Materials and Methods

Thromboembolic stroke. Experiments were approved by the Institutional Animal Care and Use Committee. Adult male C57Black/6 (Jackson Labs, Bar Harbor, ME), a2AP$^{+/+}$ mice or a2AP-deficient (C57Black/6, a2AP$^{-/-}$) mice (UC Davis, KOMP) weighing 29 -35 g were anesthetized with isoflurane. Autologous thromboemboli were made with pooled fresh frozen mouse plasma from a2AP$^{+/+}$ and a2AP$^{-/-}$ mice as described.[1] MCA thromboembolism was performed as described with monitoring by a laser Doppler flow meter (see below, ML-191, ADInstruments, Oxford Optonix, UK).[1, 2] Appropriate embolization was confirmed by a decline in hemispheric blood flow by 80%. One experimental group received purified human a2AP (4.2 mg/kg, Athens Research & Technology, Inc. Athens, GA) intravenously via the contralateral jugular vein immediately after thromboembolism. Other experimental groups received a purified monoclonal antibody inhibitor of a2AP (9.3 or 21.3 mg/kg, 4h9 whole IgG or 9.5 mg/kg 4h9 Fab, Innovative Research, Novi, MI) or TPA (10 mg/kg, 20% bolus, 80% as infusion over 1 h, Genentech, South San Francisco, CA) at the indicated times after onset of ischemia. After anesthesia, mice were supported by infusions of 250 µL of saline intraperitoneally and free access to food and water. Mice were euthanized 6 h or 7 days after thromboembolism or if there were signs of being in extremis or moribund in compliance with IACUC rules. Bederson neurologic scores were assessed in a2AP-I and TPA-treated mice 6 hours after thromboembolism as described.[3] In six hour studies, 1 mouse supplemented with a2AP and 1 TPA- treated mouse died; no control mice, a2AP$^{-/-}$ mice with a2AP$^{+/+}$ clots, a2AP$^{-/-}$ mice with a2AP$^{-/-}$ clots or a2AP-I treated mice died. After euthanasia, blood was collected by cardiac puncture and saline tissue perfusion was performed as described.[4]

Cerebral Blood Flow. Cerebral blood flow in the MCA territory was monitored by a blood flow meter (ML-191, ADInstruments, Oxford Optonix, UK) using a laser Doppler probe (MSF 100XP, ADInstruments) through a fiberoptic filament attached by use of a tissue adhesive to the intact skull 2 mm caudal to bregma and 6 mm lateral to midline of the affected hemisphere.[2] The blood flow was recorded using a Power Lab 2/26 data acquisition system (ADInstruments) and successful MCA occlusion was confirmed by ~80% drop in relative blood flow. Changes in cerebral blood flow after MCA occlusion were expressed as percentage (mean ± standard error) of the baseline value prior to thromboembolism.

Measurement of Brain Infarction, Hemorrhage and Swelling. Analyses were performed as previously described.[1] Following euthanasia and perfusion, brains were sliced coronally into 2 mm sections in a rostral-caudal orientation. Brain slices were rapidly digitally photographed on both sides through a microscope. Then brain slices were immediately incubated in triphenyl tetrazolium chloride (TTC, 2%) to assess cellular viability followed by digital photography as above. Digital microscopic images were analyzed by a blinded observer using Image Pro Plus 6.2 software to measure areas of brain hemorrhage, TTC staining and hemisphere swelling. To determine the percent hemisphere infarction, the TTC-stained areas of the ischemic and non-ischemic hemispheres were measured on both faces of each brain slice.[5] The percent infarction was calculated for each brain by the formula: infarct percentage = $100 \times (V_C - V_L / V_C)$, where V_C = TTC-stained area in the control hemisphere × slice thickness, V_L = TTC-stained area in the infarct hemisphere x slice thickness. The percent brain hemorrhage in the infarct hemisphere was determined by measuring the area of hemorrhage in digital microscopic images on both sides of each brain slice for the ischemic and contralateral, unaffected control hemisphere (in which there was no hemorrhage). The percent hemorrhage = 100 × (volume of hemorrhage in the infarcted hemisphere/ volume of the control hemisphere). The swelling in the ischemic hemisphere was determined by comparing the volume of the ischemic hemisphere and the contralateral hemisphere for

both faces of each brain slice. The percent swelling was determined for each brain by the formula: swelling percentage = 100 × (volume of the infarcted hemisphere – volume of the control hemisphere)/ volume of the control hemisphere).

Thrombus formation, dissolution and immunoblotting. *In vivo studies.* A fibrin-rich thrombus was formed with citrated pooled fresh frozen mouse plasma from $a2AP^{+/+}$ and $a2AP^{-/-}$ mice using calcium and thrombin, with and without trace amounts of ^{125}I-fibrinogen as described.[1] Pooled fresh frozen mouse plasma (5 ul) and ^{125}I-fibrinogen (~12,500 cpm) were mixed with 1ul of (premixed thrombin 0.25 U and $CaCl_2$ 100 mM) on ice. Immediately 2 ul (5,000 cpm) of plasma mix was drawn into 40 cm PE-10 tubing and clotted overnight at 4°C. The PE-10 tubing containing the clot was cut into a 40 cm length and attached to two syringes filled with sterile PBS with 1% BSA. The clot was washed by back and forth movement in the tubing by alternate syringe aspiration for 5 minutes. The clot was expelled and stained in 0.5% Evans blue in PBS for 10 minutes for visualization. After washing twice with 1% BSA in PBS, the clot was cut into 6 pieces to facilitate loading back into the tubing and then gently compressed and washed with sterile saline. The clot was counted in gamma counter (COBRA II, Packard) and pulled into a 30cm segment of modified PE-08 tubing filled with saline for insertion to MCA. At the conclusion of the study, the brain was gamma counted and the amount of thrombus dissolution was determined by the formula, thrombus dissolution % = 100 × (cpm of the original clot – residual brain thrombus cpm)/ cpm of the original clot). [1]

In vitro studies of clot dissolution. Clots were formed at 37 °C for 1 hour in 5 ml tubes (Sarstedt Inc. Newton, NC.) from pooled mouse plasma or from individual mouse plasmas by mixing 30 ul mouse plasma labeled with trace amounts of ^{125}I-fibrinogen, a2AP-I or buffer (20 ul) and thrombin and calcium (10 ul, 0.05 u thrombin, 8.3 uM calcium, final). Then 90 ul of Tris-buffered saline was added together with TPA (10ul, 1 nM final). Test tubes were placed at 37 °C and 10 ul samples of the supernatant were sampled hourly and subjected to gamma scintillation counting (COBRA II, Packard) to determine the rate of clot dissolution according to the thrombus dissolution formula above. A standard curve was formed that related the concentration of a2AP-I to the amount clot dissolution. Plasma clots made from mice receiving a2AP-I were compared to a standard curve which, by linear regression, related the effect on fibrinolysis of different concentrations of a2AP-I added to pooled mouse plasma (r^2= 0.98).

Immunoblotting studies. For immunoblotting, $a2AP^{+/+}$ and $a2AP^{-/-}$ thrombi were made as above, washed and subjected to SDS-PAGE under reducing conditions. After electrophoretic transfer to polyvinylidene fluoride membranes and blocking with 1% BSA, membranes were incubated with pooled monoclonal antibodies specific for mouse a2AP (monoclonal antibodies 173, 766 as well as 4h9, 27c9; 5 ug/ml; Molecular Innovations, Inc. MI.) or rabbit polyclonal antibody (1:100) directed to the NH_2 and C-terminal peptides of a2AP as described.[6] Bound antibodies were detected with a secondary goat anti-mouse antibody IRDye 800CW or anti-rabbit labeled with IRDye-680 (Li-COR Biosciences, Lincoln, NE) and imaged with an infrared imager (LI-COR ODYSSEY).

Measurement of a2AP levels. Levels of human a2AP were measured in the plasma of mice given supplemental a2AP by intravenous infusion. At the conclusion of experiments, six hours after a2AP administration, citrated blood samples were obtained anesthetized mice undergoing euthanasia. Plasma was obtained by differential centrifugation at 4 °C (1800 rpm for 20 minutes followed by repeat plasma centrifugation at 8000 rpm for 5 minutes). Wells of a microtiter plate were coated with a human chimeric monoclonal antibody specific for human a2AP (10 ug/ml) for 1 hour at 37'C. Then wells were washed and blocked with 1% goat serum for 1 hour. Wells were washed four times with 0.1% Tween and three times with PBS. Then mouse plasma containing various amounts of human a2AP was diluted 1:100 in PBS containing 1% goat serum was added to wells in duplicate in seven, two-fold dilutions or no mouse plasma, to create a standard curve. For comparison samples from mice administered a2AP

were added in various dilutions to mouse plasma (1:100) in PBS for 1 hour. After washing, a mouse antibody to human a2AP was added for an hour. Then wells were washed again and goat antimouse-horse radish peroxidase (1:5000 diluted in 1% BSA; Santa Cruz Biotechnology, Dallas, TX) was added for an hour. After washing four times with 0.1% Tween and three times with PBS, wells were developed developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Thermo Scientific, Rockford, IL) and monitoring in a microtiter plate reader at A370 nm. The level of human a2AP in mice given intravenous human a2AP was determined by an ELISA. The standard curve related human a2AP levels spiked into mouse plasma with the ELISA signal ($r^2 = 0.95$).

Brain tissue analyses. For immunohistochemistry and histochemical analyses, brains were fixed in 4% paraformaldehyde and paraffin-embedded. Sections were deparaffinized and hydrated in serial dilutions of ethanol and water. Antigen was retrieved with sodium citrate buffer (pH 6.0) using heat induction at 98°C (20 min) followed by room temperature (20 min). Sections were washed in PBS and blocked with 10% normal donkey serum in PBS at room temperature (45 min). MMP-9 expression (anti-MMP-9 antibody, AF909, R&D systems) was localized relative to blood vessels by double immunofluorescence staining for collagen IV (rabbit anti-mouse type IV collagen, Karlan Research Products Corporation). Sections were incubated with primary antibody diluted in 2% normal donkey serum in PBS overnight at 4°C. After washing with PBS, secondary antibodies, DyLight549–conjugated donkey anti-goat and DyLight488-conjugated donkey anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, PA) were applied for 45 minutes at room temperature. Total nuclei were counterstained with DAPI using Vectashield mounting media (Vector Laboratories, Burlingame, CA). Slides were examined by fluorescence, confocal or scanning microscopy (Aperio ScanScope, Vista, CA) and digital image analyses were performed using Image-Pro Plus 6.2 software (Media Cybernetics, Bethesda, MD). For quantitative analysis of MMP-9 immunostaining, MMP-9 staining density on red channel images of ten fields (40x) from each stroke hemisphere were analyzed. The MMP-9 staining density in each group represents the average of individual brains and was expressed in arbitrary units by Image-Pro Plus software. Microvascular thrombus formation was identified by the Martius-Scarlet-Blue stain (Atom Scientific) and quantitated by measurement of fibrin positive area (fresh red area) in 12-15 random 40x fields from each individual mice brain stroke hemisphere using Image-Pro Plus software.

In situ zymography assays were performed to evaluate MMP activity in freshly frozen brain slices isolated 6 hours after cerebral thromboembolism. Quenched fluorescein-labeled gelatin (Invitrogen) was used as a substrate to identify gelatinase activity by the development of fluorescence. For in situ zymography, 6 µm brain cryosections were first incubated with reaction buffer (0.05M Tris-HCl, 0.15 M NaCl, 5 mM CaCl2, and 0.2 mMNaN3, pH 7.6) for 10 min and then mixed with the reaction buffer containing 40 µg/ml DQ gelatin at 37˚C in a humidified chamber for 5 hours . After incubation, sections were rinsed 3 times for 5 min. intervals in PBS followed by fixation with 4% paraformaldehyde for 10 min at room temperature. Slides were then washed twice for 5 min. intervals in PBS and mounted with Vectashield mounting media with DAPI (Vector Laboratories). Negative controls were obtained by pre-fixation of the sections in 4 % paraformaldehyde solution. Whole brain images were obtained by Zeiss 710 Confocal scanning at 10x magnification.

Analysis of complete blood counts. The complete blood counts of $a2AP^{+/+}$ and $a2AP^{-/-}$ male mice, three mice per group, were analyzed by the UT Endocrinology Clinical Laboratory (Memphis, TN) on a Beckman-Coulter AcT diff Hematology Analyzer (Miami, FL). Blood was collected from anesthetized mice into EDTA containing tubes during euthanasia (BD Vacutainer, K2 EDTA 7.2mg, REF 367681, LOT 6341632).

Antibody binding. The binding of the a2AP-I to mouse a2AP or BSA was compared to a control monoclonal antibody (anti-digoxin 40-160) in an ELISA. Wells of a microtiter plate were coated with purified recombinant mouse a2AP (25 ul, 5 ug/ml for 1 hour) or nothing.[6] After washing, non-specific binding sites were blocked with 250 ul of 1% BSA for 30 min. After rinsing, purified a2AP-I (5 ug/ml, 25 ul), control monoclonal antibody (anti-digoxin 40-160, 5 ug/ml, 25 ul) or no MAb (25 ul) were added to the wells for 1 hour in duplicate. After washing , goat anti mouse- horse radish peroxidase (1/2000 in 1% BSA, 25 ul Santa Cruz Biotechnology Inc. Dallas, Texas.) was added for 30 min. at RT. Wells were washed and developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (Thermo Scientific. Rockford, IL) with detection by a microtiter plate reader at A370 nm.

Statistics. Normally distributed data were analyzed by an unpaired Student's t-test or a one way ANOVA using the Neuman-Keuls correction for multiple statistical inference. Non-Gaussian data were analyzed by a Mann-Whitney test, Spearman's rank correlation coefficient (r_s) or a one way Kruskal Wallis analysis using Dunn's correction. Survival data were analyzed by the Mantel Cox log rank test. Data are expressed as the mean ± standard error. A two-tailed $p \leq 0.05$ was considered statistically significant.

References

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