Blood Cells, Molecules and Diseases 50 (2013) 252–258

Contents lists available at SciVerse ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

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

Laurent Burnier *, José A. Fernández, John H. Griffin

The Scripps Research Institute, Department of Molecular and Experimental Medicine, 92037 La Jolla, CA, USA

ARTICLE INFO

Article history:
Submitted 18 December 2012
Available online 4 February 2013

(Communicated by U. Seligsohn, Ph.D.,
27 December 2012)

Keywords:
Activated protein C
Protein C
Coagulation
Inflammation
Thrombosis

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.

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 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]. Besides 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 potently neutralizes APC enzymatic activities *in vitro* and blocks the PC system in two murine injury models.

Materials and methods

Mice

This study was approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute and complied with

* Corresponding author at: The Scripps Research Institute, Department of Molecular and Experimental Medicine, MEM180, 10550 North Torrey Pines Rd, La Jolla, CA 92037, USA. Fax: +1 858 784 2243.
E-mail address: burnierl@scripps.edu (L. Burnier).

1079-9796/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcmd.2013.01.002

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 $CaCl_2$, 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 μM) 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 Na_2CO_3 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 (1 h) 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 $CaCl_2$ 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 $CaCl_2$ 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 μl) 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 2000*g* 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 μl/well the anti-PC mAb 29H5 (5 μg/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 μl aliquots of plasma were incubated with 20 μl 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