politécnico La Fe, Valencia, Spain

²Unidad de Trombosis y Hemostasia, Servicio de Hematología, Hospital Universitario y Politécnico La Fe, Valencia, Spain

³Department of Biomedical Engineering, Oregon Health and Science University, Portland, Oregon, United States

⁴Department of Molecular Medicine, The Scripps Research Institute, La Jolla, California, United States

Abstract

Background—Activated protein C (APC) is a major regulator of thrombin formation. Two major plasma inhibitors form complexes with APC, protein C inhibitor (PCI) and α_1 -antitrypsin (α_1 AT), and these complexes have been quantified by specific enzyme-linked immunosorbent assays (ELISAs). Also, complexes of APC with α_2 -macroglobulin (α_2 M) have been observed by immunoblotting. Here, we report an ELISA for APC: α_2 M complexes in plasma.

Methods—Plasma samples were pre-treated with dithiothreitol and then with iodoacetamide. The detection range of the newly developed APC: α_2 M assay was 0.031 to 8.0 ng/mL of complexed APC. Following infusions of APC in humans and baboons, complexes of APC with α_2 M, PCI and α_1 AT were quantified. These complexes as well as circulating APC were also measured in 121 patients with a history of venous thromboembolism (VTE) and 119 matched controls.

Address for correspondence Pilar Medina, Hospital Universitario y Politécnico La Fe, Torre A, 5^a Planta, Lab. 5-09, Avenida Fernando Abril Martorell 106, 46026 Valencia, Spain (medina_pil@gva.es). Dr. Silvia Navarro, Hospital Universitario y Politécnico La Fe, Torre A, 5^a Planta, Lab. 5-18, Avenida Fernando Abril Martorell 106, 46026 Valencia, Spain (navarro_silros@gva.es).

*These authors contributed equally to this study.

Authors' Contributions

S.N. performed the experiments, analysed the data and critically revised the manuscript. L.M. performed the experiments and analysed the data. L.A.R. performed the experiments. J.O. performed the experiments. A.F.P. performed the experiments. S.B.S. performed the plasma collection from VTE patients and controls and revised the manuscript. A.R.C. performed the plasma collection from VTE patients and controls and revised the manuscript. A.G. designed the experiments and revised the manuscript. J.H.G. designed the experiments and critically revised the manuscript. F.E. designed and performed the experiments, analysed the data and wrote the manuscript. P.M. designed and performed the experiments, analysed the data and wrote the manuscript.

Conflict of Interests

The authors state that they have no conflict of interest.

Results—In all the in vivo experiments, $\alpha_2\text{M}$ was a significant APC inhibitor. The VTE case–control study showed that VTE patients had significantly lower APC: $\alpha_2\text{M}$ and APC levels than the controls ($p < 0.001$). Individuals in the lowest quartile of APC: $\alpha_2\text{M}$ or the lowest quartile of APC had approximately four times more VTE risk than those in the highest quartile of APC: $\alpha_2\text{M}$ or of APC. The risk increased for individuals with low levels of both parameters.

Conclusion—The APC: $\alpha_2\text{M}$ assay reported here may be useful to help monitor the in vivo fate of APC in plasma. In addition, our results show that a low APC: $\alpha_2\text{M}$ level is associated with increased VTE risk.

Keywords

activated protein C; α_2 -macroglobulin; ELISA; baboon; complexes

Introduction

Activated protein C (APC) is a serine protease that circulates in plasma as an inactive precursor, protein C (PC), which is activated by thrombin on the surface of the endothelial cell.^{1–3} APC is an important regulator of thrombosis since homozygous PC-deficient infants suffer fatal thrombotic disease^{4,5} and low levels of PC or circulating APC are prevalent and independent risk factors for venous thromboembolism (VTE).^{6,7} Free APC circulates in small amounts in healthy individuals⁸ and its concentration is altered in various clinical conditions.^{9–13}

Polymorphisms in components of the PC activation pathway modify the levels of circulating APC and the risk of thrombosis.^{14–18} Plasma contains two major APC inhibitors, the heparin-dependent PC inhibitor (PCI) and α_1 -antitrypsin ($\alpha_1\text{AT}$).^{19–21} Both are physiological regulators of APC as complexes with APC have been detected in plasma from patients with different diseases.^{22,23} However, other APC complexes with an auxiliary inhibitor were observed by immunoblotting during infusion of APC into baboons, and were identified as APC: α_2 -macroglobulin ($\alpha_2\text{M}$).²⁴ APC: $\alpha_2\text{M}$ complexes were also identified in human and chimpanzee blood by immunoblotting^{25,26} but preferentially in the presence of metal ions,²⁵ suggesting that the complex formation is metal ion-dependent.

In this article, we developed a specific assay for the measurement of APC: $\alpha_2\text{M}$ complexes and demonstrate the presence of complexes of APC with $\alpha_2\text{M}$ in baboon and human plasma formed during in vivo infusion of APC. In addition, a case–control study showed that a low level of APC: $\alpha_2\text{M}$ is associated with the risk of VTE.

Materials and Methods

Reagents

Dithiothreitol (DTT) and iodoacetamide (IA) were from Sigma Aldrich (St. Louis, Missouri, United States). Chromogenic substrate S-2366 was from Werfen (Barcelona, Spain). Biotin-NHS, human $\alpha_2\text{M}$ and anti- $\alpha_2\text{M}$ antibody were from Calbiochem (La Jolla, California, United States). Human PC,²⁷ APC,²⁸ monoclonal antibody to the light chain of PC (C3)^{29,30} and PC-immunodepleted plasma were prepared as described earlier.²⁷

Study Subjects

For the case–control study, all patients with at least one episode of objectively diagnosed VTE of the lower extremities were identified from the records of patients referred to our hospital for thrombophilia examination over a 6-year period. A total of 132 Caucasian patients were identified. Plasma samples from 11 subjects were not available for the study and 121 patients participated in the study with their informed consent (60 women and 61 men). The mean age at the first thrombotic event was 39 years (women, 39 years; men, 40 years). Twenty-five patients (21%) also had pulmonary embolism (PE). VTE was established by ultrasonography or venography, and PE was diagnosed by ventilation–perfusion lung scanning or pulmonary angiography. Spontaneous VTE was defined as VTE without known precipitating risk factors (use of oral contraceptives, surgery, pregnancy, puerperium, trauma, immobilization). Sampling took place at least 10 months after the last thrombotic event (range: 10–24 months; median: 14 months).

The control group was recruited along with the cases and comprised 119 unrelated Caucasian age- and sex-matched volunteers from the same geographical area as the VTE patients. They were apparently healthy and none had a personal or familial history of thromboembolic disease. In all cases, plasma was stored in aliquots at -70°C until used within 4 years.

All subjects participated after giving written informed consent according to protocols approved by the Ethics Review Board of the La Fe University Hospital. The study was performed according to the declaration of Helsinki, as amended in Edinburgh in 2000.

Determination of APC and APC:PCI and APC: α_1 AT Complexes

Circulating APC,³¹ APC:PCI³² and APC: α_1 AT²² were measured as reported earlier and have a detection limit of 0.1 ng of APC/mL for APC and 0.1 ng of complexed APC for APC:PCI and APC: α_1 AT.

Preparation of Purified APC: α_2 M

Three hundred microliters of α_2 M (2 mg/mL) or buffer (0.05 M Tris–HCl, pH 7.0, 0.1 M NaCl) were incubated with 50 μL APC (8 $\mu\text{g}/\text{mL}$) and 3 μL Ca_2Cl (0.5 M) during a total of 9.5 hours at 37°C . The concentration of APC in the complex was estimated by measuring the concentration of free APC in the incubation mixtures A (α_2 M + APC) and B (buffer + APC). More than 99% of APC was complexed to α_2 M and the preparation of APC: α_2 M contained an estimated concentration of 1,133 ng/mL APC.

To see whether the complexed APC still had some amidolytic activity, we incubated 50 μL of different concentrations of APC: α_2 M or purified APC with 450 μL of 1 mM S-2366. A concentration of 400 nM in complex APC with α_2 M gave the same amidolytic activity as 45 nM purified APC.

APC: α_2 M Assay

Previous studies showed that when purified prostate-specific antigen (PSA) is added to plasma, most of the active PSA complexes to α_2 M and α_1 -antichymotrypsin, and that the

immunoreactivity of the PSA complexed to α_2 M is lost.³³ The same apparent loss of immunoreactivity occurred when we tried to measure APC: α_2 M complexes in plasma by enzyme-linked immunosorbent assay (ELISA). The enzyme in the complex is engulfed into the α_2 M molecule so that it is sterically hindered from cleaving high-molecular-weight substrates.^{34,35} Therefore, many of the antibodies used in the ELISAs are not able to bind to the enzyme in the complexes. However, different studies have reported that when a protease is bound to α_2 M, this binding is irreversible.^{36,37} This prompted us to investigate whether it would be possible to expose APC epitopes by treating the complex with a reducing agent.

We diluted 15 μ L sample with 123.4 μ L casein-EDTA buffer (0.05 M Tris-HCl, pH 7.4, 0.14 M NaCl, 2% casein, 5 mM EDTA and 0.05% sodiumazide) and the mixture was incubated with 5.8 μ L of 0.24 M DTT for 20 minutes at room temperature (RT) to open the α_2 M molecule and expose the APC epitopes. Thereafter, 5.8 μ L of 0.6 MIA was added and the mixture was incubated for 30 minutes at RT to stabilize the reduced complex. Thus, the final plasma dilution was 1/10. The APC: α_2 M calibrator was also pre-treated with DTT and IA and diluted with casein-EDTA buffer also pre-treated with DTT and IA, and used at final concentrations of APC complexed to α_2 M from 16 to 0.031 ng/mL.

The ELISA was performed as follows. Microtiter plates were coated with 100 μ L/well of 10 μ g/mL C3 antibody and incubated overnight at 4°C. After washing and blocking with casein-EDTA buffer, 100 μ L/well of calibrator, pre-treated plasma samples or pre-treated buffer was added and plates were incubated for 1 hour at RT. After washing, 100 μ L/well of a 1/1,000 dilution of biotin-anti- α_2 M antibody was added, and plates were incubated for 1 hour at RT. After washing with conjugate buffer, 100 μ L/well of a 1/1,000 dilution streptavidin horseradish peroxidase in conjugate buffer was added and plates were incubated for 1 hour at RT. Colour was developed by adding 100 μ L/well OPD and the reaction was stopped with 50 μ L/well 4 M H₂SO₄. Values are expressed as ng/mL of APC complexed to α_2 M.

To determine the precision of the assay, plasma from a control showing no circulating APC: α_2 M was spiked with APC: α_2 M complexes to reach final concentrations of APC complexed to α_2 M from 0.06 to 2.0 ng/mL.

In Vivo APC Complex Formation in Baboons

APC: α_1 AT, APC:PCI and APC: α_2 M complexes were measured in baboon plasma collected following infusion of purified APC as reported.²⁴ Baboons bearing chronic A-V shunts were used as described²⁴ and blood samples used in the present study are companion plasma aliquots to those that had been extracted in that previous study, which was approved by the Scripps Animal Research Committee.²⁴ Briefly, baboons were infused with 0.25 or 1.0 mg APC/kg in 1 hour and arterial blood samples were collected in 0.13 M sodium citrate containing 0.3 M benzamidine before, during and after distal infusion of APC, and plasma was stored at -70°C for 10 years. Thus, the samples used in the present study for the measurement of APC: α_2 M were matching plasma aliquots derived from the blood that was also used for determination of APC:PCI and APC: α_1 AT.²⁴ The half-life of APC: α_2 M complexes was calculated as indicated earlier for APC:PCI and APC: α_1 AT.²⁴

In Vivo APC Complex Formation in Humans

APC: α_1 AT, APC:PCI and APC: α_2 M complexes were also measured in plasma samples obtained from subjects included in a phase 1 study of XIGRIS (drotrecogin alfa; FIK-LC-GUAD; www.fda.gov/ohrms/dockets/ac/01/briefing/3797b102_FDA briefing.doc). Forty-four healthy adults (>40 years of age) received XIGRIS for two dosing periods, 6 or 24 hours of IV infusions, separated by at least 5 days, at 12 ($n = 12$), 24 ($n = 18$) or 36 ($n = 14$) μ g/kg/hour recombinant human APC (rhAPC). Venous blood was collected into duplicate tubes holding 1/10 volume of 3.8% sodium citrate, one containing 50 mM benzamidine and 50 μ M PPACK, and the other containing 100 U/mL heparin. After incubation at 37°C for 30 minutes, plasma was stored in aliquots at -70°C for 2 years until use.

Calculation of the Limit of Detection of the APC: α_2 M Assay

The limit of detection (LoD) of the APC: α_2 M assay was calculated from the equation:

$$\text{LoD} = \text{LoB} + 1.645 (\text{SD}_{\text{low concentration sample}}),$$

where LoB is the limit of blank ($\text{mean}_{\text{blank}} + 1.645 \times \text{SD}_{\text{blank}}$), and $\text{SD}_{\text{low concentration sample}}$ is the standard deviation of replicates of a sample containing 0.0625 ng/mL APC complexed to α_2 M.

Statistical Analysis

Analyses were performed using SPSS BASE 11.5.0 (SPSS, Chicago, Illinois, United States). Results were expressed as mean $\pm$ 1 SD or as median and interquartile ranges (25th to 75th percentiles). Parameter levels were compared with the Mann–Whitney *U*-test. A 5% significance level was used for all the statistical tests.

Results

Characterization of the ELISA for APC: α_2 M

Fig. 1 shows a calibration curve for the APC: α_2 M complex assay. The calibration curves of APC: α_2 M complex were linear within the range of concentrations between 0.031 and 8.0 ng/mL APC complexed to α_2 M under the assay conditions used. Purified α_2 M or APC at concentrations of 0.05 to 400 μ g/mL (α_2 M) or 0.01 to 4.0 μ g/mL (APC) used as negative control provided no signal in the ELISA (data not shown). The LoD in the purified system (calibrator) was 0.06 ng/mL APC complexed to α_2 M. However, the LoD for plasma was 0.6 ng/mL APC complexed to α_2 M because plasma samples become diluted 1/10 after pre-treatment with dithiothreitol (DTT) and IA.

The intra-assay coefficient of variation (CV), using concentrations of APC complexed to α_2 M of 0.8, 1.5 and 3.0 ng/mL ranged from 5 to 10% ($n = 10$), whereas the inter-assay CV, obtained with 10 runs over a period of 2 weeks, ranged from 8 to 14%.

When APC: α_2 M complex (0.6–2.0 ng/mL of APC complexed) was incubated with different dilutions of normal pooled plasma (in pre-treated casein-EDTA buffer), the recovery of the complex was 80% at a 1/5 dilution and 95 to 104% at 1/10 to 1/40 dilution. This indicates

that, at 1/10 plasma dilutions, the concentration of PC in the sample does not interfere in the measurement of the complex. To see at what concentration PC or APC will disturb measurement of the complex, purified PC or APC was added to PC-depleted plasma to reach final concentrations between 0 and 10 $\mu\text{g/mL}$, and then 0.6 to 2.0 ng/mL of pretreated APC: $\alpha_2\text{M}$ complex was added. Final concentrations of PC or APC below 6.4 $\mu\text{g/mL}$ did not significantly alter the recovery of the APC in the complex. Also, we added different concentrations of pretreated complex to PC-depleted plasma. In all cases, the complexed APC concentration obtained was similar to that obtained when the pretreated complex was added to normal pooled plasma, indicating that the assay was specific for the complexed APC.

The recovery in the assay after addition of several concentrations (0.6–10 ng/mL) of APC complexed with $\alpha_2\text{M}$ to pooled plasma ranged from 93 to 110%. To study dilution linearity, we diluted two plasma samples containing 25.1 and 10.0 ng/mL of APC complexed to $\alpha_2\text{M}$ from 2-fold to 32-fold using the standard casein-EDTA buffer. There was essentially a linear relationship between complexed APC concentration and dilution.

The stability of the APC: $\alpha_2\text{M}$ complex was analysed in purified complexes and in plasma samples to which purified APC: $\alpha_2\text{M}$ had been added to give 1 to 10 ng/mL APC complexed to $\alpha_2\text{M}$. After 15 years at -70°C , the purified complex at different dilution yielded ODs in the ELISA similar to that observed earlier. In the plasma system, the recovery was 93 to 106% after 10 years and 95 to 104% after 1 year at -70°C , 90 to 109% after 4 days at 4°C , 70 to 87% after 4 days at RT, and 92 to 109% after freezing at -70°C and thawing–refreezing the samples four times.

Therefore, the ELISA provided a highly sensitive and reproducible method for quantifying the APC: $\alpha_2\text{M}$ complex. Results were expressed as ng/mL of APC complexed to $\alpha_2\text{M}$.

In Vivo APC: $\alpha_2\text{M}$ Complexes in Baboons

Fig. 2 shows the mean values of APC: $\alpha_2\text{M}$ obtained from two different experiments using two baboons receiving two different APC doses. Before APC infusion, no APC: $\alpha_2\text{M}$ complexes were detected. During infusion, complexes of APC with $\alpha_2\text{M}$ were detectably formed and increased in concentration with time (see Fig. 1 in the study of Mezzano et al²³). At the low APC dose (0.25 mg APC/kg in 1 hour), the level of APC complexed to $\alpha_2\text{M}$ was 0.18 $\mu\text{g/mL}$ after 1 hour of infusion, compared with 1.4 and 0.8 $\mu\text{g/mL}$ of APC complexed to PCI and $\alpha_1\text{AT}$, respectively, measured previously. At the high APC dose (1.0 mg APC/kg in 1 hour), the concentration of APC complexed to $\alpha_2\text{M}$ was 0.75 $\mu\text{g/mL}$ compared with 2.9 and 4.1 $\mu\text{g/mL}$ APC complexed with PCI and $\alpha_1\text{AT}$, respectively.²⁴ From the low and the high APC dose used, we estimated a half-life of approximately 30 minutes for APC: $\alpha_2\text{M}$ complexes, compared with the half-life of 40 minutes for APC:PCI and of 140 minutes for APC: $\alpha_1\text{AT}$ previously reported.²⁴

In Vivo APC: $\alpha_2\text{M}$ Complexes in Healthy Subjects

Fig. 3 shows the mean or median values of APC, APC: $\alpha_1\text{AT}$, APC:PCI and APC: $\alpha_2\text{M}$ obtained during 6- and 24-hour infusions of rhAPC, at three different doses, in 44 healthy adults from the phase 1 study of XIGRIS. In all cases, $\alpha_1\text{AT}$ appeared to be the major APC

inhibitor, followed by α_2 M and PCI. Furthermore, when increasing the APC dose infused, the concentration of these three APC:inhibitor complexes also increased and their relative abundance remained constant. Table 1 shows the mean $\pm$ SD or median (25–75% interquartile range, for APC: α_2 M) concentration of different parameters measured at basal conditions, before infusion of rhAPC, in samples from the phase 1 study of XIGRIS. Twenty-five subjects had detectable APC: α_2 M complexes (> 0.6 ng/mL). At basal conditions, individuals with undetectable and detectable APC: α_2 M complexes had similar APC levels (4.1 ± 2.0 ng/mL vs. 4.2 ± 2.3 ng/mL; $p = 0.88$), as well as similar α_2 M levels ($p = 0.85$).

Case (VTE)–Control Study

As a low level of circulating APC is a risk factor for VTE, we analysed whether a low level of APC: α_2 M is also a risk factor for VTE in a case–control study that included 121 VTE patients and 119 age- and sex-matched healthy controls. Table 2 shows the characteristics of the study subjects. Percentages of male sex and presence of factor V Leiden were significantly higher in VTE patients than in controls. The median level of APC complexed to α_2 M was lower in VTE patients (0.7 ng/mL; 0.0–1.3) than in controls (1.3 ng/mL; 0.6–2.0; $p < 0.001$). When these levels were distributed by quartiles of APC: α_2 M levels in controls, individuals in the first quartile had approximately four times more VTE risk than those in the fourth quartile. Adjusting for age, sex, APC levels, PC or protein S deficiency, the presence of factor V Leiden or prothrombin 20210A did not significantly changed the OR (Table 3). As expected, the median level of APC was also lower in patients (1.05 ng/mL; 0.82–1.32) than in controls (1.22 ng/mL; 0.98–1.49; $p < 0.001$), and individuals in the first quartile of APC had approximately four times more VTE risk than those in the fourth quartile (Table 4).

As low levels of both APC and APC: α_2 M are associated with increased risk of VTE, we investigated whether a combination of low levels of both APC and APC: α_2 M further increases the VTE risk. Thus, the OR for VTE for the subgroup of individuals in the lower quartile for both APC and APC: α_2 M (30 VTE patients and 17 controls) compared with the subgroup in the highest quartile for both APC and APC: α_2 M (1 VTE patient and 14 controls) was 25 (95% CI: 3–204), indicating that the combination of low APC and low APC: α_2 M significantly increased the risk of VTE.

There was a positive correlation between the levels of APC: α_2 M and the levels of APC both in controls ($r = 0.496$, $p < 0.001$) and in VTE patients ($r = 0.192$, $p = 0.034$).

Discussion

Pre-treatment of plasma samples with DTT, and then with IA to stabilize the reduced complex, resulted in exposition of at least one APC epitope, as we could detect and measure APC: α_2 M complexes with an ELISA using as capture antibody a monoclonal anti-PC antibody that binds the EGF1 domain of APC, and for the second detecting antibody a polyclonal anti- α_2 M Ab. The assay performed well both in purified system (data not shown) and in plasma. A linear response was obtained in the range from 0.031 to 8.0 ng/mL, and the

LoD in plasma was 0.6 ng/mL. The assay was used to assess the relevance of α_2 M as an inhibitor of APC.

In a previous study, during infusion into baboons, APC formed complexes with two major inhibitors, PCI and α_1 AT, that could be measured by sandwich ELISAs.²⁴ Also, apparent complexes of APC with an auxiliary inhibitor were observed by immunoblotting, were identified as APC: α_2 M^{23–25} and were divalent metal ion dependent.²⁵ Scully et al also reported the presence of APC: α_2 M complexes during the clinical course of DIC in three patients by qualitative Western blotting.³⁸ Here, with our assay, we have demonstrated the formation of APC: α_2 M in baboon and human plasma during in vivo infusions of APC.

Given that plasma samples used here for the measurement of APC: α_2 M in baboons were companion samples for those used for the determination of APC:PCI and APC: α_1 AT in a previous study,²⁴ we were able to compare the concentration of the three APC complexes. At the lower APC dose used in baboons (0.25 mg/kg/hour), approximately 61% of the complexed APC was in the form of APC:PCI, 33% as APC: α_1 AT and 6% as APC: α_2 M at the time of blood sampling. At the higher APC dose used in baboons (1.0 mg APC/kg/hour), these percentages were 36, 52 and 12%, respectively.

To assess the relevance of α_2 M as an APC inhibitor in humans, we measured the three APC complexes in 44 healthy adults included in a phase 1 study who received rhAPC at different dosages. At baseline ($t = 0$), 25 of these healthy adults showed detectable APC: α_2 M levels ranging from 0.6 to 7.0 ng/mL of APC complexed to α_2 M (Table 1), showing that α_2 M is a physiological inhibitor of APC. During infusion of APC, α_1 AT was the major APC inhibitor. However, at any APC dosage, the contribution of α_2 M to APC inhibition was higher than that of PCI, in contrast to the results in baboon studies. It has been reported that incubation of PCI with APC results in the formation of APC:PCI complexes and also in partial degradation of PCI in a free form, which increases when increasing the enzyme/inhibitor molar ratio,²⁰ indicating that APC uses PCI as both a pseudo-substrate and an inhibitor. This could also explain the relative decrease in APC:PCI concentration observed at the highest dose of APC used. It is interesting to note that, in baboons, the half-life of APC: α_2 M appears to be shorter (30 minutes) than the half-life of APC: PCI (40 minutes) and APC: α_1 AT (140 minutes). Our study design does not allow us to calculate the half-life of the complexes of APC with its inhibitors in humans. However, if these half-lives were similar to those in baboons, the real contribution of α_2 M to the inhibition of APC may be more important than that calculated from the observed in vivo concentrations at the time of blood sampling.

A reduced concentration of circulating, free APC is a strong, prevalent, independent risk factor for VTE.⁶ The present results are in line with those previous data (Table 4). We hypothesized that low APC: α_2 M levels in plasma could also be associated with VTE. The results in the present case-control study indicate that a reduced concentration of APC: α_2 M is associated with the risk of VTE. Reduced APC: α_2 M levels (below 0.6 ng/mL, i.e. 25th percentile of controls) was found in 47% of our VTE patients (OR, 3.9), and this OR did not significantly change after adjusting for age, sex, APC levels, PC or protein S deficiency, and the presence of factor V Leiden or prothrombin 20210A (Table 3).

Besides its anticoagulant functions, the PC system displays multiple cytoprotective activities including anti-apoptotic, anti-inflammatory, neuro-protective and stabilization of endothelial barrier function.^{39–43} Thus, the APC: α_2 M assay described here may be useful to monitor the fate of infused APC in plasma during the treatment with APC, when treatment with wild-type APC or with new modified APC variants (e.g. 3K3A-APC) with preserved cytoprotective activity but with low or absent anticoagulant activity comes into clinical use.
44–47

Some enzymes bound to α_2 M still may express biological activity. Thus, α_2 M-bound proteases purified from plasma are able to degrade fibrinogen⁴⁸ and α_2 M-bound proteases purified from serum are able to clot fibrinogen.⁴⁹ Atkinson et al⁵⁰ developed a novel immuno-activity assay to measure multiple protease– α_2 M complexes with potential clinical applications. These findings raise the possibility that, in addition to its activity as a relevant plasma proteolytic enzyme inhibitor, α_2 M may modulate enzyme–substrate interactions, providing a mechanism for the preservation and protection of a portion of the enzymatic activity in the presence of other circulating inhibitors.⁴⁸ Although it has been reported that the anticoagulant activity of APC bound to α_2 M is inhibited,²⁶ we do not know whether APC in the APC: α_2 M complex retains cytoprotective activity.

A limitation of this study could be that plasma samples from the baboon studies were more than 10 years old, though notably kept to -70°C . However, this reinforces the in vivo relevance of α_2 M as an APC inhibitor, since any storage induced loss of complexes in plasma would have led to an underestimation of the amount of APC: α_2 M complex. Furthermore, studies on the stability of the APC: α_2 M complex in purified complexes and in plasma samples to which purified APC: α_2 M had been added to give 1.0 to 10 ng/mL APC complexed to α_2 M showed that after 15 years at -70°C , the results were similar to that observed earlier. Another limitation is that the number of VTE cases and controls is relatively small to draw definitive conclusions regarding the association of low APC: α_2 M levels with the risk of VTE.

In conclusion, these results establish the feasibility of an ELISA for measuring APC: α_2 M complexes that are formed in vivo and show that α_2 M is a physiologically significant APC inhibitor. The study also shows that a low APC: α_2 M level is associated with the risk of VTE and that this association is stronger in individuals with low levels of both APC and APC: α_2 M. However, the biological mechanisms that lead to these reduced APC: α_2 M levels and its clinical relevance need to be elucidated.

Acknowledgments

This research was supported in part by research grants from ISCIII (PI12/00027, RD12/0042/0029, PI14/00512, PI14/00079, FI14/00269), FEDER una manera de hacer Europa, Generalitat Valenciana (PrometeoII/2015/017) and NIH (R01 HL052246 and R01 HL133728 to J.H.G.). S.N. is an IIS La Fe researcher. P.M. is a Miguel Servet researcher (FISCP09/00065 and CPII15/00002). We are grateful to L. Harker and S. R. Hanson for their advice in the course of baboon experiments, and to Lilly Co. for providing us the plasma samples from subjects included in a phase 1 study of XIGRIS (drotrecogin alfa; F1K-LC-GUAD; www.fda.gov/ohrms/dockets/ac/01/briefing/3797b102_FDA briefing.doc).

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What is known about this topic?

- Infusion of activated protein C into baboons results in the formation of complexes of APC with PCI and α_1 AT.
- Complexes of APC with α_2 M were identified by immunoblotting.
- No assay for the quantitative measurement of APC: α_2 M complexes has been described.

What does this paper add?

- A quantitative assay for APC: α_2 M complexes is described that is useful to quantify complexes in healthy and patient samples. Significant amount of APC complexed to α_2 M is detected during infusion of APC into baboon and healthy individuals.
- The data show that, besides PCI and α_1 AT, α_2 M is also a major APC plasma inhibitor.
- A low level of plasma APC: α_2 M is a risk factor of venous thromboembolism.

Fig. 1.

Calibration of the ELISA for APC:α₂M complex. APC:α₂M complex was diluted with pre-treated casein-EDTA buffer as indicated in section 'Materials and Methods' to reach the final concentrations of APC complexed to α₂M indicated, and samples were assayed for APC:α₂M as indicated in section 'Materials and Methods'.

Fig. 2.

Time course for complexation of APC with α_2M during APC infusion into baboons. 0.25 (●—●) or 1.0 (○—○) mg APC/kg in 1 hour (30% initial bolus; remainder by continuous infusion) was infused into two different baboons on separate days. Blood samples were withdrawn at different time intervals and anticoagulated with 1/9 volume citrate containing 0.3 M benzamidine before, during and after APC infusion, and APC: α_2M complexes were measured as indicated in section 'Materials and Methods'.

Fig. 3.

Time course of complexation of APC during i.v. APC infusion in humans. Forty-four adult men and postmenopausal or surgically sterile females were infused i.v. with recombinant human APC (XIGRIS) in two dosing periods, 6 (**A, C, E**) and 24 hours (**B, D, F**) separated by at least 6 days, at 12 (**A and B**; $n = 12$), 24 (**C and D**; $n = 18$) and 36 (**E and F**; $n = 14$) $\mu\text{g/kg/hour}$. Blood samples were withdrawn at different time intervals as indicated in section 'Materials and Methods', before and during or after APC infusion, and APC (—) and its

complexes with PCI (●—●), α1AT (○—○) and α2M (▲—▲) were measured as indicated in section 'Materials and Methods'.

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Table 1

Mean $\pm$ SD or median (25–75% interquartile range, for APC: α_2 M) concentrations of different parameters measured at baseline conditions, before infusion of APC, in 44 adult subjects

Parameter	Controls
PC (%)	117 $\pm$ 29
PCI (%)	128 $\pm$ 26
α_1 AT (%)	104 $\pm$ 20
α_2 M (%)	83 $\pm$ 20
APC, ng/mL	4.2 $\pm$ 2.1
APC:PCI, ng/mL of APC complexed	0.5 (0.3–0.8)
APC: α_1 AT, ng/mL of APC complexed	11.4 $\pm$ 3.7
^a APC: α_2 M, ng/mL of APC complexed	0.7 (0.0–1.75)

Abbreviations: APC, activated protein C; PCI, protein C inhibitor.

^aFor APC: α_2 M, 19 subjects had levels below detection limit (<0.6 ng/mL APC complexed to α_2 M); for median calculation, they were arbitrarily given a concentration of 0 ng/mL.

Table 2

Characteristics of the study subjects

	VTE	Controls	Statistical significance, <i>p</i>
<i>N</i>	121	119	
Age (y, mean $\pm$ SD)	43 $\pm$ 11	43 $\pm$ 11	0.808
Male sex, <i>N</i> (%)	61 (50)	57 (48)	0.701
Recurrent thrombosis, <i>N</i> (%)	23 (19)	-	-
Family thrombosis, <i>N</i> (%)	35 (32)	-	-
Spontaneous thrombosis, ^a <i>N</i> (%)	59 (49)	-	-
FV Leiden, <i>N</i> (%)	16(13)	4(3)	0.017
PT G20210A, <i>N</i> (%)	14(12)	5(4)	0.03

Abbreviation: VTE, venous thromboembolism.

^a After excluding circumstantial risk factors (pregnancy and puerperium, trauma, surgery, immobilization, oral contraceptives).

Table 3

ORs (95% CI) of VTE by quartiles of APC: α_2 M levels (ng/mL APC complexed to α_2 M) in controls

APC: α_2 M	VTE patients	Controls	OR (95% CI)	
Quartiles	(<i>n</i> = 121) <i>n</i> (%)	(<i>n</i> = 119) <i>n</i> (%)	Crude	Adjusted ^a
Q1 (0.6)	57 (47)	36 (30)	3.9 (1.7–8.8)	3.2 (1.3–7.9)
Q2 (>0.6– 1.3)	30 (25)	26 (22)	2.8 (1.2–6.8)	1.8 (0.7–4.9)
Q3 (>1.3– 2.0)	23 (19)	30 (25)	1.9 (0.8–4.6)	1.6 (0.6–4.0)
Q4 (>2.0)	11 (9)	27 (23)	1 ^b	1 ^b

Abbreviations: APC, activated protein C; CI, confidence level; OR, odds ratios; VTE, venous thromboembolism.

^a Adjusted for age, sex, APC and the presence of thrombophilic risk factors (factor V Leiden and prothrombin 20210A). Forty-eight VTE patients (40%) and 26 controls (22%) had levels below detection limit (<0.6 ng/mL APC complexed to α_2 M), and they were arbitrarily given a concentration of 0 ng/mL.

^a Reference group.

Table 4

ORs (95% CI) of VTE by quartiles (Q) of APC levels (ng/mL) in controls

APC	VTE patients	Controls	OR (95% CI)	
Quartiles	(<i>n</i> = 121) <i>n</i> (%)	(<i>n</i> = 119) <i>n</i> (%)	Crude	Adjusted ^a
Q1 (0.98)	57 (47)	30 (25)	3.9 (1.8–8.6)	2.4 (1.0–5.8)
Q2 (>0.98– 1.22)	31 (26)	31 (26)	2.1 (0.9–4.6)	1.5 (0.6–3.6)
Q3 (>1.22– 1.49)	19 (16)	29 (24)	1.4 (0.6–3.2)	1.0 (0.4–2.7)
Q4 (>1.49)	14(12)	29 (24)	₁ ^b	₁ ^b

Abbreviations: APC, activated protein C; CI, confidence level; OR, odds ratios; VTE, venous thromboembolism.

^a Adjusted for age, sex, APC:α₂M and the presence of thrombophilic risk factors (factor V Leiden and prothrombin 20210A).^b Reference group.