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Antibody SPC-54 provides acute in vivo blockage of the murine protein C system

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Abstract

Multiple protective effects of pharmacological activated protein C (APC) are reported in several organ pathologies. To help evaluate the endogenous murine PC system, we characterized a rat monoclonal anti-mouse PC antibody, SPC-54, which inhibited the amidolytic and anticoagulant activities of murine APC by > 95%. SPC-54 blocked active site titration of purified APC using the active site titrant, biotinylated FPR-chloromethylketone, showing that SPC-54 blocks access to APC's active site to inhibit all enzymatic activity. A single injection of SPC-54 (10 mg/kg) neutralized circulating PC in mice for at least 7 days, and immunoblotting and immuno-precipitation with protein G-agarose confirmed that SPC-54 in vivo was bound to PC in plasma. Pre-infusion of SPC-54 in tissue factor-induced murine acute thromboembolism experiments caused a major decrease in mean survival time compared to controls (7 min vs. 42.5 min, $P=0.0016$). SPC-54 decreased lung perfusion in this model by 54 % when monitored by vascular perfusion methodologies using infrared fluorescence of Evans blue dye. In LD50 endotoxemia murine models, SPC-54 infused at 7 hr after endotoxin administration increased mortality from 42 % to 100 % ($P<0.001$). In summary, monoclonal antibody SPC-54 ablates *in vitro* and *in vivo* APC protective functions and enzymatic activity. The ability of SPC-54 to block the endogenous PC/APC system provides a powerful tool to understand better the role of the endogenous PC system in murine injury models and in cell bioassays and also to neutralize the enzymatic activities of murine APC in any assay system.

Keywords

activated protein C; protein C; coagulation; inflammation; thrombosis

Introduction

Blood coagulation is a complex process where different factors interact in both positive and negative reactions. One major negative feedback regulation system involves the protein C (PC) system [1]. PC, a vitamin K-dependent plasma glycoprotein, is a zymogen that binds to

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Authorship

L. Burnier, J.A. Fernández and J.H. Griffin designed experiments. L. Burnier and J.A. Fernández conducted experiments. L. Burnier and J.H. Griffin wrote the manuscript.

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the endothelial PC receptor (EPCR) where it is activated by thrombin in complex with thrombomodulin [1,2]. APC is a potent inactivator of activated coagulations factor V (FVa) and VIII (FVIIIa), and APC functions in humans include reducing risks for inflammation, infection and venous thrombosis, as shown by the severe or partial PC deficiencies in humans [3–5]. The PC system is also an important part of the host defense system and APC is a homeostatic protease with multiple biologic activities [1,6–10]. Beside its anticoagulant activity, APC is also cytoprotective in multiple ways, by binding and signaling via different receptors, such as protease activated receptor 1 (PAR1), EPCR, apolipoprotein E receptor 2 (ApoER2), and Mac-1 integrin on different cells [1,2,11,12].

Basic and preclinical knowledge about the PC system have been based on pharmacologic studies of wild-type and mutant APCs [1,7–10,13]. Additional insights have come from several studies using antibodies to block the PC system in baboons [13–15] and mice [7,16,17] and using genetically modified mice [18–21]. The monoclonal anti-murine PC monoclonal antibody (mAb), MPC1609, was selected to block binding of PC to endothelial cells and it inhibits APC's signaling and anticoagulant activity most likely due to its binding the Gla-domain which binds EPCR or phospholipid surfaces [17]. The mAb MPC1591 was selected for its binding to APC but not to PC; although MPC1591 inhibits APC's anticoagulant activity, it does not inhibit full APC enzymatic activity as it permits some of APC's signaling actions [17]. These two mAbs have been useful for showing that inhibition of anticoagulant activity does not necessarily impair cytoprotective signaling, as more directly shown by APC mutagenesis studies. However, none of the previously described anti-PC mAbs blocks *per se* all of the enzymatic activity of APC. The intrinsic utility of a mAb that unequivocally blocks murine APC's enzymatic activity *in vitro* and *in vivo* stimulated us to seek a novel anti-APC mAb with such properties. Here we report the *in vitro* and *in vivo* characterization of the rat anti-murine-PC mAb SPC-54 and show that SPC-54 potentially neutralizes APC enzymatic activities *in vitro* and blocks the PC system in two murine injury models.

Material and Methods

Mice

This study was approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute and complied with National Institutes of Health guidelines. C57BL/6J mice were bred in the institute.

Recombinant murine APC

Recombinant murine APC was produced using HEK293 as described [6] with the following modifications. Mouse PC in media containing 10 mM EDTA was loaded onto a Fast-Flow Q column (Sigma-Aldrich, Saint-Louis, MO), washed with 20 mM Histidine, 100 mM NaCl, pH 6.5, 0.02% Na-azide, and then eluted with 20 mM Histidine, 100 mM NaCl, 50 mM CaCl₂, pH 6.5, 0.02% Na-azide. Fractions containing PC were pooled, dialyzed against 20 mM Histidine 50 mM NaCl, pH 6.5, 0.02% Na-azide and loaded onto an UNO-Q column (Bio-Rad, Hercules, CA), washed with 4 column volumes of loading buffer, and then PC was eluted with a linear 100 to 500 mM NaCl gradient. Fractions containing PC were pooled and dialyzed against 20 mM sodium citrate, 50 mM NaCl, pH 6.0. For activation, mouse PC (diluted to 20 μ M) was incubated 4 hours at 37°C with 100 U/ml human recombinant thrombin (Recothrom, ZymoGenetics, Seattle, WA). After the activation, the incubation mixture was loaded onto a Mono Q column in the activation buffer and eluted with a 50–650 mM NaCl gradient.

Biotinylated FPR-chloromethylketone (b-PPACK, Haematologic Technologies, Inc., Essex Junction, VT) was used to quantify APC active site concentration. Samples were incubated with a 20-fold molar excess of this reagent, and after 60–90 min, APC amidolytic activity was reduced > 98 %. Then aliquots received SDS-DTT and were boiled and loaded onto SDS-PAGE gels (TGX AnyKd gels, Bio-Rad), electrophoresed and transferred onto nitrocellulose membranes (Li-Cor) using a semi-dry apparatus (Bio-Rad). Membranes containing biotinylated PC heavy chain were exposed to IRdye800-conjugated-streptavidin (Li-Cor) for 30 min. After washing, membranes were scanned at 800 nm with the Odyssey IR fluorescent scanner (Li-Cor) and quantification was done using Odyssey Image Studio 2.0 (Li-Cor). Values for murine APC active site concentration were made using human recombinant APC of known concentration.

Production of rat anti-mouse protein C monoclonal antibodies

Lewis rats were immunized by intraperitoneal injection of recombinant mouse PC in complete Freund's adjuvant (Difco). After 2 months, animals received a second PC injection. Four days later, the spleen was removed and fused to hybridoma SP2/0 cells for generation of clones using standard protocols. Hybrid cells secreting antibodies that were positive for binding murine PC or APC were further screened for their ability to inhibit APC amidolytic activity. Cells of the desired specificity were cloned and recloned at least once by limiting-dilution methods at one hybrid cell per well. Clones used for this study were designated SPC-54, 29H5 and 6B9.

Solid phase assay

Mouse PC 10 µg/ml was immobilized onto 96 wells plates (Nunc Maxisorp, Nunc International, Rochester, NY) using 0.1 M Na₂CO₃ pH 9. After blocking the wells with 2% BSA in Tris buffered saline (TBS) (0.02 M Tris, 0.15 M NaCl, pH 7.35), serial dilutions of SPC-54 (0 to 380 nM) were added to wells and incubated for 1 h. Following washing, bound antibodies were measured by incubating with 1 µg/ml biotinylated anti-rat IgG (1h) followed by 1 µg/ml streptavidin horseradish peroxidase (30 min). Then octo-phenyl diamine substrate hydrolysis was measured at 450 nm for 5 min using a microplate reader (Molecular Devices).

Amidolytic activity assay for APC

Mouse recombinant APC amidolytic activity was measured using 0.4 mM Pefachrome PCa colorimetric substrate (Pentapharm, Basel, Switzerland) in Tris-buffered saline (TBS; pH 8.0) containing 5 mM CaCl₂ and 2 mg/mL BSA. The increase on absorbance change at 405 nm due to hydrolysis was measured using a Thermomax microplate reader (Molecular Devices) and activity was calculated using SoftMax Pro Software (version 5.3, Molecular Devices). For SPC-54 inhibition studies, APC was used at 54 nM or 175 nM.

Thrombin generation in mouse plasma

To permit comparison of SPC-54 effects on APC anticoagulant activity, we used aliquots of a large pool of citrated plasma obtained by cardiac puncture (C57BL/6 mice, Bioreclamation, LLC, Hicksville, NY). Quality of the plasma was identical with our own pool of citrated plasma obtained by inferior vena cava puncture. Plasma, phospholipids (4 µM final, 20 % phosphatidylethanolamine, 60 % phosphatidylcholine and 20 % phosphatidylserine), SPC-54 (serial dilutions starting at 625 nM) and APC (10, to 0 nM) were incubated for 10 min at 37°C in HBS-BSA buffer (20 mM HEPES, 140 mM NaCl, 5 mg/ml BSA, pH 7.35). Then the reaction was started by simultaneous addition of tissue factor (TF) (1.5 pM, Innovin, Dade Behring, Marburg, Germany), 16.4 mM CaCl₂ and 0.25 mg/mL of the fluorescent thrombin substrate, I-1140 (Bachem, Bubendorf, Switzerland).

The changes in fluorescence (Excite:Emit, 360 nm:460 nm) due to substrate hydrolysis by thrombin were measured using a microplate reader (Molecular Devices). Area under the curve (AUC) and peak height for thrombin generation were calculated from the first derivative using Prism (version 5.0, GraphPad Software).

SPC-54 infusion and normal plasma sample collection

SPC-54 (in 50 μ L) was infused by retro-orbital injection in mice at a dose of 10 mg/kg in Dulbecco phosphate buffered saline (PBS). At different time points, blood was taken by retro-orbital puncture. Blood was then centrifuged at 2,000g for 5 min and plasma was aliquoted and kept at -80°C .

Quantification of plasma PC

To quantify free PC in plasma by immunocapture enzyme assay, 100 μ L/well the anti-PC mAb 29H5 (5 μ g/ml) was adsorbed onto a 96 wells plate (Nunc Maxisorp). After washing, diluted plasma aliquots were incubated 1 h. After washing, immuno-captured PC was activated using 1 U/ml of Protac (Pentapharm) diluted in 20 mM Tris, 50 mM NaCl, pH 7.4, 0.1 % BSA buffer for 1 h. After washing, APC amidolytic assays were used to quantify the amount of immuno-captured PC.

For quantification of PC antigen in plasma by Western blot, 10 μ L aliquots of plasma were incubated with 20 μ L of 50% slurry Protein G-agarose beads (Calbiochem), and then centrifuged, and subsequently supernatants were subjected to SDS-PAGE analysis as above using TGX AnyKd gels. To recover proteins attached to beads, beads were washed and incubated in Sample Buffer (Li-Cor) with DTT before centrifugation and SDS-PAGE analysis. After transfer of samples onto nitrocellulose membrane, PC antigen was detected with a biotinylated Rat anti-mouse PC mAb (clone 6B9) and IRdye800CW-conjugated streptavidin. Membranes were scanned using the Odyssey IR fluorescence scanner and quantification was done using the Odyssey Image Studio 2.0 (Li-Cor). For non-denatured samples analyzed using PAGE without SDS, 7.5 % precast gels (Bio-Rad) were used and equilibrated with native running buffer (standard Tris/glycine) for 45 min before the samples were electrophoresed.

TF-induced pulmonary embolism

The venous thromboembolism model was adapted from previous reports [22] with the following modifications. Mice were anesthetized by injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Human recombinant TF (Innovin) (50 μ L, diluted 1:8 in 0.9% NaCl) was injected in the inferior vena cava. The time to respiratory arrest (sustained for at least 2 min) was recorded, and experiments were terminated at 45 min. At that time, but heart still beating, 2% Evans blue dye (10 mg/kg in sterile 0.9 % NaCl) was retro-orbitally injected. Lungs were excised, photographed, and scanned with Odyssey IR fluorescence scanner. The Evans blue dye deposited in the lung, which is inversely related to the amount of thromboembolism since thrombi block blood flow into the lung, was quantified using Odyssey Image Studio 2.0 software.

Endotoxemia model

Mice received intraperitoneally LPS (25 mg/kg, O111:B4, Sigma-Aldrich) or vehicle (sterile 0.9% NaCl). After 7 h, mice were infused with a retro-orbital injection of 10 mg/kg SPC-54 in PBS. Boiled SPC-54 antibody was used as a negative control. Mortality was checked for 7 days.

Statistics

Values were expressed as the mean $\pm$ SEM. Survival data were plotted using the method of Kaplan-Meier. Log-rank (Mantel-Cox) test was used to compare statistically the curves (Prism 5.0d, GraphPad Software, La Jolla, CA). The other data were analyzed, using two-tailed Student *t* test or one-way ANOVA and Bonferroni multiple comparison test (Prism 5.0d). A P-value < 0.05 was considered statistically significant (***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$).

Results

SPC-54 antibody blocks the enzymatic activities of mouse APC

Assays were performed to determine if SPC-54, a rat IgG2a anti-PC monoclonal antibody that was made by immunizing rats with intact murine recombinant PC, blocks APC's enzymatic activities by blocking its active site. In amidolytic assays, SPC-54 was a potent inhibitor of APC amidolytic activity (Fig. 1A). Inhibition of APC amidolytic activity was similarly shown when we used another APC concentration (data not shown). Because the tripeptide amidolytic substrate is small and does not involve exosite interactions distant from the active site, we hypothesized that SPC-54 binds in the immediate vicinity of the active site, possibly in a way to impair entry of a small molecule into APC's active site. To test this idea, we studied reaction of APC with the irreversible active site reagent, biotinylated FPR-chloromethylketone which covalently biotinylates the active site in the heavy chain of APC along with the inactivation of the enzyme. As revealed by Western blotting (Figs. 1B and 1C), SPC-54 blocked reaction of this reagent with APC's active site. Thus, SPC-54 inhibited the enzymatic active site of wild-type murine APC. Moreover, further studies showed that SPC-54 was similarly a potent inhibitor of a well-characterized mutant of murine APC, namely 5A-APC (Fig. 1D) which has the five mutations of RR229/230AA plus KKK191–193AAA. This murine 5A-APC displays less than 10 % of normal anticoagulant activity but normal cytoprotective activities and shows normal neuroprotective activities and a normal ability to reduce mortality in murine sepsis [10,23–25]. The apparent K_d for APC-54 for murine PC was 8 nM in binding assays (data not shown). SPC-54 did not recognize or neutralize recombinant human APC (data not shown) and did not recognize PC antigen in rat plasma in Western blots, suggesting this rat mAb recognizes mouse PC but not human or rat PC. Thus, SPC-54 is a potent inhibitor of APC's enzymatic activity against small substrates and appears to do this by blocking APC's active site.

Thrombin generation assays were performed using mouse platelet-poor plasma to assess the ability of SPC-54 to block APC's anticoagulant activity in plasma. SPC-54 inhibited APC's anticoagulant activity when either the peak height or the AUC parameters were analyzed in thrombin generation assays (Figs. 2A and 2B).

SPC-54 binds and neutralizes PC *in vivo*

Because SPC-54 blocks *in vitro* multiple activities of APC including APC in mouse plasma, we next assessed if this mAb would bind murine PC *in vivo* in the circulation and thereby reduce its free concentration in blood. When SPC-54 was injected in C57BL/6J mice and plasma was collected at different times, Western blot analysis revealed that PC antigen levels increased with the PC plasma antigen level very remarkably at 24 and at 48 hours after injection (Fig. 3A). We hypothesized that SPC-54 binds PC and that SPC-54:PC antibody:antigen complexes are not cleared from the circulation as fast as PC itself due to the long half-life of antibodies versus the likely short 6–8 h half-life of murine PC (if it resembles that of human PC). We measured total functional PC using an immunocapture assay for PC in which the captured PC is then activated by Protac, followed by determination of APC amidolytic activity using a fluorogenic substrate. If SPC-54 is bound

to the immunocaptured PC, it would not manifest any amidolytic activity in this assay, whereas if PC is free in plasma, it would be converted and measured as APC. Overall, the data suggest that functionally available PC levels remained significantly decreased by infusion of a single dose of SPC-54 (10 mg/kg) for at least 7 days (Fig. 3B). The PC level was immediately decreased at only 1 hour after SPC-54 injection (data not shown). Therefore, SPC-54 decreases functionally viable PC, but not PC antigen, in blood over days after a single dose of SPC-54. However, in this assay, we cannot exclude that SPC-54 impaired binding to 29H5 antibodies.

To confirm that antibody:antigen complexes of SPC-54:PC formed *in vivo* and would explain the decrease in available functional PC in plasma despite an increased PC antigen level, Western blot analysis of plasma samples was made using non-denaturing PAGE in the absence of SDS. As shown in Fig. 3C, at day 1, 3 and 7 after SPC-54 infusion, the migration of the major observed PC antigen band was much slower than that of free PC which was seen for samples from days 0 (before SPC-54 infusion) and at day 14 after a single SPC-54 dose. The more slowly migrating bands corresponded to the position expected for PC in complex with one IgG molecule, presumably representing a complex of SPC-54:PC. Moreover, the increased PC band intensities for days 1, 3 and 7 correlated with the increased PC antigen levels seen in Fig. 3A, consistent with the idea that formation of antibody:antigen complexes prolonged the PC antigen half-life. To show directly the presence of antibody:antigen complexes of SPC-54:PC in mouse plasma after SPC-54 infusion, we performed IgG pull-down assays using protein G-agarose beads. Western blot analyses (Fig. 3D) showed that in absence of infused SPC-54 (hour 0), pull down of immunoglobulin by protein G-agarose didn't decrease PC antigenic level, whereas for plasma at 48 hours after SPC-54 infusion, the PC antigen in plasma was markedly depleted by protein-agarose. As correspondingly expected, elution of protein bound to protein G-beads revealed a strong, clear PC band at 48 hours but none from beads' eluate from the 0 hour sample. Before incubation with antibodies, membranes have been stained with Ponceau red and photographed as internal loading control (not shown). Thus, our data show that SPC-54 forms a complex with PC and that this antibody:antigen complex circulates in mouse blood as a high molecular weight complex while this mAb appears to block potential for generating functional APC, suggesting that PC/APC system knockdown by SPC-54 is sustained for days.

SPC-54 infusion increases mortality in a thrombosis model

APC functions *in vivo* as a major antithrombotic factor in man and in animals [1–5,14,16,26]. So we did studies to test the hypothesis that SPC-54 would increase mortality in a murine model of venous pulmonary thromboembolism. As shown in Fig. 4A, SPC-54 decreased survival after initiation of thromboembolism by tissue factor (TF) injection into the inferior vena cava, suggesting that SPC-54 blocks APC's *in vivo* antithrombotic activity. When Evans blue dye was injected immediately after a sustained arrest of breathing, or after a maximum of 45 min, visualization of blood vessels that are not obstructed by thrombi can be assessed both in visible and infrared fluorescence spectra (Fig. 4B), allowing quantification (Fig. 4D) of lung perfusion as a marker of embolism. Measurement of dye uptake by lungs was comparable both in wet (Fig. 4D) and dried lungs, and slight variation of lung's weight did not significantly influence results (data not shown). Since thrombi occlude lung vessels, dye uptake in lungs is inversely related to thrombotic occlusion. The data in Fig. 4 revealed a dramatic increase of emboli in mice injected with SPC-54. Thus, SPC-54, by blocking the endogenous PC system, increased the procoagulant state *in vivo*, leading to an increased mortality in this murine lung TF-induced thromboembolism model.

SPC-54 exacerbates mortality in murine endotoxemia

To test the hypothesis that SPC-54 infusion would block the endogenous PC system and its ability to modify protectively the response to endotoxemia, we performed a murine endotoxemia LD₅₀ model. When a single bolus of 10 mg/kg of SPC-54 was injected 7 h after LPS challenge, this antibody dramatically increased mortality from 50 % to 100 % within 36 h (Fig. 5) whereas controls, which involved either boiled antibodies or PBS vehicle alone, showed the expected mortality of about 50%. In absence of LPS, SPC-54 did not increase mortality, change animal behavior, or visibly alter significantly clinical signs.

Thus, it appears that SPC-54 is a potent *in vivo* inhibitor of the endogenous PC system of host defense, decreasing dramatically the survival of mice in different models where PC and APC are thought to be key protective factors.

Discussion

APC is a circulating serine protease with abilities to maintain homeostasis in the face of serious challenges. APC plays multiple roles in the tangled web of host defense and exerts both anticoagulant and cytoprotective activities [1,2,7–10]. Despite intense research to understand the mechanisms of action of APC, and despite conflicting data about the utility or futility of low dose infusions of wild-type APC for human adult severe sepsis [27], new basic knowledge is needed. Insights into the endogenous PC system as well as studies of pharmacologic APC mutants have the possibility of being translated to the clinic and could lead to development of drugs in different domains such as stroke [10], acute myocardial infarction [28,29], or whole body radiation toxicity [30]. Murine injury model studies are invaluable for future translational research into the PC systems. Here we characterize in detail the properties of a rat anti-mouse PC monoclonal antibody, SPC-54, which is shown to block *in vitro* and *in vivo* APC activities. SPC-54 decreased APC amidolytic activity of wild type murine APC as well as the murine 5A-APC mutant with multiple mutations in the protease domain. The active site irreversible titrant, biotin-FPR-chloromethylketone, was also blocked from reaction with APC, suggesting that SPC-54 epitope is likely in the immediate vicinity of the active site, or should bind in a manner to impair entry of very small substrates into the active site. SPC-54 also completely neutralized APC anticoagulant activity in mouse plasma. SPC-54 was bound to PC *in vivo* in plasma and the free PC plasma level was considerably decreased, and this phenomenon was sustained for up to 7 days after a single infusion of SPC-54. As expected, infusion of SPC-54 was procoagulant as shown by the increased mortality in a TF-induced thromboembolism model, and SPC-54 infusion decreased dramatically survival after LPS challenge. Hence, SPC-54 potently blocks APC's enzymatic activities *in vitro* and *in vivo*. SPC-54, by totally neutralizing the endogenous PC/APC system *in vivo*, could be an extremely valuable tool to study the endogenous actions of APC or to block endogenous murine PC and APC while giving human or rat APC to mice to permit unambiguous interpretations of studies that employ mAb-blocking strategies particularly because SPC-54 doesn't recognize human APC. However, further studies should evaluate whether PC or APC, once bound to SPC-54, could still bind to other receptors such as ApoER2 and abrogate signaling.

In conclusion, SPC-54 blocks the active site of APC from small molecules and kills APC enzymatic activities. These properties distinguish SPC-54 from the interesting mAbs, MPC1609 and MAPC1591, which likely recognize, respectively, APC's N-terminal Gla domain and some epitope on the APC protease domain located distant from the enzyme's active site [17]. These three anti-murine PC/APC antibodies thus have complementary profiles and each may have a unique utility for blocking selectively one or all of APC's activities in future studies of the PC system.

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Figure 1.

Anti-PC monoclonal antibody SPC-54 inhibits enzymatic activities of APC. Panel (A) shows the inhibition of murine APC amidolytic activity by SPC-54. APC (175 nM) was incubated with different concentrations of SPC-54 and amidolytic activity was determined. Data are mean values $\pm$ SEM of 3 independent experiments. Panels (B) and (C) show the inhibition by SPC-54 of reaction of biotinylated FPR-chloromethylketone with the active site of murine APC. Panel (B) shows data for one typical experiment in which APC (175 nM) was incubated with the indicated concentrations of SPC-54 and then with a molar excess of biotinylated FPR-chloromethylketone. When the amidolytic activity of the sample without antibody was > 98 % decreased, the samples were analyzed using reduced SDS-PAGE. The bands visualized represent biotinylation of APC's heavy chain. Panel (C) shows

data from 3 independent experiments for the relative intensity of the biotinylated PC heavy chain, indicating that SPC-54 blocked reaction of APC with the irreversible active site reagent. Panel **(D)** shows that SPC-54 inhibited similarly the amidolytic activity of murine wild-type (WT) and 5A-mutant APC (mAPC-WT and mAPC-5A, respectively).

Figure 2.

Anti-PC monoclonal antibody SPC-54 inhibits the anticoagulant activity of APC in thrombin generation assays in mouse plasma. **(A)** Peak height and **(B)** area under the curve (AUC) of thrombin generation in plasma was determined using different concentrations of exogenously added mouse recombinant APC (0.5 to 10 nM) and of SPC-54. SPC-54 increased the amount of thrombin generated in a dose dependent manner. Values represent mean values $\pm$ SEM of 3 independent experiments.

Figure 3.

Anti-PC monoclonal antibody SPC-54 forms a complex with PC *in vivo* and neutralizes PC. (A). Western blot using reduced SDS-PAGE of plasma samples collected at different times after SPC-54 injection. Total PC antigen was revealed using an anti-PC antibody. (B). Immunocapture of PC from plasma at different times after SPC-54 injection. PC was immunocaptured with anti-PC antibodies, then activated with Protac, and finally APC amidolytic activity was quantified. Data are represented with median, box and whiskers (5 to 95 percentile). Each day is represented by values for 4 to 16 mice and each value was quantified in duplicates. ANOVA $P < 0.001$, Bonferroni multiple comparison test, asterisk denotes a statistical significant difference with day 0 (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). (C). Western blots of plasma samples on nondenaturing PAGE analyzed for PC antigen using an anti-PC antibody. At days 0 and 14, the lower band corresponded to the position for free PC in plasma. At days 1, 3 and 7, free PC bands were almost not visible whereas strong PC bands appeared at the apparent Mr corresponding to the 1:1 complex of PC with SPC-54. This image is representative of three independent experiments. (D).

Immunoprecipitation of PC antibody complexes at different time points after SPC-54 injection. Immunoglobulins were adsorbed from plasma using Protein G-agarose beads. The residual adsorbed supernatants and eluates from the protein G-agarose beads were analyzed using reduced SDS-PAGE and Western blotting to visualize PC antigen. Protein G-agarose beads didn't adsorb PC out of plasma at baseline (0 h) whereas at 48 h after SPC-54 administration, the plasma PC band intensity was greatly decreased compared to control (absence of Protein G-agarose). Pictures show representative images of three experiments.

Figure 4.

Anti-PC monoclonal antibody SPC-54 increases mortality in a lung thromboembolism model in which tissue factor is injected in the inferior vena cava. **(A)**. Time to respiratory arrest (sustained for at least 2 min). Experiments were terminated at 45 min in the absence of respiratory arrest. $P = 0.0016$. **(B)**. At the time of respiratory arrest or at 45 min, Evans blue was retro-orbitally injected. Lungs were photographed and scanned using infrared fluorescence. **(C)**. Quantification of the amount of Evans blue in lungs (inversely related to the amount of thromboembolism) is represented as integrated intensity of infrared fluorescence intensity with mean and SEM ($n = 6$).

Figure 5.

Anti-PC monoclonal antibody SPC-54 increases mortality of mice in an endotoxemia model. Plots represent survival data for mice ($n = 12$ per group) that received endotoxin (LPS) or vehicle (at time 0 h). At 7 h, mice received SPC-54, boiled SPC-54, or PBS. SPC-54 alone was not lethal, but in conjugation with LPS, mortality increased severely. LPS+SPC-54 *versus* LPS+PBS Log-rank test, $P < 0.001$.