Online Data Supplement

Urokinase-Type Plasminogen Activator Inhibits Efferocytosis of Neutrophils

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Methods

Purification and culture of mouse bone marrow neutrophils. Purification and culture of mouse bone marrow neutrophils was performed as previously described (1-4). Briefly, the femur and tibia of a mouse were flushed with cold RPMI to obtain bone marrow cells that were resuspended in cold RPMI medium. The cells were then passed through a 0.45 μm filter, pelleted, and re-suspended in 900 μl of cold PBS after which 35 μl of custom primary antibody cocktails against the cell-surface markers F4/80, CD4, CD45R, CD5, and TER119 were added and incubated for 15 minutes with rotation at 4°C, followed by addition of 100 μl of anti-biotin tetrameric antibody complexes and incubation for 15 minutes at 4°C. Finally, 60 μl of colloidal magnetic dextran iron particles were added to the suspension and incubated for another 15 minutes at 4°C with rotation. The entire cell suspension was then loaded into a column, surrounded by a magnet. The T cells, B cells, RBC, monocytes, and macrophages were captured in the column, allowing the neutrophils to pass through by negative selection. The isolated neutrophil population was consistently more than 98% pure, as determined by both flow cytometry analysis (CD11b+GR-1+) and Wright-Giemsa staining following cytospin and was kept in RPMI medium with 1% FBS.

Purification and culture of peritoneal macrophages (PMΦ). Peritoneal macrophages (PMΦ) were obtained from peritoneal exudates collected 4 days after intraperitoneal injection of 1 ml of sterile 4% thioglycollate. The cells in the peritoneal exudates were washed 3 times and plated in 24 well plates with microscope cover slips (Fisherbrand,

12-545-82 12CIR-1D) at 0.3×10^6 cells per well. After incubation at 37°C for 1 h, the wells were washed three times to remove non-adherent cells. The remaining PMΦ were cultured in RPMI with 5% inactivated FBS and were used for phagocytosis assays within 4 days of isolation. More than 94% of PMΦ were Mac-1 positive, as determined by flow cytometry (data not shown).

Induction of neutrophil apoptosis. Neutrophils (10×10^6 cells/ml) were suspended in RPMI medium with 1% FBS. When comparing the effect of uPA on the phagocytosis of wild-type or Vn-/- neutrophils, Vn-/- neutrophils were re-suspended in Vn-/- mouse serum and control WT neutrophils in WT mouse serum. To induce apoptosis, neutrophils were heated at 43°C for 1h, followed by culture at 37°C in 5% CO_2 for 2.5 h. This method routinely yielded a population of neutrophils that included approximately 40% cells that were annexin V positive (early apoptotic) and less than 10% of cells that were propidium iodide (PI) and annexin V positive (late apoptotic).

***In vitro* efferocytosis assay.** *In vitro* efferocytosis assays were performed as previously described (5, 6). Briefly, 3×10^6 apoptotic neutrophils suspended in RPMI medium with 1% FBS were added to 0.3×10^6 macrophages cultured on coverslips. After culture at 37°C for 2 h, the cover slips were washed 3 times with ice-cold PBS and stained with HEMA 3. Phagocytosis was evaluated by a blinded observer by counting for 4 times from duplicate experiments. The phagocytosis index was calculated as the percentage of macrophages containing at least one engulfed neutrophil.

***In vitro* phagocytosis of beads**. To determine the phagocytosis of carboxylate modified beads by macrophages, beads (2 μm) were suspended in 1% FBS RPMI medium at a concentration of 30×10^6/ml. 15×10^6 beads were added to 0.3×10^6 macrophages cultured on coverslips and co-incubated with 1 μg/ml scuPA or HSA at 37°C for 2 h. The coverslips were washed 3 times with cold PBS, stained with HEMA 3, and the phagocytic index determined.

***In Vivo* efferocytosis Assay.** To determine the effect of scuPA on the phagocytosis of apoptotic neutrophils by alveolar macrophages *in vivo*, mice were anesthetized with isofluorane. 10×10^6 apoptotic neutrophils and 2 μg of scuPA or HSA were re-suspended in 50 μl sterile PBS and injected intratracheally. Two hours later, the mice were sacrificed and bronchoalveolar lavage (BAL) performed using 1 ml sterile PBS containing 5 mM EDTA. Two cytospin slides were prepared using 300 μl and 500 μl of the BAL. The slides were fixed, stained with HEMA 3, and phagocytosis index determined.

uPA activity assay. Protease activity in uPA and uPA mutants was determined using an activity kit (Millipore) according to the manufacturer's instructions. Briefly, 0.2 μg of uPA samples and a gradient of active uPA standards were added to the 96-well plate. Deionized water was added to bring the total volume to 160 μl. Assay buffer (20 μl) and chromogenic substrate were then added to each well. The plate was incubated at 37°C for 15 minutes. The optical density (O.D.) of each sample was measured by reading the plate at a wave length of 405 nm. A standard curve for uPA activity was performed and the

protease activity of each uPA sample calculated as U/μg uPA protein.

Immunoprecipitation and immunoblotting. Wild type and mutant uPA was preincubated with soluble $\alpha_v\beta_3$ at 4°C with rotation for 1 h. Anti-$\alpha_v\beta_3$ antibody (1 μg/ml) was then added to each sample and incubated at 4°C overnight with rotation. Protein G-agarose beads were then added to each sample and incubated for 2 h. In designated experiments examining the competition between the binding of uPA and MFG-E8 to $\alpha_v\beta_3$, uPA or MFG-E8 were pre-incubated with $\alpha_v\beta_3$ protein for 30 minutes followed by addition of uPA or MFG-E8 to the protein mixtures for another 30 minutes. Finally, $\alpha_v\beta_3$ antibody was added into each sample of the protein complexes and immunoprecipitation with anti-$\alpha_v\beta_3$ antibody performed. Immunoprecipitated proteins were resolved by Western blots with anti-uPA antibodies.

Rac-1 activation assay. Determination of Rac-1 activation using a commercially available kit was performed according to the manufacturer's instructions. In brief, peritoneal macrophages were preincubated with 1 μg/ml scuPA or HSA for 1 hour following which 10×10^6 apoptotic mouse thymocytes were added to 2.5×10^6 macrophages in a 6 well plate and incubated for 30 minutes. The macrophages were then washed 5 times with cold PBS and lysed with Mg^{2+} lysis buffer. Cell lysates were cleared by centrifugation and the supernatants incubated with 10 μL GST tagged PAK-1 PBD agarose slurry at 4°C for 1 h. The agarose beads were then pelleted by centrifugation and washed 5 times with Mg^{2+} lysis buffer. Active Rac-1 bound to the GST tagged PAK-1 PBD agarose was eluted with 2×SDS loading buffer, resolved by SDS-PAGE, and

detected by anti-Rac-1 antibody. The blots were then stripped and blotted with anti-GST antibody to demonstrate equal pull-downs of GST tagged PAK-1 PBD.

Supplementary Figure Legends

Supplementary Figure 1. Human scuPA does not activate macrophages to produce pro-inflammatory cytokines. Macrophages were treated with LPS, HSA, MSA, or scuPA at the indicated concentrations for 6 hours. The levels of TNF-α and IL-6 in the medium were determined by ELISA. Data are representative of two independently performed experiments.

Supplementary Figure 2. Macrophages were pre-treated for 1 hour with 100 μl BAL fluid obtained 24 hours after intratracheal LPS instillation in wild-type or uPA-/- mice. The cells were then washed with PBS five times and apoptotic neutrophils added into the cultures. Phagocytosis assays were then performed. n=4 for wild-type mice and n=3 for uPA-/- mice. *** $P<0.001$ compared to wild-type group.

References

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