Cancer Cell, Volume *14*

Supplemental Data

Article

Plasminogen Activator Inhibitor-1 Protects

Endothelial Cells from FasL-Mediated Apoptosis

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Supplemental Experimental Procedures

Cell Culture and Treatments

The human neuroblastoma cell line SK-N-BE(2) was obtained from Dr. J. Biedler (Memorial Sloan Kettering Cancer Center, New York, NY). A clone SK-N-BE(2) cl10 that stably expressed an enhanced green fluorescent protein (EGFP) was selected for the experiments. HBMECs were isolated from surgically resected brain tissues of children with seizure disorders as previously described (Stins et al., 1997). Both cell lines were maintained in RPMI 1640 supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml), 2 mM of L-glutamine and 10% fetal bovine serum (FBS). Human fibrosarcoma HT1080 cell lines were grown in DMEM with penicillin (100U/ml), streptomycin (100 μg/ml) and 10% FBS. Active stable rPAI-1 was kindly provided by Prof. P. Declerck (Katholieke Universiteit Leuven, Leuven, Belgium). A recombinant sFasL extending from aa 103 to 281 and fused to the N-terminus to a linker peptide and a Flag-tag and produced in HEK 293 cells was purchased from Alexis Biochemicals. A monoclonal antibody against the proteolytic activity of uPA was purchased from American Diagnostics Inc. (clone 394) and a monoclonal function blocking anti-Fas antibody (clone ZB4) from Upstate. Aprotinin and leupeptin were obtained from Sigma. The MMP inhibitor AG3340 was kindly provided by Dr. D. Shalinsky (Agouron-Pfizer).

Tumor Analysis by Angiography and Immunofluorescence

Biotinylated *Lycopersicon esculentum* (tomato) lectin (50 μl) and Texas red avidin D (100 μl; Vector Laboratories, Inc.) were mixed for 20 min before being injected i.v. in tumor bearing mice (150 μl per mouse). Five min after injection, animals were anesthetized with 2% Avertine, the chest was opened, the right atrium incised to drain the blood and the vasculature washed free of blood by slow perfusion of 20 ml of 0.9% NaCl into the left ventricle. The tumors were harvested and examined unfixed under fluorescent confocal microscopy with a 488 nm argon ion laser and RSP500 beam splitter as previously described (Chantrain et al., 2004). Cryostat sections (5 μm in thickness) were fixed first in acetone at -20°C followed by 80% methanol at 4°C and then incubated with a rat monoclonal antibody against mouse PECAM-1/CD31 (Pharmingen; dilution 1:20). The microvessel area for each tumor was determined by measuring the surface in each field that stained for PECAM-1/CD31 using Metamorph 4.6 software as previously described (Chantrain et al., 2003). To evaluate tumor cell proliferation mice were injected intraperitoneally with 5'-bromo-2'deoxyuridine (BrdU) (4 mg/mouse) 2 hr prior to

sacrifice and sections were incubated with an anti-BrdU mouse monoclonal antibody (Becton Dickinson). After being washed three times in PBS for 10 min each, sections were incubated with a fluorescein-isothiocyanate (FITC)-conjugated horse anti-mouse IgG (Vector Laboratories, dilution 1:100) secondary antibody for 30 min in the presence of 1 μg/ml of DAPI solution. After three washes in PBS for 10 min, coverslips were mounted and the sections were examined under a fluorescence microscope (Leica DMRA microscope). To evaluate apoptosis in these tumors we used a death detection kit (Roche Diagnostics GmbH). Mouse ECs were detected with a rat monoclonal anti-mouse PECAM-1/CD31 as primary antibody (Pharmingen, dilution 1:20) and a Texas red-conjugated goat anti-rat IgG as secondary antibody (Vector Laboratories, dilution 1:100). The percentage of proliferative or apoptotic tumor cells was then determined by counting the number of BrdU or dUTP nick end labeled (TUNEL) positive cells in 10 fields (20x magnification) from a total of 3 histological sections. To determine the level of apoptosis in ECs, sections were stained for PECAM-1/CD31 and TUNEL and the percentage of cells with dual stain was determined. Tumors were also examined for type IV collagen, uPA, uPAR, Fas, FasL and PECAM-1/CD31 expression by dual immunofluorescence on frozen sections fixed with methanol. The following primary antibodies were used: a goat anti-PECAM-1/CD31, polyclonal antibody (Santa Cruz Biotech, dilution 1:500), a rabbit polyclonal anti-type IV collagen antibody (dilution 1:200), a mouse anti-human Fas monoclonal antibody (IPO-4, Santa Cruz Biotech, dilution 1:50), a mouse anti-rat FasL monoclonal antibody (BD Biosciences, dilution 1:500), a rabbit polyclonal anti-mouse uPA antibody (American Diagnostica Inc., dilution 1:100), and a goat anti-mouse uPAR (R&D Systems, dilution 1:50). As secondary antibody we used a Cy-3 conjugated donkey anti-goat antibody, a FITC conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratory, dilution 1:1000) or a FITC conjugated donkey anti-mouse IgG (Vector Laboratories), dilution 1:1,000). Slides were stained with DAPI and mounted with crystal mount (Biomedia Corp).

Immunoprecipitation and Western Blot

Cells were harvested in extraction buffer (0.1M Tris, 0.4% Triton X-100, pH 8.1) containing a cocktail of protease inhibitors (Roche Diagnostics GmbH). After electrophoresis on 0.1% SDS polyacrylamide gels, the proteins were transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat dry milk in PBS-Tween (0.1%) overnight at 4°C, and incubated in the presence of the primary antibody for 2 hr at room temperature. The following antibodies were used: mouse monoclonal anti-uPA (American Diagnostics, Inc.), mouse monoclonal anti-uPAR (R&D Systems), rabbit anti-PARP, p-Bcl_2, Bcl-X_L, Bak, Bax, p-Akt, Akt, p-Erk 1/2 , Erk and cleaved caspase-8 (Asp 374) and total and cleaved caspase-9 (Cell Signaling Technology), mouse monoclonal anti-FLAG M2 (Stratagene), mouse monoclonal anti-His G-HRP antibody (Invitrogen), mouse monoclonal anti-FasL (G247-4, BD Biosciences) and rabbit anti-FasL (clone C20, Santa Cruz Biotechnology, Inc), followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature (Cell Signaling Technology). Immunoreactive bands were detected using enhanced chemiluminescence (ECL, Amersham Biosciences) after exposure to light-sensitive films. For immunoprecipitation, we used a mouse anti-FasL monoclonal antibody (clone NOK-2, BD Biosciences) that was incubated at 2 μg/ml in 6x concentrated conditioned medium overnight at 4°C. The immunocomplexes were collected by centrifugation after incubation with protein A Sepharose beads (Pharmacia), washed in PBS and resuspended in loading buffer containing 2.5% p-mercaptoethanol and heated at 95°C before being analyzed by SDS-PAGE.

Fas Activation

Fas activation was examined by testing for its association with FADD by immunoprecipitation. Eighty percent confluent HBMECs were treated with FLAG-sFasL (500 ng/ml) or 6xHisLys145FasL (500 ng/ml) for 3 hr. After washing 3x in PBS, the cells were lysed in RIPA buffer, and the lysates (200 µg) incubated overnight at 4°C with 2 µg of an anti-Fas (M20, Santa Cruz Biotechnology) antibody. The immunocomplexes were then collected in the presence of protein A Sepharose beads, heated at 95°C in loading buffer for 5 min and examined by SDS-PAGE and Western blot analysis as above described, using a mouse anti-human FADD antibody (BD Transductions Laboratories, dilution 1:500).

Recombinant Soluble FasL Protein

The cDNA of *FasL* extending from the cleavage site for plasmin Lys145 to the C-terminus of FasL was amplified by PCR using a forward primer with an internal BamHI site 5'-GCCGGGGGATCCAAAGTGGCCCATTTAACAGGC-3' (nucleotides 433-452) and a reverse primer with an internal HindIII site 5'-CGCGGGAACTTTCATTAGAGCTTATATAAGCCG-3' (nucleotides 828-846) and the *FasL* pcDNA 3.1 plasmid as template. The PCR reaction was performed with the Taq PCR Core Kit (Qiagen) using the following conditions: 94°C, 3 min; 94°C, 1 min for 3 cycles; 60°C, 1 min; 72°C, 1 min for 30 cycles. The 400 bp DNA PCR product was purified by agarose gel electrophoresis, digested with BamHI and HindIII, and subcloned into the pRSET plasmid downstream of a 6xHis sequence (Invitrogen) which was used to transform competent *E. coli* cells. Clones containing the expression vector were isolated and one was sequenced to confirm that it contained the correct FasL sequence. This plasmid was then used to transform BL21 (DE3) pLysS-strain of *E. coli* which were grown in the presence of ampicillin (100 µg/ml). The expression of the recombinant protein (6xHisLys145FasL) was induced at 37°C for 5 hr in the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified from the cell lysate by passage through a ProBond nickel chelating column (Invitrogen) following the instructions of the manufacturer. Identification of the protein that eluted from the column in the presence of 250 mM imidazole was done by SDS-PAGE and Western blot in the presence of an anti-His antibody. The protein suspension was dialyzed in PBS and concentrated by filtration (Millipore) before being tested for its activity. As control, we used a similar preparation from *E. coli* expressing a tagged LaZ protein.

Supplemental References

Chantrain,C.F., DeClerck,Y.A., Groshen,S., and McNamara,G. (2003). Computerized Quantification of Tissue Vascularization Using High- resolution Slide Scanning of Whole Tumor Sections. J. Histochem. Cytochem. *51*, 151-158.

Stins,M.F., Gilles,F., and Kim,K.S. (1997). Selective expression of adhesion molecules on human brain microvascular endothelial cells. J. Neuroimmunol. *76*, 81-90.

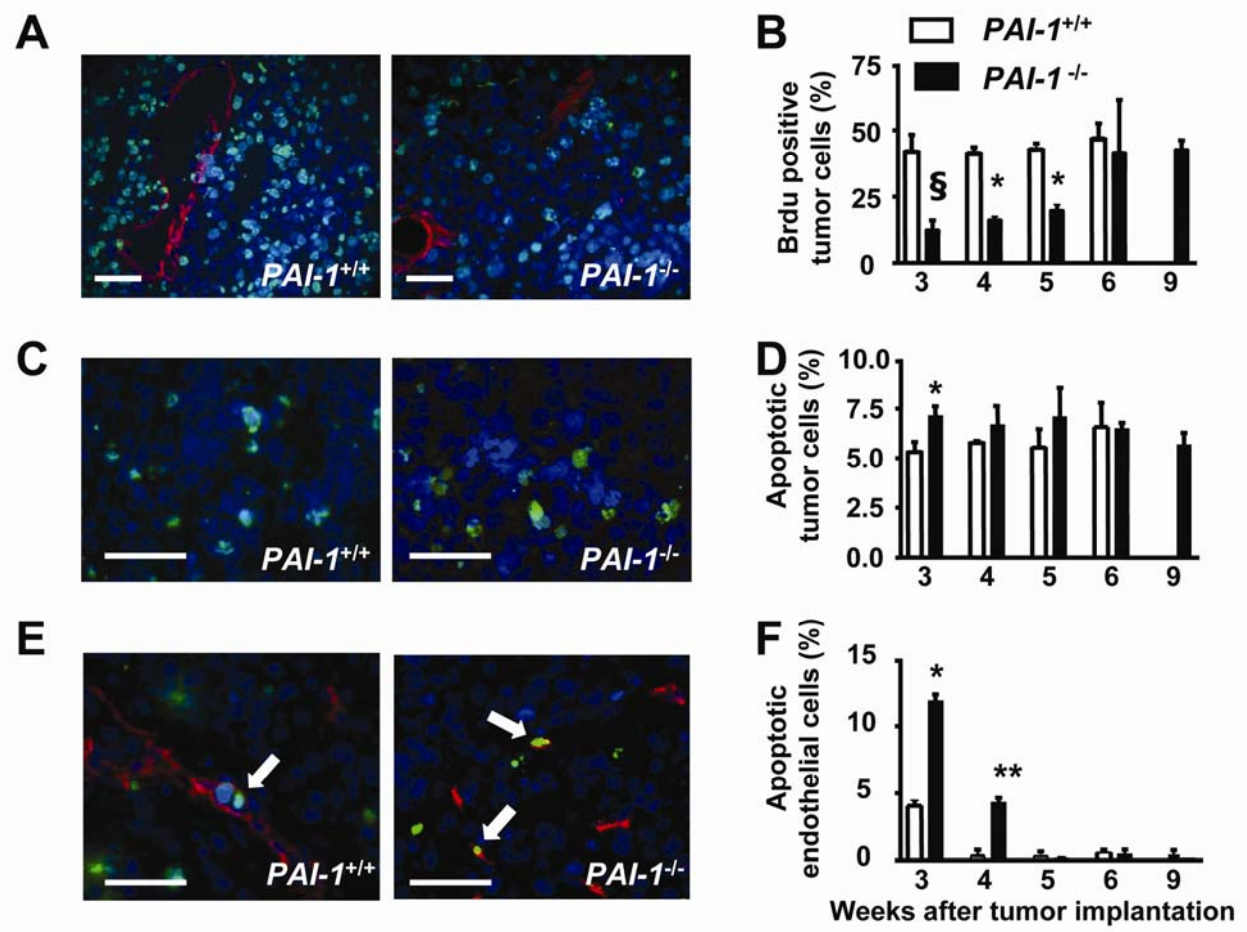

Figure S1. Increase in Tumor Cell Proliferation and in EC Apoptosis in the Absence of Host-derived PAI-1

(A, C, and E) Representative photomicrographs of tumor sections obtained 4 weeks after tumor implantation and stained for BrdU (A), TUNEL (C) and PECAM-1 (red) and TUNEL (green) (E) as indicated under Supplemental Procedures. Scale bar is 50 μm. White arrows indicate the presence of apoptotic ECs.

(B, D, and F) The percentage of BrdU positive cells (B), apoptotic cells (D) and apoptotic ECs (F) in tumor sections was determined at indicated time. The data represent the means (± SD) from a total of 6 sections examined in each of 6-8 tumors per time point (*$p < 0.05$, **$p < 0.01$).

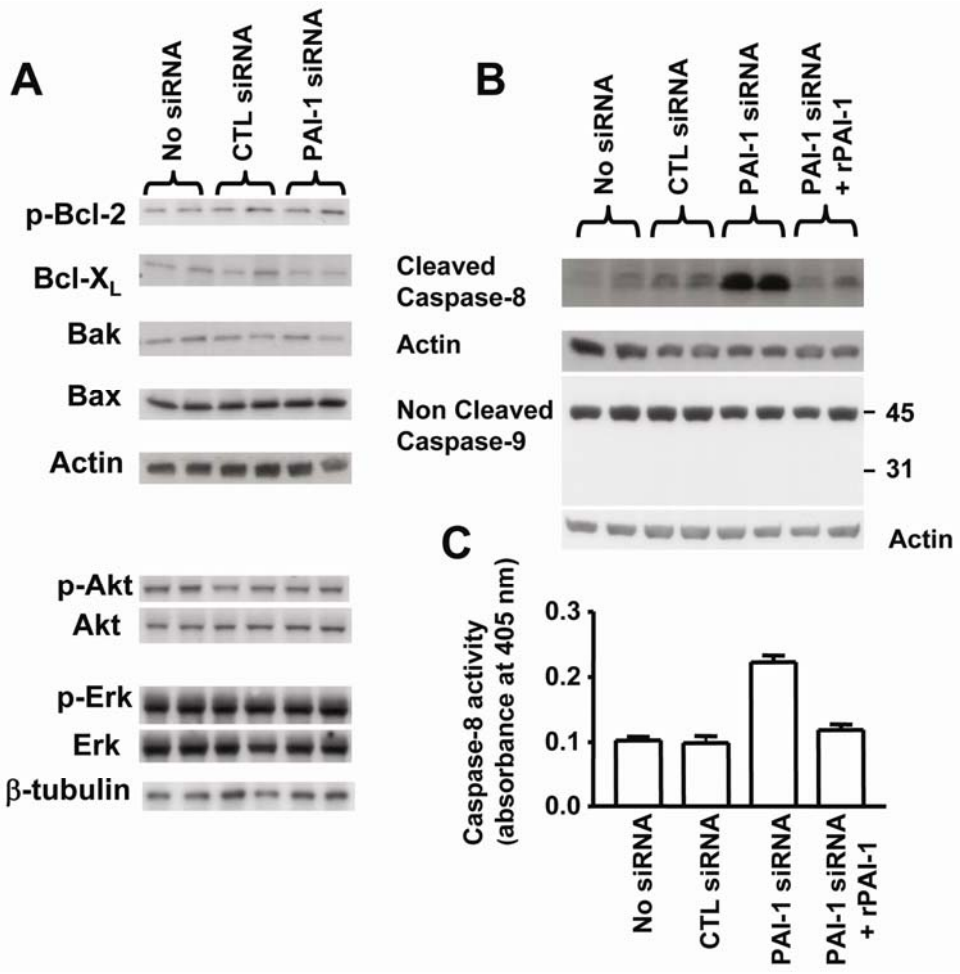

Figure S2. Absence of Changes in Mitochondrial-dependent Apoptosis and Increase in Caspase-8 Activity upon *PAI-1* Downregulation

(A) Western blot analysis of the expression of p-Bcl2, Bcl-$X_{L,}$ Bak, Bax, pAkt, Akt, p-ERK 1/2, and ERK 1/2 in HBMECs transfected with siRNA as shown on top.

(B) Western blot analysis for the presence of cleaved caspase-8 and uncleaved caspase-9 in cell extracts of HBMECs treated as shown on top.

(C) Caspase-8 activity in HBMECs transfected with siRNA was determined by colorimetric assay as described in Experimental Procedures. rPAI-1 (2.5 μg/ml) was added 24 hr before analysis. The data represent the mean (±SD) values of triplicate samples (*$p < 0.001$).

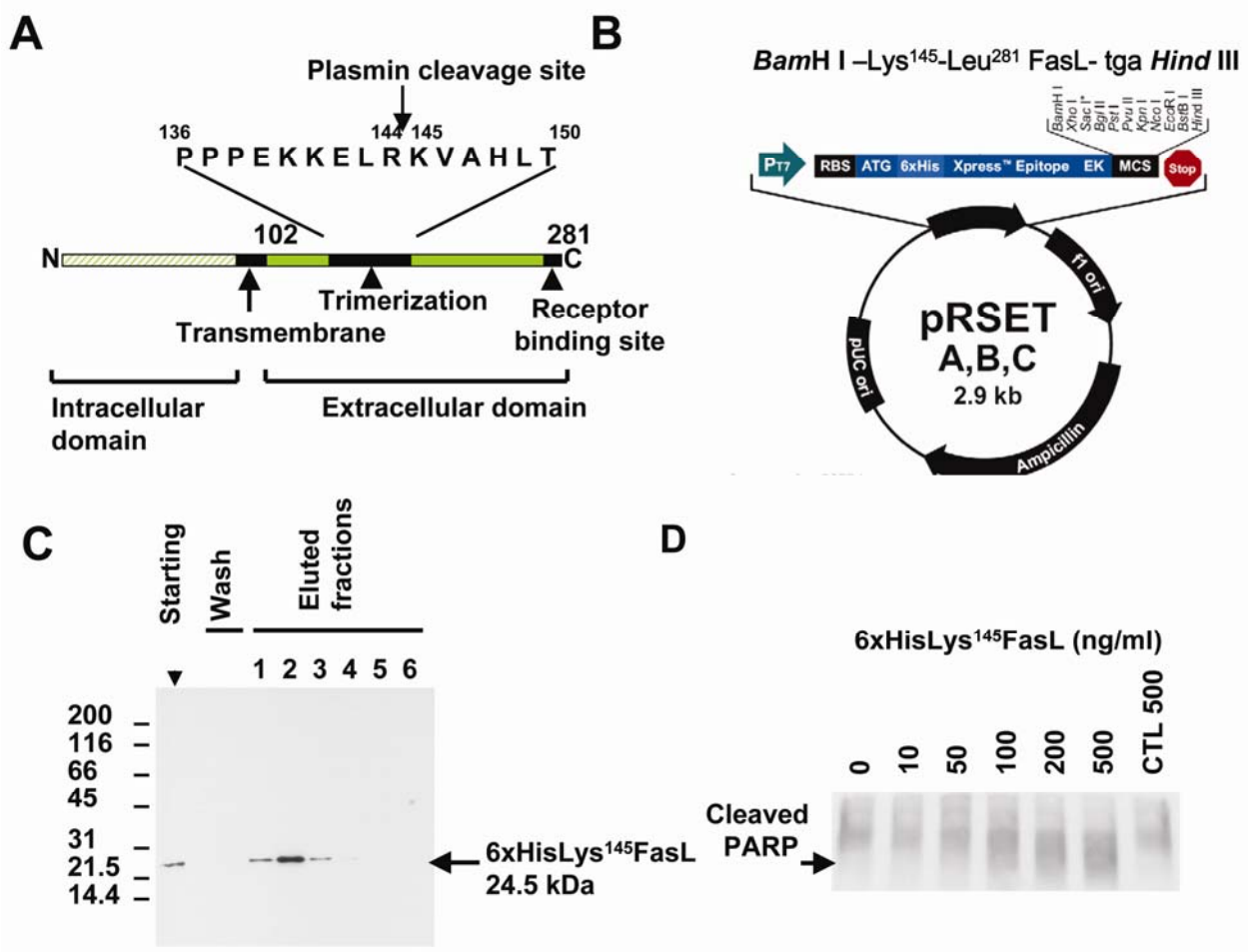

Figure S3.

(A) The cleavage site of FasL by plasmin was determined by N-terminal amino acid sequencing of the 21.5 kDa fragment isolated from the gel shown in Figure 6C. The sequence identified was the KVAHLT sequence that locates the cleavage site at Arg144-Lys145 in the trimerization domain.

(B) Construct used to make recombinant 6xHisLys145FasL (from Invitrogen), as described in Supplemental Procedures.

(C) Plasmid pRSET - 6xHisLys145FasL was transfected into *E. coli* and the recombinant protein purified in a single step by affinity chromatography on a nickel column. The presence of the 6xHis recombinant protein in the eluting fractions was monitored by Western blot with an anti-His antibody.

(D) Western blot analysis of PARP cleavage in HBMECs treated with 6xHisLys145FasL at indicated concentrations. CTL = 500 ng of recombinant LacZ protein.

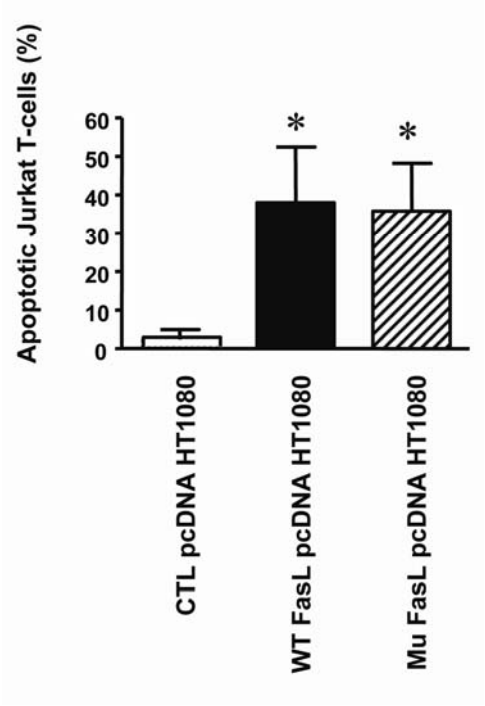

Figure S4. mu *FasL* Is Active

Jurkat T-cells were cultured on monolayers of HT1080 cells overexpressing WT *FasL* or mu *FasL* cells for 24 hr. The Jurkat T-cells were then harvested and examined for apoptosis by TUNEL staining and flow cytometry. The data represent the average percent (± SD) of apoptotic Jurkat T-cells from triplicate samples (*$p < 0.05$).

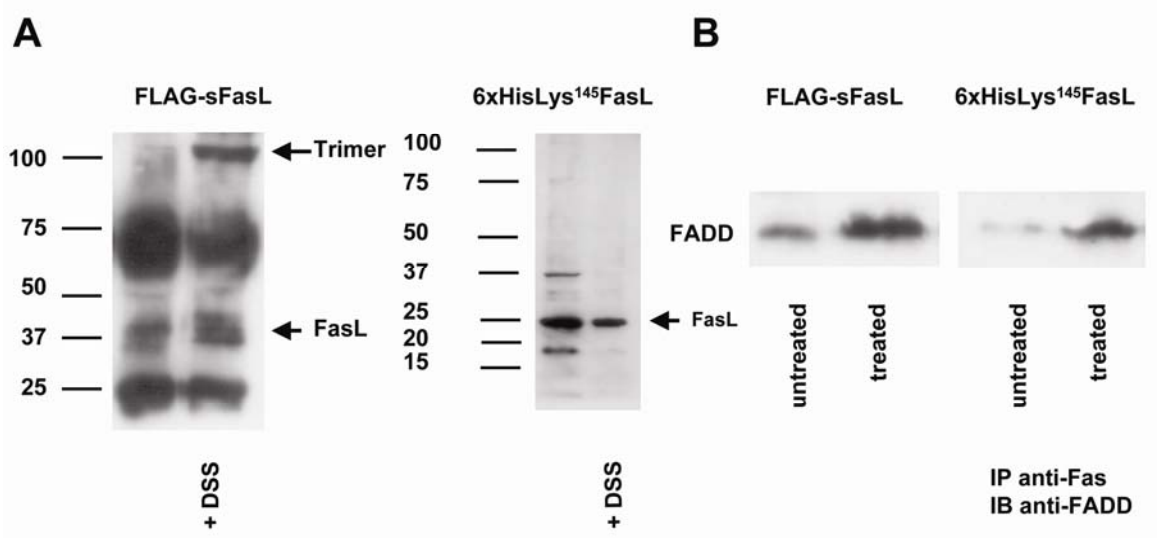

Figure S5. 6xHisLys145FasL Does Not Form Trimers but Activates Fas

(A) Analysis of FLAG-sFasL and 6xHisLys145FasL proteins incubated in vitro in the absence or presence of DSS (1.25 mM) for 30 min and examined by SDS-PAGE and Western blot with an anti-FLAG (left panel) and anti-His (right panel) antibody.

(B) Immunoprecipitation and Western blot analysis of cell lysates of HBMECs treated with FLAG-sFasL or 6xHisLys145FasL. Immunoprecipitation was done with an anti-Fas antibody and Western blot with an anti-FADD antibody as described in Supplemental Procedures.

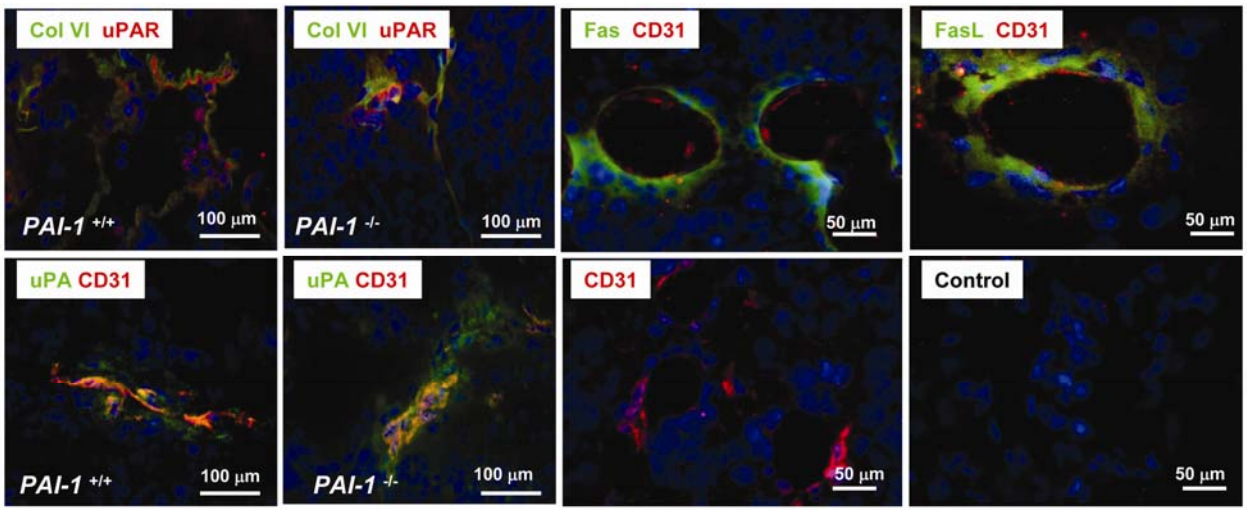

Figure S6. uPA/uPAR and FasL/Fas Are Coexpressed in ECs in the Vasculature of Neuroblastoma Tumors Orthotopically Implanted in Mice

Immunofluorescence analysis of frozen sections from neuroblastoma tumors orthotopically implanted in WT mice or *PAI-1*$^{-/-}$ mice (KO) for the co-expression of type IV collagen (green) and uPAR (red), and co-expression of PECAM-1/CD31 (red) and uPA, Fas and FasL (green).

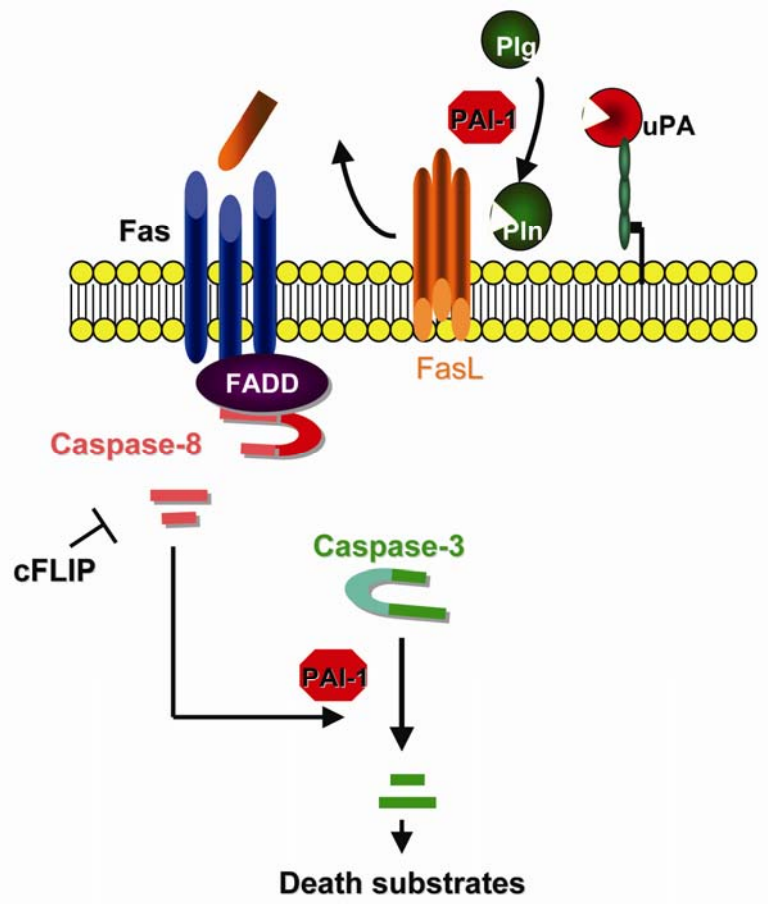

Figure S7. PAI-1 Protects ECs from sFasL-Mediated Apoptosis

Model describing the mechanism of action of PAI-1 in protecting ECs from sFasL/Fas-mediated apoptosis. The presence of PAI-1 in the pericellular space inhibits activation of plasminogen into plasmin by cell-associated uPA at the surface of ECs. As a result, it prevents the cleavage of membrane-associated FasL by plasmin and the release of proapoptotic sFasL.