

MMP-9 modulates Pigment Epithelium-Derived Factor control of angiogenesis via proteolysis

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Purpose: To determine the effect of matrix metalloproteinase (MMP)-mediated proteolysis on the anti-angiogenic activity of pigment epithelium-derived factor (PEDF) *in vitro* and determine the MMP-mediated cleavage sites within the primary sequence of PEDF. Furthermore, we have determined the effect of inhibiting MMPs on the intraocular levels of PEDF protein expressed from an adenoviral expression system in a mouse eye.

Methods: To determine the site at which PEDF is cleaved by MMP-9, purified PEDF was incubated with and without MMP-9 (1:200 mass ratio MMP to PEDF) at 37°C for 24h then with trypsin (1:40 trypsin to PEDF) overnight. The peptides were separated by reverse-phase HPLC on a C-18 column, collected, treated with 2-sulfobenzoic acid anhydride, and sequenced by MALDI-MS. The anti-angiogenic activity of MMP-treated PEDF was quantified using the mouse aortic ring bioassay and a standard invasion assay. The effect of MMP inhibition on PEDF levels was evaluated by immunoassay 1 day after intravitreal injection of 1x10⁹ particles of adenovector carrying the gene for human PEDF into the right eye of C57-B16 mice that had been pretreated with or without 2 ng of the MMP-2 and -9 inhibitor, SB-3CT.

Results: We have found proteolytic digestion of PEDF by the pro-angiogenic proteinase MMP-9 eliminates the anti-angiogenic activity of PEDF as measured in the mouse aortic ring assay, and converts PEDF into an endothelial chemoattractant factor, as the products formed from the digestion of PEDF with MMP-9 increase the invasiveness of cultured endothelial cells *in vitro*. PEDF is proteolytically processed by MMP-9 at the serpin reactive center loop of PEDF as well as near the N-terminal, anti-angiogenic region of the protein. Finally, pretreatment of mouse eyes with intravitreal injection of the MMP-2 and -9 inhibitor, SB-3CT, tripled the observed amount of expressed PEDF in mouse eyes intravitreally transduced with an adenovector containing the gene for human PEDF.

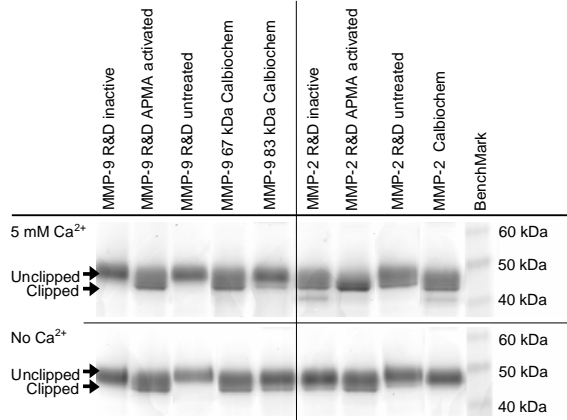


Figure 1. Purified PEDF was incubated with the stated MMPs for 2 hours at 37°C in TNTZ buffer with and without 5 mM CaCl₂. PEDF was cleaved from a diffuse band of approximately 48 kDa to a sharp band at ca. 44 kDa. MMP-2 and -9 from two manufacturers was tested. MMP from R&D systems was either used untreated, activated as suggested by the manufacturer with APMA, or incubated under the same conditions as the activated enzyme without APMA. Control PEDF is visually identical to PEDF in lane one and is not shown. Note that the addition of calcium had no effect on MMP-9, but strongly activated MMP-2.

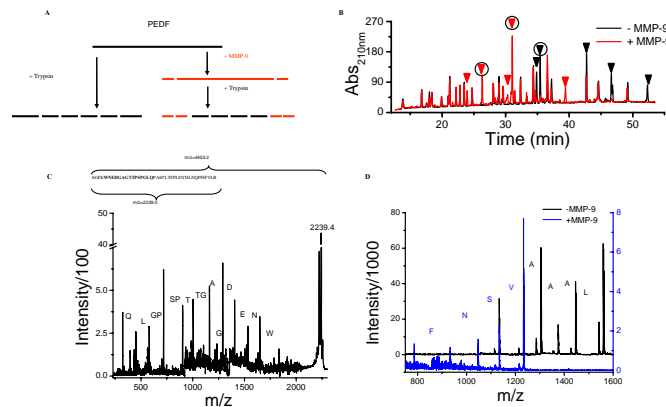


Figure 2. Determining the MMP-9 mediated cleavage sites within the primary sequence of PEDF. **A:** Schematic representation of the method used (see Methods for full description). Full-length PEDF was digested either sequentially with MMP-9 and then trypsin, or with trypsin alone. The horizontal line represents the intact protein, and the dashed lines reflect the predicted peptide fragments. The peptides predicted to be different after MMP-9 and trypsin treatment versus trypsin alone are in red. **B:** C-18, reversed-phase HPLC traces after PEDF digest with trypsin alone (black trace) or sequential treatment with MMP-9 and then trypsin (red trace). The arrows indicate peaks that were different between the two traces. The mass spectra of part C and D derive from peaks marked with circled arrows. The other marked peaks either contained insufficient material to analyze, or were simply tryptic peptides. **C:** PSD MALDI fragment ion mass spectrum of a derivatized tryptic peptide obtained from MMP-9 treated PEDF (circled arrow in B). The peak at 2239.4 m/z is the parent peptide. The amino acid residues that match the difference in mass between adjacent peaks are shown. The predicted tryptic peptide that corresponds to this sequence is 4453.2 Da. The observed tryptic fragment parent ion with a m/z ratio of 2239.0 could only arise from PEDF cleaved by MMP-9 at PAH¹L¹T (cleavage indicated with the *) in the RCL. **D:** PSD MALDI mass spectrum of derivatized tryptic peptides isolated from PEDF pretreated with (blue trace), or without MMP-9 (black trace). The MMP-9 and trypsin processed sample contained the peptide VSNFGYDLYR while the trypsin only PEDF contained the peptide LAAAVSNFGYDLYR. Therefore, MMP-9 cleaved PEDF at the sequence LAAAVSNF.

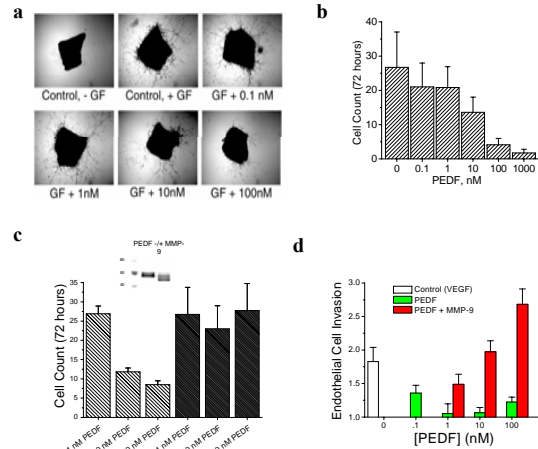


Figure 3. We tested the anti-angiogenic activity of PEDF with the mouse aortic ring assay. In this assay, 0.5-0.8 mm rings from the aortas of C57-B16 mice were placed on liquid Matrigel in 24 well plates, and the Matrigel was allowed to harden at 37°C for 10 minutes. Human Basal Endothelial Cell media supplemented with 0.25% mouse serum and antibiotic/antimycotic was then added above the rings. The endothelial growth factors bFGF and VEGF were added at 2 ng/mL and 25 ng/mL, respectively, to elicit endothelial cell outgrowth. Various concentrations of purified PEDF were also added to the media. **A:** Representative photomicrographs of the aortic rings after approximately 120 hours of incubation under various conditions; Ring control - GF contains no bFGF or VEGF or PEDF; +GF control contains bFGF and VEGF but no PEDF; GF + concentration contains bFGF and VEGF and the stated concentration of PEDF. **B:** Quantitation of endothelial cell outgrowth by cell count at 72 hours showing the potent anti-angiogenic activity of PEDF. **C:** PEDF was incubated with or without MMP-9 for 24h at 37°C (mass ratio 1:40 PEDF to MMP) then used in the aortic ring assay. MMP-9 treatment of PEDF eliminated the PEDF anti-angiogenic activity. **D:** MMP-9 processed PEDF was assayed on the commercially available angiogenic invasion assay based on Fluoroblok transwells. In brief, cultured, primary dermal microvascular endothelial cells were placed on a Matrigel cushion above a porous-membrane transwell in un-supplemented media. Below the transwell, was supplemented (50 ng/ml VEGF) media to act as a pro-angiogenic chemoattractant. Also included in the supplemented media below the transwell was various concentrations of PEDF, PEDF treated with MMP-9 as above, and MMP-9 alone in concentrations identical to that in the MMP-9-treated PEDF. Cells were allowed to invade for 24 hours after the relative number of cells that passed through the membrane was measured via labeling with the fluorescent dye Calcein AM. Fluorescence below each transwell was compared to the -VEGF control. These data show that while untreated PEDF strongly inhibited endothelial cell invasion, MMP-9 treatment of PEDF produced a contrawise pro-angiogenic effect.

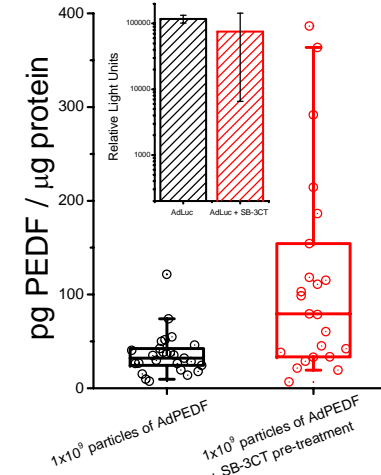


Figure 4. 1x10⁹ particles of AdPEDF were injected into the right eye of C57BL/6 mice with or without 24 hour pre-treatment with 2 ng of the MMP-2 and -9 inhibitor, SB-3CT. The eyes were enucleated after 24 hours, and the amount of PEDF was measured by ELISA. The data is represented with box (25-50-75 quartile) and whiskers (5-95 quartile) with overlaid data from each animal. The mean amount of PEDF detected from AdPEDF alone was 36.2 ± 4.5 pg/mg protein (n=26) while from AdPEDF plus MMP inhibitor was 114.3 ± 22.5 pg/mg protein (mean ± SEM, n=23; p=0.00075). The amount of PEDF in naive and vehicle injected eyes was below the level of detection of the assay. Inset: 1x10⁹ particles of an adenovector containing the luciferase gene (AdLuc) were injected into mouse eyes with and without pretreatment with 2 ng of SB-3CT. There was no difference in the amount of detectable luciferase between the two groups (error bars are 1 SD; n=5).

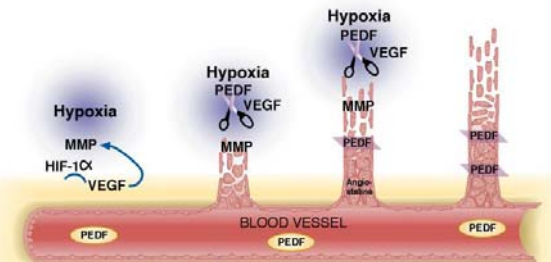


Figure 5: The model that we propose based on our data is shown above (moving left to right). In the first condition on the left, prolonged hypoxia, known to play a role in retinal neovascularization, protects hypoxic response factor HIF-1 α from degradation. The increased concentration of HIF-1 α leads to VEGF expression and thus MMP expression and activation. While abundant PEDF in tissues and in blood maintain zero-vessel growth in normal physiology, MMPs locally eliminate this inhibitory activity by proteolytic cleavage of PEDF thus allowing endothelial cells to proliferate and invade. Once a vessel is formed and begins allowing blood to flow, PEDF returns to actively blocking neovascularization in the absence of MMPs (far right).

Conclusions: PEDF levels are decreased in the human eye during proliferative disease, while MMP activity is increased. Cleavage of PEDF by MMP-9 eliminates the anti-angiogenic activity of PEDF, while the inhibition of MMP within the mouse eye correlates with higher levels of expressed PEDF. Our results suggest that pro-angiogenic MMPs modulate PEDF activity by proteolysis at the serpin reactive center loop or the N-terminal, anti-angiogenic region of the protein and this may be important in ocular disease progression.