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Species specific anticoagulant and mitogenic activities of murine protein S

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ABSTRACT

Background
The protein C pathway downregulates thrombin generation and promotes cytoprotection during inflammation and stress. For preclinical murine injury model studies (e.g., sepsis and ischemic stroke), murine protein S may be required due to restrictive species specificity.

Design and Methods
We prepared and characterized recombinant murine protein S using novel coagulation assays, immunoassays, and cell proliferation assays.

Results
Purified murine protein S had good anticoagulant cofactor activity for murine activated protein C (APC), but not for human APC, in mouse or rat plasma. In human plasma, murine protein S was a poor cofactor for murine APC and had no anticoagulant effect with human APC, suggesting protein S species specificity for factor V in addition to APC. We estimated that mouse plasma contains 22±1 µg/mL protein S and developed quantifiable assays to measure APC cofactor activity of the protein S in murine plasma. APC-independent anticoagulant activity of murine protein S was demonstrable and quantifiable in mouse plasma, and this activity was enhanced by exogenous murine protein S. Murine protein S promoted the proliferation of mouse and human smooth muscle cells. The potency of murine protein S was higher for mouse cells than for human cells and similarly, human protein S was more potent for human cells than for mouse cells.

Conclusions
Recombinant murine protein S exhibits a spectrum of bioactivities with mouse plasma and smooth muscle cells similar to those of human protein S. Thus, studies of the protein C pathway in murine disease models will benefit from in vitro and in vivo studies using mouse protein S. This study extends to mouse previous observations regarding the remarkable species specificity of protein S.

Key words: anticoagulant, mitogenic activities, murine protein S.


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Smooth muscle cell cultures

Human vascular smooth muscle cells (Human VSMC) were isolated from human brain pial arteries, as described. These cultures expressed smooth muscle α-actin, myosin heavy chain, calponin and SM22α and were negative for CD11b (microglia), glial fibrillary acidic protein (astrocytes), prolyl 4-hydroxylase (collagen-synthesizing fibroblasts) and endothelial cell von Willebrand factor. Mouse vascular smooth muscle cells (Mouse VSMC) from aorta were obtained from the American Type Culture Collection, Manassas, VA, USA (ATCC, CRL-2797). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FBS) at 37°C, in a 5% CO2 humidified environment.

Construction and expression of murine protein S cDNA

Mouse liver Marathon-Ready cDNA was purchased from Clontech (Mountain View, CA, USA). Two primers MPSN (CGCGCTAGCGCCAAGCCAGG) and MPS4 (CAGCTGTGATAGGAATGTG) were designed to span the whole murine protein S cDNA from nt 1 to nt 2158 according to Genebank sequence data by Chu et al. PCR was performed using primers MPSN and MPS4 and mouse liver cDNA as template at 60°C annealing temperature for 30 cycles and 0.25 U of high fidelity PfuTurbo polymerase (Stratagene, La Jolla, CA, USA) was added to the reaction to limit the replication error. The obtained ~2158-bp PCR product was cloned into the Invitrogen pcDNA3.1 expression vector (~2158-bp PCR product was cloned into the Invitrogen pcDNA3.1 according to manufacturer’s instructions. The sequence of murine protein S cDNA was confirmed by sequencing. Stable transfection into a K293 cell line was performed using Effectene transfection reagent from Qiagen (Germantown, MD, USA) according to the manufacturer’s instructions. The transfected cells were selected in DMEM/F-12 supplemented with 10% fetal bovine serum containing 0.8 mg/mL antibiotic G418 and were selected in DMEM/F-12 supplemented with 10% fetal bovine serum containing 0.8 mg/mL antibiotic G418. The G418-resistant colonies were picked and grown in serum-free medium containing 10 µg/mL vitamin K1. Conditioned media were collected and tested for protein S antigen level by ELISA and Western blot. The positive colonies were chosen and used to produce conditioned media containing murine protein S.

Purification of recombinant wild type murine protein S

Protein S was collected in serum free DMEM/F12 media, then 5 mM benzamidine and 5 mM EDTA were added and the pooled media was loaded on a fast flow Q-Sepharose column (10 cm x 2 cm) in 50 mM Tris, 150 mM NaCl, 1 mM EDTA pH 7.4. Protein S was eluted with the same buffer but containing 30 mM CaCl2 in place of NaCl, 1 mM EDTA pH 7.4. This protocol selected for properly γ-carboxylated protein S since under-carboxylated vitamin K-dependent proteins are not eluted by CaCl2. A second anion exchange chromatography step was performed using a mono Q column (Amersham/Pharmacia). In this step, a NaCl gradient (0-400 mM) was used to purify murine protein S. After this step the fractions of interest were pooled and dialyzed against 50 mM Tris, 100 mM NaCl, pH 7.4. The protein S concentration was determined by monitoring absorbance at 280 nm, using an ε(1%, 1 cm) value of 9.5 and a molecular mass of 75,000 Da.

ELISA for protein S in mouse plasma

Costar half-well microplates (Corning, NY, USA) were coated 1 h at 37°C with 5 µg/mL of IgG of rabbit anti-human protein S (Dako) in 0.1 M sodium carbonate, pH 9.0 (20 µL/well) and then blocked 1 h with 170 µL/well 1-Block in TBS (Tropix, Bedford, MA, USA). Plasma (0.15 to 1.25 µL) or purified mouse protein S (0.25 to 2 µg/mL) was diluted in 20 µL HBS, 0.5% BSA, 0.005% Tween-20 and was incubated in the wells overnight at room temperature. Bound protein S was detected with 10 µg/mL goat anti-protein S IgG, followed by 1 µg/mL biotinylated mouse anti-goat IgG (Pierce, Rockford, IL, USA), 2 µg/mL streptavidin-horseradish peroxidase (Pierce) and o-phenylenediamine substrate (Sigma). Washes between steps were with TBS, 0.005% Tween-20, 2 mM CaCl2. The reaction was stopped upon appropriate color development (~10 min) with an equal volume of 1 M HCl and the product was measured at 490 nm.

Anticoagulant activity assays using purified protein S

Activated partial thromboplastin time (APTT) clotting assays were performed by incubating a mixture of 25 µL murine APC (180, 90, 45, 22.5, 11.2, 0 nM), 25 µL murine protein S (260 nM) or buffer, 10 µL mouse plasma, 25 µL of human fibrinogen (2 mg/mL), and 25 µL of Platelin LS (bioMerieux, Durham NC, USA) for 180 sec at 37°C. Reagents were diluted as needed in 50 mM NaCl, pH 7.4 containing 0.1% BSA. The clotting times were then recorded after adding 25 µL of 30 mM CaCl2. For FXa one-stage clotting assays, citrated mouse plasma (10 µL) was incubated for 180 sec at 37°C with 25 µL of 80%/PC/20%/PS vesicles (68 µM), 25 µL of human fibrinogen (2 mg/mL), 25 µL of FXa (34 nM), 25 µL of mouse or human APC (27 nM) and 25 µL of mouse or human protein S at varying concentrations. Clotting was initiated with 25 µL of 30 mM CaCl2.

APC cofactor activity of protein S in murine plasma

Plasmas, as well as purified prothrombin, mouse APC, FVa, and fibrinogen at the concentrations to be used were frozen in multiple aliquots and thawed just before use. Plasma aliquots were thawed and treated for 20 min with PAPMSF just before use. Mouse test plasma (12 µL) was mixed with 2 µL of 3 µM prothrombin, 10 µL of 9 mg/mL fibrinogen, and 45 µL of 105 µM phospholipid vesicles (20%/PS/80%/PC) in Heps-buffered saline (HBS), 0.5% BSA in cuvettes of a coagulometer. Murine APC (25 µL of 2 µg/mL in HBS-BSA) or HBS-BSA was added, followed by 33 µL of 0.3 µg/mL FVa in HBS-BSA. After 2 min incubation at 37°C with mixing, 25 µL of 50 mM CaCl2 was added to initiate clotting, and clotting time was measured. For standard curves in this assay, rat or mouse PSdP was mixed in various proportions with pooled mouse plasma so that protein S in mouse plasma increased from 0-100% of normal, resulting in a linear increase in clotting time prolongation upon addition of APC.
Introduction

In translational research, investigators commonly use murine genetic manipulations and injury models to generate preclinical information about pathogenic mechanisms and to assess potential drugs under development. Recombinant activated protein C (APC) is approved for therapy of severe sepsis in humans and is promising for other indications, including ischemic stroke. Mice are used extensively as experimental animals for investigations of the antithrombotic and cytoprotective activities of APC. Most of the proteins involved in the protein C anticoagulant and cytoprotective pathways have been successfully targeted for genetic deletion in mice with the exception of protein S, an anticoagulant cofactor of APC. Homozygous protein C-deficient mice undergo fetal development, but they present a phenotype with brain abnormalities, secondary edema, and bleeding as major contributors to premature death, often occurring in utero. Human APC has a half-life of 20-30 min in human blood in vivo that is determined by its irreversible inactivation by protease inhibitors such as protein C inhibitor and α1PI.

These protease inhibitors irreversibly neutralize APC enzymatic activity by forming a covalent acyl enzyme complex with APC.

APC displays significant species specificity and murine APC is superior to human APC for translational research studies in mice. The anticoagulant species specificity of APC may be due primarily to protein S-APC interactions. Protein S in human or rhesus monkey plasma serves well as a cofactor to human APC, and protein S in bovine, rabbit, or porcine plasmas serves optimally as a cofactor to bovine APC in anticoagulant activity assays. Purified rat protein S is a notably inefficient cofactor for human APC, in contrast to purified rabbit protein S.

Human protein S is present in plasma at 25 µg/mL, or 350 nM, and functions as a non-enzymatic cofactor for APC in the proteolytic inactivation of FVa and FVIIa. The molecular mechanisms involved in the cofactor function of protein S are incompletely understood. Protein S increases by 10-fold the affinity of APC for negatively charged phospholipids and also alters the orientation of the active site of membrane-bound APC. About 60% of circulating human protein S is in a non-covalent complex with C4b-binding protein (C4bp), a complement regulatory factor. However, complex formation between protein S and C4bp protein does not occur in mouse plasma.

Human protein S also has direct, APC-independent anticoagulant activity, by virtue of direct binding and inhibition of FXa, FVa, and FVIIa, and it may enhance the ability of tissue factor pathway inhibitor to inhibit the FVIIa/tissue factor complex. In a baboon thrombosis model, human protein S was antithrombotic independently of APC, but no information about protein S direct anticoagulant activity in other species is available.

In this study, we produced recombinant murine protein S and compared the cofactor activity of murine protein S versus human protein S in plasma clotting assays using mouse, human, and bovine APC. In cell assays, we determined the potency of murine protein S for stimulating cell proliferation. We determined the half life of murine APC in plasma and showed a strong interaction of murine APC with murine α1PI. We also developed an assay for APC cofactor activity of murine plasma protein S and a novel assay to investigate whether the protein S in murine plasma exerts direct anticoagulant activity. These new data and methods will help define significant aspects of the murine protein C pathway components and show that recombinant murine protein S is a valuable tool for future murine injury model studies.

Design and Methods

Reagents

Mouse recombinant protein C and human protein C were prepared and activated as described. Human FV was purified and activated, and goat anti-protein S was prepared and purified, as described. FXa, fibrinogen, human protein S and prothrombin were from Enzyme Research Laboratories, South Bend, IN, USA. Bovine APC was purchased from Haematologic Technologies (Essex Junction, VT, USA). p-APMSF was from Sigma, St. Louis, MO, USA. Synthetic phospholipid vesicles consisting of 80% 1,2-dioleoyl-sn-glycerol-3-phosphatidylcholine, 20% 1,2-dioleoyl-sn-glycero-3-phosphatidylserine (80%PC/20%PS) were prepared as described. Rat monoclonal antibodies, anti-mouse protein C TVM1 and 6B9, were prepared in house using purified recombinant mouse protein C as antigen. Cell culture media and supplements were obtained from Invitrogen (Carlsbad, CA, USA). Rabbit anti-human Ki-67 was from DBS (Pleasanton, CA, USA) and rat monoclonal anti-mouse Ki-67 from DAKO (Carpinteria, CA, USA). Secondary antibodies for mitogenic studies were Cy3-labeled anti-rabbit IgG and anti-rat IgG. For nuclear staining TO-PRO-3 was purchased from Invitrogen. [methyl-3H]thymidine was from Amersham/Pharmacia (Arlington Heights, IL, USA). Trichloracetic acid (TCA) was obtained from Sigma.

Plasmas

Mouse, rat and bovine plasma were obtained from Bioreclamation, Hicksville, NY, USA. Murine blood was also acquired from male CD-1/ICR mice or C57/BL6J mice by cardiac puncture. The blood was mixed with 3.8% sodium citrate and immediately centrifuged at 2,000 g for 20 min. The plasma was pooled and aliquots of 50-200 µL were frozen at -80°C until used. Normal pooled human plasma was obtained from George King Inc., Overland Park, KS. Rat or murine protein S-depleted plasma (Psdp) was prepared from 1.5 mL plasma by treatment with 20 µM pAPMSF, followed by adsorption on a 2 mL column of 4CNBr-Sepharose (GE Healthcare, Piscataway, NJ, USA) coupled with 4 mg of Dako rabbit anti-human protein S according to manufacturer’s instructions. The column was pre-equilibrated and the plasma was eluted in 0.2 mL fractions with Tris-buffered saline, pH 7.4 (TBS) containing 1 mM trisodium citrate. Fractions that were nearly undiluted were pooled and frozen in 50 µL aliquots. Dot immunobinding showed that >90% of protein S was removed.
Direct anticoagulant activity of murine plasma protein S

Mouse plasmas (15 µL) were pretreated for 15 min with 20 µM pAPMSF just before use and mixed with 5 µL of 3 µmolar prothrombin and 6 µL of 0.93 mg/mL neutralizing monoclonal antibody (TVM1) against murine and rat APC in HBS-BSA for 5 min. Phospholipids and CaCl2 in 15 µL of HBS-BSA was added to final concentrations of 15 µM and 9 mM, followed by 60 µL of fluorogenic substrate Z-GGR-aminomethyl coumarin (Bachem, Torrance, CA, USA) in HBS-BSA. To measure thrombin generation, fluorescence was read every 20 s over a 45 min period at an excitation of 460 nm and an emission of 460 nm. The change in fluorescence/min was calculated to monitor thrombin generation. This assay is a modification of endogenous thrombin potential (ETP) assays. For standard curves, pooled normal mouse plasma and rat or mouse PSdP were mixed in various proportions as described above. For validation of the assay, the effects of exogenous mouse protein S and rabbit anti-protein S were evaluated.

Cell proliferation assays

Immunofluorescence microscopy

To study the effect of murine protein S on proliferation of human- and mouse-VSMC, 6x104 cells were seeded into each well of 4-well chambered slides (Lab-Tek, Nunc, Rochester, NY, USA). After 24 h, cells were synchronized in G0 phase after transfer into DMEM containing 0.2% fetal FBS for 18 h. Cells were then treated with various doses of protein S (2.5 to 250 nM) and cultured for 72 h. Next, cells were washed with phosphate buffered saline (PBS) and fixed with 4 % paraformaldehyde. Immunostaining for mitotic marker Ki-67 was performed by using anti-Ki-67 antibodies (1:100) followed by detection with Cy3-labeled secondary antibodies, as explained under Reagents. Cells were then double stained with TO-PRO-3, a fluorescent nuclear marker. Fluorescence images were taken using a Zeiss 510 confocal microscope.

Cell counting

Human and mouse VSMC were cultured in 48-wells culture plates and treated with protein S for 72 hours as mentioned above. For quantitative assessment of cell proliferation, cells were trypsinized and the total number of cells in each well was determined by trypan blue-excluding cells in a hemocytometer.

[3H]Thymidine incorporation assay

To determine cell proliferation by de novo DNA synthesis, cells were grown in 48-well culture plates in DMEM with 0.2% FBS, as above. Cells were next treated with protein S for 48 h and labeled with 1 µCi of [methyl-3H]thymidine for 24 h. Cells were then washed with PBS and incubated with cold 20% TCA for 30 min at 4°C. TCA-soluble material was removed and monolayers were washed with PBS and extracted with 0.5 N NaOH/0.5 % SDS. TCA-precipitable counts were determined by scintillation counting using a Packard TRI-CARB 2100TR liquid scintillation analyzer.

Data analysis

Graph Pad Prizm 5 software (San Diego, CA, USA) was used for statistical comparisons by two-tailed t tests. Differences with p<0.05 were considered to be statistically significant. For the proliferation assays data were analyzed by one-way ANOVA followed by Tukey’s post hoc test. p<0.05 was considered as statistically significant.

Results

Recombinant murine protein S

Since the species specificity of protein S had been proposed to explain the major cause of reduction of human APC activity in animal plasma, we produced recombinant murine protein S. The nucleotide sequence of our murine cDNA for protein S (data not shown) was identical to the sequence of Chu et al.21 Comparing our murine cDNA protein S sequence to that of Lu et al.17 we found Phe instead of Leu at residue 493. Murine protein S mature protein sequence has one amino acid less (634 residues) than human protein S (655 residues). There are two potential glycosylation sites at residues 499 and 509 in murine protein S while in human protein S there is a third potential glycosylation site at residue 530. Recombinant murine protein S was expressed in stably transfected HEK293 cells and purified from conditioned serum-free medium via ion-exchange chromatography by a modification of the described protocol.22-25 SDS-PAGE analysis demonstrated that murine protein S was ~95% pure. In spite of having 80% sequence identity with human protein S, most antibodies available against human protein S did not recognize murine protein S. However two polyclonal antibodies recognized murine protein S sufficiently for their use: goat anti-human protein S prepared in house, and Dako rabbit anti-human protein S. We were unsuccessful in producing high-affinity anti-mouse protein S antibodies despite attempts made in nine rats and two rabbits. The low-affinity antibodies raised did not perform as well as the anti-human protein S antibodies and did not neutralize mouse protein S activity.

Activities of various species of APC

Protein C activities exhibit notable species specificity. We compared the anticoagulant activity of human, bovine and murine APC in several plasma species (Figure 1). Using a modified APTT assay and bovine plasma (Figure 1A), we found that the anticoagulant response of bovine APC was 4.2-fold higher than that of murine APC and 1.8-fold higher than that of human APC, based on the initial slopes of dose response curves. In human plasma (Figure 1B), the anticoagulant activity of human APC was 6-fold higher than that of murine APC and 17-fold higher than that of bovine APC. In rat plasma (Figure 1C), the anticoagulant activity of murine APC was 5-fold higher than that of bovine APC and 10-fold higher than that of human APC. Human APC anticoagulant activity was also remarkably low in rat plasma in earlier reports.16 In mouse plasma (Figure 1D), the anticoagulant activity of murine APC was 3.5-fold higher than that of human APC and 1.8-fold higher than that of bovine APC.
Comparison of APC cofactor activities of purified murine and human protein S

The activity of human or murine APC was assayed in the presence or absence of human and murine protein S in modified APTT assays using mouse plasma (Figure 2A). Murine protein S at 48 nM increased by 2-fold the anticoagulant activity of murine APC. However, the cofactor activity of human protein S for human APC in mouse plasma was very weak. For APTT assays using human plasma, 48 nM human protein S enhanced the activity of human APC by 1.7-fold, but murine protein S had almost no effect on murine APC in human plasma (Figure 2B). For murine APC, the cofactor activity of murine protein S in FXa-1-stage clotting assays was at least 4 times greater than that of human protein S in mouse plasma whereas human protein S was a very weak cofactor for murine APC (Figure 2C). When assayed in human plasma under similar conditions, the cofactor activity of human protein S for human APC was 6-times more potent than that of murine protein S (Figure 2D). Notably, murine protein S had essentially no anticoagulant cofactor activity for human APC at the concentrations tested.

Exogenous murine protein S exerted good cofactor activity for murine APC in mouse plasma, but not in human plasma, and exogenous human protein S exerted good cofactor activity for human APC in human plasma, but not mouse plasma (Figure 2A,B). It is suggested that other sources of species specificity must exist in addition to APC-protein S interactions.

APC cofactor activity of murine plasma protein S

Addition of murine APC to mixtures of mouse plasma and rat PSdP resulted in a linear prolongation of clot time as the proportion of mouse plasma increased in mixtures of mouse plasma and rat PSdP (Figure 3). APC had little effect in the PSdP, thus the dose response was due to the increasing amounts of protein S in increasing doses of murine plasma. The parent rat and mouse plasmas used to prepare PSdP had similar clotting times in the absence of APC.

The base clot time of the frozen and thawed aliquots of rat PSdP was stable, but the base clot time of mouse PSdP increased markedly over time and could no longer be used. It is recognized that differences other than protein S in mouse and rat PSdP could have contributed to the dose-response curves observed, but when mouse PSdP was initially prepared, it behaved similarly to rat PSdP. Mouse and rat protein S share 93% sequence identity and 98% sequence similarity. In the absence of APC, clotting time in PSdP was shorter than clotting time in normal mouse plasma (Figure 3). This difference was unaffected by inclusion of neutralizing antibody (TVM-1) against mouse and rat APC (not shown). This suggested that mouse plasma protein S had direct anticoagulant activity, as was explored in studies described below.

Direct anticoagulant activity of murine plasma protein S

In a modified ETP assay, plasmas were pretreated with neutralizing antibody against murine or rat protein C (TVM-1) to prevent any APC cofactor activity of protein S. As part of the validation of the assay, recalcified murine plasma with phospholipids was tested alone, or supplemented with partially purified recombinant mouse protein S (Figure 4A). Exogenous mouse protein S had an anticoagulant effect, causing a 2.3-fold prolongation of the lag time, 47% increase in time to peak, and 32% decrease in peak thrombin generation. Addition of Dako rabbit anti-protein S to murine plasma caused a decrease in lag time and time to peak, and an increase in area under the curve for thrombin generation (Figure 4B). When normal murine plasma was mixed in varying proportions with rat PSdP, area under the curve for thrombin generation and peak thrombin generation decreased in a linear manner with increasing doses of murine plasma (Figures 4C,D). Other proteins in normal mouse plasma besides protein S could vary in the rat
PSdP/mouse plasma mixtures and affect thrombin generation. However, freshly prepared mouse PSdP gave similar results as rat PSdP in the mixtures (data not shown), and response to mouse protein S was anticoagulant (Figure 4A). Thus, these data are consistent with the existence of direct anticoagulant activity of protein S in murine plasma.

**Physical characterization of mouse protein S and quantification of protein S in mouse plasma**

Murine and human protein S were subjected to 4-12% NuPAGE (Invitrogen) under reducing conditions and stained with Simple Blue (Invitrogen). The proteins appeared ~95% pure on SDS-PAGE (Figure 5A). Murine protein S had slightly faster migration under reducing and non-reducing conditions than human protein S, consistent with a known difference in the number of glycosylation sites. Under native conditions, murine protein S migrated slightly slower than human protein S and a small proportion of multimers was observed (Figure 5B). When human C4bp was preincubated in a 1.4 molar excess with mouse protein S, protein S-C4bp complexes were formed, as seen near the top of the gel (Figure 5B).

The ELISA for protein S was dose-responsive in the range of 0.25-2.0 µg/mL purified murine protein S (Figure 5C insert). Parallel slopes were obtained for purified murine protein S and mouse plasma when the data were plotted on a log-log scale in the range of 0.25-1.0 µg/mL protein S (Figure 5 C). The curves were superimposable at dilutions of mouse plasma that indicated a plasma concentration of 22±1 µg/mL protein S. These ELISA assays will be useful for quantifying differences in protein S antigen among experimental mouse plasmas in the dilution range of 0.25-1 µg/mL protein S. These ELISA assays will be useful for quantifying differences in protein S antigen among experimental mouse plasmas in the dilution range of 0.25-1 µg/mL protein S. We used quantitative immunoblotting as another means to quantify protein S in mouse plasma (Figure 5D). By this method, we determined a value of 15±2 µg/mL protein S in pooled plasma of C57/BL6J mice, though other plasma components probably caused a slight shift in mobility and a decrease in sharpness of the protein S band. Thus, estimates for C57/BL6J mouse plasma range from 15-22 µg/mL protein S, with the ELISA calculation of 22 µg/mL as probably the more accurate value.

**Protein S promotes proliferation of smooth muscle cells**

Protein S stimulates smooth muscle cell proliferation. Addition of exogenous murine or human protein S led to cell proliferation and [3H]-thymidine incorporation in mouse aortic smooth muscle cells and in human pial arterial smooth muscle cells (see Figure 6 for quantification and Online Supplementary Figure S2 for immunostaining).

Murine protein S was more potent than human protein S on the mouse cells (Figure 6A,C). Conversely, when human VSMC were studied, human protein S was more potent than mouse protein S (Figure 6B,D). Thymidine incorporation showed the same pattern with protein S as cell proliferation.
Half-life of APC in plasma

We previously characterized recombinant murine APC. To determine if mouse plasma protease inhibitors play important roles for regulation of the protein C pathway, the in vitro half-life of APCs in pooled plasmas from different species were measured (Online Supplementary Figure S1). APC in human plasma is inhibited by several members of the family of SERine Proteinase INhibitors (SERPINs) and to a lesser extent, by α2-macroglobulin. Here we show that in human plasma, the rate of inactivation of human, bovine and murine APC was similar, with a plasma half-life of ~20 minutes. In mouse plasma, the half-life of mouse and human APC (~15 min) was significantly shorter than in human plasma.

The high concentration of α1PI in mouse plasma (2-fold compared with human plasma) could contribute to inhibition of APC activity in mice. Indeed, we identified complexes of murine APC with α1PI in mouse plasma. However, bovine APC had a longer half-life, possibly because bovine APC has been shown to be resistant to inhibition by α1PI in vitro. It should be noted that in all cases, the half-life of APC in plasma is considerably longer than the half-lives of most coagulation proteases, such as thrombin and FXa.

Discussion

Murine models are ideal for in vivo thrombosis studies due to the animal size, frequency of breeding, and the potential for generating genetically altered animals. However, functional implications of species differences have been an obstacle to either design or confident interpretation of in vivo protein C pathway studies in mice. That human versus mouse species differences constitute a particular problem was shown earlier by our group when murine APC was used in human plasma clotting assays. The impaired cofactor activity of human protein S for murine APC was proposed as the reason for this species incompatibility.

Studies using human APC in vivo in antithrombotic rat models are controversial, as Smirnov reported antithrombotic effects of low doses of human APC in the rat, whereas others failed to demonstrate antithrombotic effects of human APC in rat arterial thrombosis models. Human and rabbit APC can function with protein S from certain species and not others. Clearly there is a need to define better the reactions of components of the murine protein C system.

Since protein S modulates the anticoagulant activity of APC, we expressed and purified recombinant murine protein S for functional studies using in vitro clotting assays.

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Figure 4. Coagulation assays for the direct anticoagulant activity of murine plasma protein S. In all cases, plasma was treated with neutralizing antibody (TVM-1) against murine APC to exclude any contribution of protein S cofactor activity for APC. (A) Thrombin generation profile for mouse plasma. Following addition of calcium and phospholipids to mouse plasma, thrombin generation was monitored over time in a modified ETP assay as described in Design and Methods. Normal mouse plasma, solid line; mouse plasma with addition of partially purified recombinant murine protein S (0.1 µg), dashed line. (B) Thrombin generation in normal mouse plasma with and without addition of 2 mg/mL neutralizing rabbit anti-human protein S. (C) Thrombin generation in mixtures of normal mouse plasma and rat PSdP. See figure 3 legend for mixtures. (D) Variation in ETP parameters with increasing dose of mouse normal plasma. In mixtures of pooled normal mouse plasma with rat PSdP area under the curve and peak thrombin generation in the ETP assay decreased in a linear manner with increasing dose of murine normal plasma. 12 µL plasma was used in (B); 15 µL was used in other panels.
The amino acid sequence of the mature form of murine protein S shows close to 90% identity with that of human protein S and differs by one residue in length. Despite the species specificity of the APC cofactor activity of murine protein S, murine protein S is structurally very similar to human protein S and is likely biologically equivalent with regard to its role in the protein C pathway. Recombinant murine protein S showed good anticoagulant cofactor activity for murine APC in APTT and FXa-one-stage assays using mouse plasma. These assays had residual activity for murine protein S in the presence of 30 ng mouse protein S in the absence and presence of 300 ng human C4bp (lanes 2, 3). ELISA for murine protein S. Standard dilutions of purified murine protein S were used to calculate the concentration of murine protein S in dilutions of pooled murine plasma, using a log-log plot. The plots were superimposed at dilutions of mouse plasma that indicated an initial concentration of 22 μg/mL mouse protein S. The insert shows a linear plot of extended concentrations of purified mouse protein S. Quantitative immunoblot for protein S in mouse plasma, using a 4-12% Bis-Tris gel. The first 3 lanes contain purified murine protein S (45-15 ng), mixed with 10 μg BSA/lane; the next 4 lanes contain pooled mouse plasma (3-0.5 μL). Blots in (B) and (D) were developed with 10 μg/mL goat anti-protein S, 1 μg/mL biotin-mouse anti-goat protein S (Pierce), 1 μg/mL streptavidin-horseradish peroxidase (Pierce) and chemiluminescent detection.

Figure 5. Protein and antigen analysis. (A) Denaturing SDS-PAGE electrophoresis of non-reduced (2 μg) purified protein S stained with Simple Blue. (H) Human plasma-derived protein S. (M) Murine recombinant protein S. The faster migration of mouse protein S is due to the lack of a carbohydrate side chain at residue 489. (B) Native PAGE immunoblot using 8% Tris-Glycine gel of 0.5 ng human protein S (lane 1) and 30 ng mouse protein S in the absence and presence of 300 ng human C4bp (lanes 2, 3). (C) ELISA for murine protein S. (D) Quantitative immunoblot for protein S in mouse plasma, using a 4-12% Bis-Tris gel. The first 3 lanes contain purified murine protein S (45-15 ng), mixed with 10 μg BSA/lane; the next 4 lanes contain pooled mouse plasma (3-0.5 μL). Blots in (B) and (D) were developed with 10 μg/mL goat anti-protein S, 1 μg/mL biotin-mouse anti-goat protein S (Pierce), 1 μg/mL streptavidin-horseradish peroxidase (Pierce) and chemiluminescent detection.
human plasma, human protein S, and the Dako antibodies. Note that only partially-purified mouse protein S, and not mouse protein S purified by the full method described here, had direct anticoagulant activity. This was most likely because of loss of a zinc ion from the MonoQ-purified mouse protein S, which affects direct anticoagulant activity but not APC cofactor activity of protein S. Both assays for APC cofactor activity and for direct anticoagulant activity of protein S are quantifiable and will be useful when in vivo protein S activity levels might be affected by experimental conditions.

The value of 22 µg/mL protein S in mouse plasma estimated by ELISA compares to 10 µg/mL free protein S and 15 µg/mL of protein S in C4bp complexes in human plasma. As noted, mouse plasma does not contain a form of C4bp capable of forming complexes with protein S, although we find that mouse protein S is capable of forming complexes with human C4bp. Human protein S-C4bp complexes have only weak APC cofactor activity but do have direct anticoagulant activity.

Free and C4bp-complexed forms of protein S bind to apoptotic cells, providing potential for local control of complement and coagulation systems. It was suggested that protein S binding to apoptotic cells can stimulate phagocytosis by serving as a bridging molecule between the apoptotic cell and the phagocyte. Recently, it was shown that protein S binds through the Gla domain to apoptotic cells and forms dimers that preferentially bind.
to and activate the TAM receptor tyrosine kinase Mer on macrophages, promoting phagocytosis. Like Gasα a protein S homologue, protein S emerges as a significant ligand for TAM receptors. It was reported that protein S is a mitogen for rat VSMC and that there is a receptor for protein S on human VSMC. Here, we show that murine protein S has species-specific mitogenic properties on VSMC.

In summary, murine recombinant protein S described in the present work is an important tool for in vivo studies of the protein C pathway biology. The results show species incompatibility between murine protein S and human APC in mouse plasma, which emphasizes the requirement for murine APC in murine in vivo studies of the role of the protein C pathway in experimental pharmacology. Protein S is also a regulator of VSMC proliferation, which may have implications for the treatment of a variety of proliferative vascular diseases. Moreover, extrapolation of data from murine protein S to human protein S requires careful evaluation of quantitative and qualitative inter-species differences.

Note added in proof


Authorship and Disclosures

JHG was the principal investigator and takes primary responsibility for the paper. JAF, MJH, and IS performed the laboratory work for this study. BVZ supervised the VSMC studies. JHG coordinated the research. JAF, MJH, and JHG wrote the paper.

The authors reported no potential conflicts of interest.

References


