Species-specific anticoagulant and mitogenic activities of murine protein S

José A. Fernández,[1] Mary J. Heeb,[1] Xiao Xu,[1] Itender Singh,[2] Berislav V. Zlokovic,[2] and John H. Griffin[1]

[1]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, and [2]Center for Neurodegenerative and Vascular Brain Disorders and Interdisciplinary Program in Dementia Research, University of Rochester Medical Center, Rochester, NY, USA

Citation: Fernández JA, Heeb MJ, Xu X, Singh I, Zlokovic BV, and Griffin JH. Species-specific anticoagulant and mitogenic activities of murine protein S. Haematologica 2009; 94:1721-1731. doi:10.3324/haematol.2009.009233

Supplementary methods

Inactivation of activated protein C in plasma

The inhibition of mouse, bovine and human APC by protease inhibitors in pooled plasma was studied by adding purified APC to plasma and monitoring the disappearance of APC amidolytic activity. Plasmas specimens were preincubated with 20 μM of the thrombin inhibitor I-2581 (DiaPharma, West Chester, OH, USA) for 5 min at 37 °C and then the time courses for inactivation of the APC in human and mouse plasma were determined by adding 30 μL of different APC (730 nM) to 270 μL of pooled citrated plasma at 37 °C. To assay residual APC amidolytic activity, 10 μL aliquots in duplicate were transferred to microtiter wells containing 250 μL of 1 mM Pefachrome PCa (PentaPharm, Norwalk, CT, USA) in 50 mM Tris, 100 mM NaCl, pH 7.4, 0.5% BSA, 5 mM $CaCl_2$ at 1, 5, 10, 15, 30, 45, 60, 90, and 120 min. The absorbance at 405 nm was read on an Optimax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The half-life of APC amidolytic activity in plasma was determined by nonlinear regression analysis of the decay curves generated using GraphPad Prism 5.0 software (San Diego, CA, USA) and the equation:

$$t_{1/2} = 0.69/k_1(app)$$

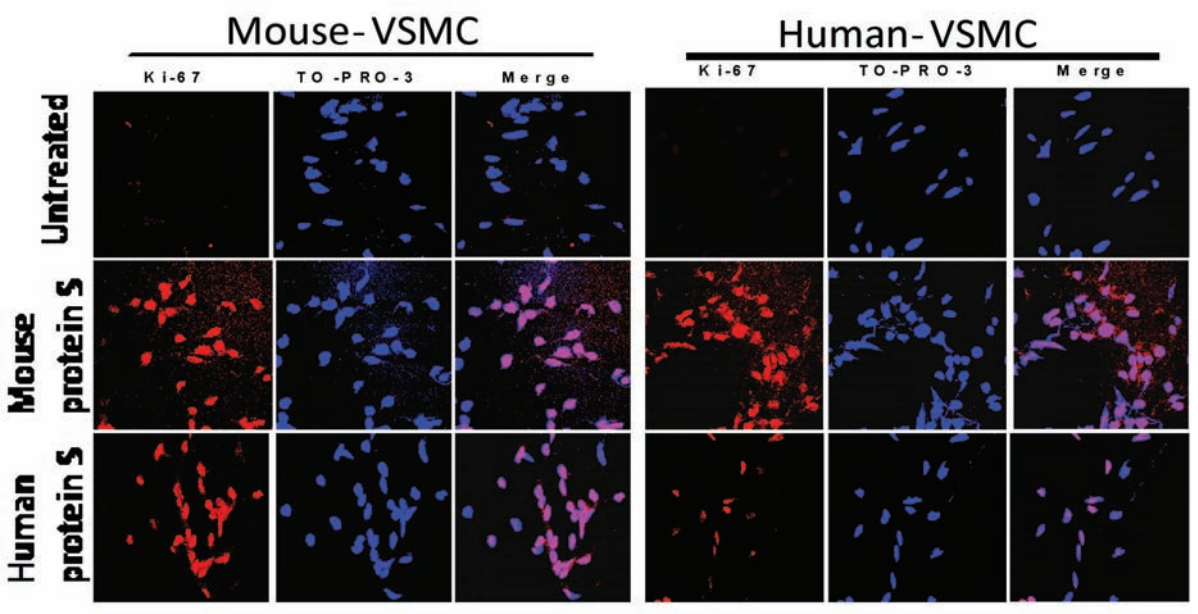
where k1(app) = the apparent first-order rate constant for APC inactivation

Gel electrophoresis and western Blots

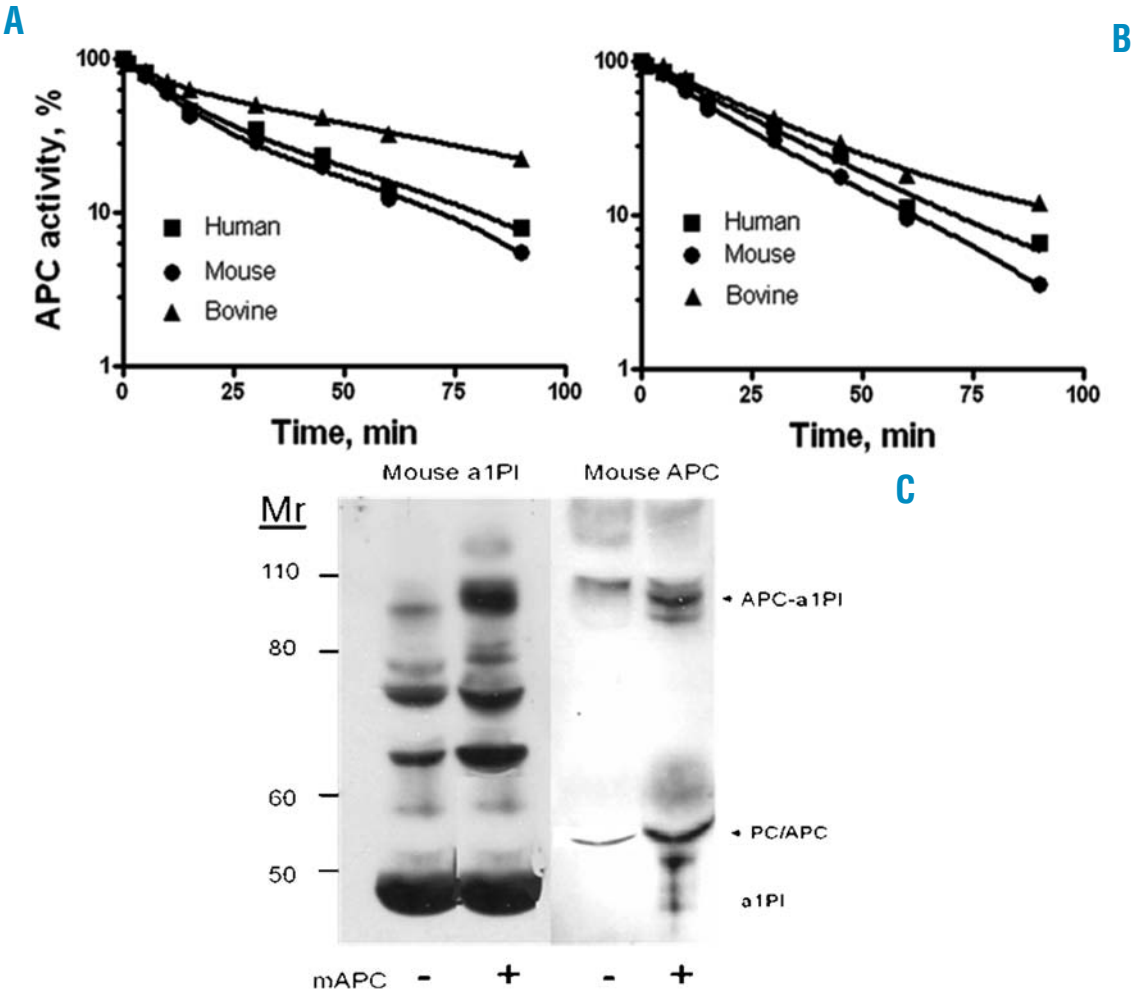
To study the complex formation between recombinant murine APC and mouse protease inhibitors, 4-12% NUPAGE MOPS gels (Invitrogen) were run under denaturing and non-reducing conditions. Mouse plasma was incubated with different concentrations of murine APC for 1 h at 37 °C in the presence of 5 mM $CaCl_2$ and 5 mM hirudin and then loaded on gels. Gels were transferred to PVDF membranes (0.45 μm) from Pierce on a Trans-Blot semi-dry transfer cell (Bio-Rad, Hercules, CA, USA).

Rat monoclonal anti-mouse protein C antibody (6B9) was used for detection of murine APC antigen. The antibody was peroxidase-labeled using the EZ-Link Plus activated peroxidase kit (Pierce).

Peroxidase-labeled chicken anti-mouse α1PI was purchased from Abcam Inc. (Cambridge, MA, USA). Fluorescence detection using X-ray films was performed using the ECL Plus Western Blotting detection system from GE Healthcare.



Online Supplementary Figure S1. Protein S dose-dependent increase in the proliferation of VSMC. Mouse-VSMC (left) and human-VSMC (right) were treated with or without 25 nM of mouse protein S or human protein S for 72 h. The cells were then stained with Ki-67 antibodies to determine the proliferation (red) and TO-PRO-3 to visualize the nucleus (blue). The merging of the two stains is shown in magenta.



Online Supplementary Figure S2. Inactivation of human, mouse and bovine APC in human and mouse plasma. Human, murine, and bovine APC were added to human (A) and mouse (B) plasma and the loss of APC amidolytic activity over time was measured using the APC substrate Pefachrome PCa. The values observed for APC addition were corrected by subtracting background amidolytic activity values observed for plasma specimens without addition of APC. (C) Western blot of murine APC-α1PI complex using SDS-PAGE. Murine APC–α1PI complexes formed by adding murine APC to murine plasma were analyzed by denatured inmmunoblot analysis using anti-murine protein C/APC antibodies and murine α1PI antibodies. The results for mouse plasma without added APC and treated under the same conditions are shown as a control.