Materials and Methods

Reagents
All reagents, including high molecular-weight, porcine UFH, sodium salt (specific activity 196 U/mg), unless specified otherwise, were from Sigma-Aldrich. Heparinoid ODSH (specific activity 150-160 U/mg) was a gift from ParinGenix Inc. Chromogenic substrate S2366 was from Chromogenix/diaPharma, recombinant hirudin from Calbiochem, and PRT was from American Pharmaceutical Partners. Mixed histones for in vitro studies were from Roche Diagnostic and those used for studies in vivo from Sigma-Aldrich. Histones H1 and H4 were obtained from New England Biolabs. Human soluble (s) TM was purified from TM-expressing HEK293 cells by anion-exchange chromatography and affinity chromatography on Ilα-sepharose. Non-glycosylated, low molecular weight TM was separated from high molecular weight TMCS by high performance anion exchange chromatography to yield TMCS2.3. The different forms of TM were validated by Western blot as described4. Rabbit TM was purchased from Hematologic Technologies. Human protein C, was isolated from plasma as described5, and further purified by immunoaffinity chromatography using the Ca2+-dependent monoclonal antibody HPC46, provided as a gift from Dr. C.T. Esmon, Oklahoma Medical Research Foundation.

In vitro aPC assay
Generation of aPC was assayed in 96-well plates as described4. Briefly, rabbit or human soluble (s) TMCS or sTMCS, was mixed with various amounts of histones, PF4 and/or heparinoid for 10 minutes in assay buffer (final concentration, 20 mM Tris, 100 mM NaCl, 1 mM CaCl2, 0.1% BSA, pH 7.5). Final concentrations of TMs were 0.5-40 nM. PC (final concentration, 500 nM) was added for an additional 10 minutes followed by addition of Ilα at a final concentration of 0.2 or 2 nM and then incubated for 15 minutes. All incubations were done at 37°C. The reaction was quenched with 1 mM EDTA and 100 nM hirudin. Concentrations of aPC formed in the quenched samples were inferred from initial rate measurements after the addition of 0.5 mM S2366. Initial rates of chromogenic substrate cleavage were determined by measuring absorbance at 405 nm using a Thermomax Microtiter V max plate reader (Molecular Devices).

In vivo aPC measurements
PF4 knockout (PF4KO) mice have been previously generated and characterized by us. WT mice were also on C57Bl6 background. All mice had been backcrossed ≥10 times onto a C57Bl6 background. Mice studied were 8-12 weeks of age. All animal experiments were approved by the Children’s Hospital of Philadelphia’s institutional animal care and use committee.

After mice were anesthetized with intraperitoneal injection of pentobarbital (100 mg/kg), in vivo aPC generation was assayed in plasma of mice injected via the jugular vein over 2 minutes with murine IIa at 8 U/kg (~1 pmol/20 g mouse) concurrently with histones (0-20 mg/kg) in the absence or presence of 5 mg/kg of PF4. After 10 minutes, blood was drawn from vena cava into sodium citrate/benzamidine (final concentration 5 mM/50 mM, respectively), centrifuged for 10 minutes at 5,000 rpm at 4°C and plasma frozen. In some experiments, animals were pre-injected with UFH or ODSH or buffer 10 minutes before injection of histones. Histones at 20, 50 or 75 mg/kg, was injected and blood was drawn after 30 minutes in surviving animals. Plasma aPC levels were measured by capture ELISA using an anti-mouse aPC antibody and recombinant mouse aPC as a standard (both kindly supplied by Dr. Esmon) and chromogenic substrate Spectrozyme PCa (American Diagnostica).

Activated partial thromboplastin time (aPTT) was measured in plasma of blood collected from tails of mice infused with heparinoids, followed 10 minutes later by various concentrations of histones, infused via a jugular vein. Time to clot formation was measured after mixing plasma with TriniCLOT Automated aPTT reagent (Tcoag).

Statistical analysis
Differences between groups were compared using a two-tailed Student’s t-test. Statistical analyses were performed using Microsoft Excel (Microsoft). Differences were considered significant when p values were < 0.05.

REFERENCES:


