Published in final edited form as:

Invest Ophthalmol Vis Sci. 2009 October ; 50(10): 4974–4981. doi:10.1167/iovs.09-3619.

Plasminogen activator inhibitor -1 (PAI-1) Facilitates Retinal Angiogenesis in a Model of Oxygen-Induced Retinopathy

Anupam Basu¹, Paul McGuire¹, Gina Menicucci¹, Joann Maestas¹, and Arup Das^{2,3}

¹Department of Cell Biology and Physiology, University of New Mexico School of Medicine, Albuquerque, NM 87131

²Division of Ophthalmology, Department of Surgery, University of New Mexico School of Medicine, Albuquerque, NM 87131

³New Mexico VA Health Care System, Albuquerque, NM 87131

Abstract

Purpose—Angiogenesis, or the formation of new retinal blood vessels is a key feature of many proliferative retinal diseases including diabetic retinopathy, retinal vein occlusions, and retinopathy of prematurity. The aim of the present study was to investigate the role of the serine proteinase inhibitor PAI-1 in facilitating retinal angiogenesis.

Methods—The temporal expression of PAI-1 was examined by real time PCR, western blotting and immunohistochemistry in retinal tissues from mice with oxygen-induced retinopathy. The requirement for PAI-1 in facilitating the retinal angiogenic response in this model was examined by quantitating the angiogenic response using both wildtype and PAI-1 null mice. The mechanism by which PAI-1 mediates angiogenesis was further investigated using isolated human retinal vascular endothelial cells.

Results—PAI-1 expression is up regulated in the retina of mice with oxygen-induced retinopathy. This coincides with a significant increase in the expression of vitronectin in the retina of the experimental mice. There was significant reduction in the angiogenic response of PAI-1^{-/-} mice as compared to wild type mice. PAI-1 promotes endothelial cell migration in vitro and facilitates migration of cells on a vitronectin substrate by regulating α_v integrin cell surface expression.

Conclusions—These observations suggest a role for PAI-1 during retinal angiogenesis and point to a potential new therapeutic target in the prevention or treatment of retinal neovascularization seen in many ocular diseases.

Introduction

Ocular angiogenesis, in response to tissue ischemia, is a leading cause of vision loss in numerous clinical conditions like diabetic retinopathy, retinal vein occlusions and retinopathy of prematurity.¹ By definition, angiogenesis is a process in which new blood vessels are formed by sprouting of endothelial cells from pre-existing vessels. Angiogenesis is a multi-step process involving the activation of endothelial cells, the breakdown of the capillary basal lamina, the migration and proliferation of endothelial cells, and the maturation of new capillary tubes.^{2,3} Recent work suggests that in some circumstances the formation of new retinal vessels may also involve bone marrow derived progenitor cells that contribute to the process.^{4,5}

The activation of capillary endothelial cells is brought about by the activity of specific growth factors that are expressed in the retina in response to ischemic or hypoxic conditions. These growth factors include Angiopoietin, VEGF, HGF and others.^{6–8} Endothelial

activation involves the upregulation of specific proteinases which mediate extracellular matrix remodeling and cell migration. We have previously reported increased levels of the serine proteinase urokinase plasminogen activator (uPA) in human diabetic neovascular membranes and in the retina of mice with oxygen-induced retinopathy.^{9,10}

Urokinase binds to a receptor (uPAR) on the cell surface where it can efficiently convert plasminogen to plasmin. Through the activation of plasminogen, uPA is also capable of initiating a proteolytic cascade resulting in the activation of members of the matrix metalloproteinase (MMP) family that together with plasmin degrade components of the extracellular matrix, activate and release growth factors stored in the extracellular matrix and modify the presentation of proteins at the cell surface. The interaction of uPA with uPAR results in conformational changes within the uPAR protein leading to interactions of uPAR with other cell surface proteins. These interactions can lead to changes in gene expression, signaling events, and cell-matrix adhesion.¹¹ Inhibition of urokinase action or loss of its cell surface receptor uPAR can result in significant decreases in pathological retinal angiogenesis.¹⁰

The activity of uPA as well as the surface expression of the uPA/uPAR complex is tightly regulated by plasminogen activator inhibitor type 1 (PAI-1).¹²⁻¹³ PAI-1 is a member of the serine proteinase inhibitor (serpin) super family and is postulated to play a role in the regulation of angiogenesis.¹⁴⁻²¹ PAI-1 is a 50kDa glycoprotein found in low concentrations in the blood and is expressed in response to cytokines and growth factors. The stability and half-life of PAI-1 is increased by interaction with and binding to the extracellular matrix protein vitronectin.²² The aim of the present study was to investigate the role of the serine proteinase inhibitor PAI-1 in facilitating retinal new vessel formation in a model of oxygen-induced retinopathy.

Materials and Methods

Animal Models

Wild type C57BL/6J and genetically modified PAI-1^{-/-} mice were bred at the University of New Mexico Animal Research Facility. The homozygous PAI-1^{-/-} mice (B6.129S2-*Serpine1*^{tm1Mlg/J}) were originally obtained from The Jackson Laboratory (Bar Harbor, Maine). All experiments were consistent with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the use of animals in ophthalmic and vision research.

Oxygen Induced Retinopathy

Retinal angiogenesis was induced in wild type and PAI-1^{-/-} mice using the oxygen induced retinopathy (OIR) model described by Smith and co-workers²³ Briefly, 7 day-old pups were placed with their nursing mothers in a chamber (BioSpherix ProOx model 110; Redfield, NY) at 75% O₂ until postnatal day 12 (P12). Mice were then placed at room air until postnatal day 17 during which time angiogenesis occurred at the junction of the vascularized and non-vascularized regions of the retina. By day 17, retinal neovascularization was achieved in 100% of the experimental mice. Age matched mice, housed only in room air served as controls.

Quantitation of Vaso-oblivation and Retinal Neovascularization

To characterize the response of wildtype and PAI-1 deficient mice to the OIR protocol, blood vessels were stained in retinal whole mounts from untreated P7 and oxygen-treated P12 animals. The eyes were fixed for 2 hours with 2% paraformaldehyde in PBS and the retinas removed. The retinas were incubated in cold 70% ethanol followed by 1% triton

X-100 and finally incubated overnight in PBS containing 5µg/ml of FITC-labeled Isolectin GSA-IB4 (Invitrogen). For quantitation of retinal angiogenesis, eyes from control and experimental animals were fixed overnight in 10% neutral buffered formalin and embedded in paraffin. Serial sections (6 µm) parallel to the optic nerve were mounted on gelatin-coated slides. After deparaffinization, sections were treated with 0.0125% Trypsin at 37°C for 10 minutes. Sections were blocked with 10% normal goat serum and stained for Type IV collagen (Rockland Inc., Gilbertsville, PA) to label the vessels. The sections were mounted with Vectashield containing diamidinophenylindole (DAPI) to stain the nuclei. Nuclei associated with type IV collagen positive vessels on the vitreous side of the inner limiting membrane of the retina were counted in every third section using a Zeiss inverted fluorescence microscope.

Cell Culture

Human retinal microvascular endothelial cells (ACBRI-181) were obtained from Cell Systems (Kirkland, WA) and grown on fibronectin-coated cell culture dishes in MCDB-131 supplemented with 10 % FBS, 10ng/ml EGF, 1 µg/ml Hydrocortisone, 0.2mg/ml EndoGro and 0.09mg/ml Heparin (VEC Technologies, Rensselaer, NY).

Quantitative Real Time PCR

Retinas were collected from the oxygen-treated and room air control mice on postnatal days 12, 15, and 17. Total mRNA was extracted using Trizol and first-strand cDNA was synthesized with 2 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The following TaqMan assays for real-time RT-PCR were obtained from Applied Biosystems: PAI-1: Mm00435860 and 18s RNA Hs99999901. Amplification was performed using the Applied Biosystems 7500 Fast system. Data were derived using the comparative Ct method for duplicate reactions.²⁴

Western blot analysis

Retinal extracts were analyzed for both PAI-1 and vitronectin proteins by Western blot analysis. Retinas were collected from control and oxygen-treated mice on days 15 and 17 and extracted with 1X SDS PAGE sample buffer in a boiling water bath. Total protein was quantitated using the Micro BCA method (Pierce). Equal amounts of total protein were separated on a 10% gel and transferred to PVDF membranes. The membranes were blocked with 5 % milk in 1 X TBST and incubated with either anti- PAI-1 (BD Bioscience; San Jose, CA) or anti-vitronectin (AbCam; Cambridge, MA), antibody at 4°C overnight. After washing, the membranes were incubated with HRP-conjugated secondary antibody and developed with enhanced chemiluminescence reagents.

Immunostaining

Eyes from experimental and control mice were collected on days 15 and 17 and fixed overnight with either HistoChoice fixative for frozen sections or 4% paraformaldehyde for paraffin embedding. The sections were blocked with 10% normal goat serum for 30 minutes and incubated with either anti-PAI-1 antibody for 60 minutes or anti-vitronectin antibody. Sections were washed with 1 X TBST and incubated with a fluorescently-labeled secondary antibody for 30 minutes. After a final wash the sections were cover slipped with Vectashield and examined using fluorescence microscopy.

Cell Migration Assay

Cell migration assays were performed using Falcon cell culture inserts containing a membrane with 8.0µm pores. Human retinal microvascular endothelial cells (2×10^4 cells) were plated onto membranes coated with either vitronectin or type I collagen in serum-free

MCDB-131 media. The lower chamber contained serum-free media with or without 40 ng/ml vascular endothelial growth factor (VEGF) added as a chemo-attractant. Some wells contained anti-PAI-1 antibody (BD Bioscience; San Jose, CA) at 6.25 μ g/ml, 12.5 μ g/ml or 37.5 μ g/ml. The cells were allowed to migrate for 18 hours and the cells on the upper surface of the membrane were removed with a cotton swab. The cells that migrated to the lower chamber and attached to the lower surface of the membrane were fixed with 100% methanol for 5 minutes and rinsed with PBS. The membranes were excised, mounted on glass slides with vectashield containing DAPI to stain the cell nucleus. The number of migrated cells was determined at 10X magnification in four peripheral fields and one central field. The number of migrated cells was averaged from 5 fields per membrane.

Flow Cytometry

Human retinal microvascular endothelial cells were plated at a subconfluent density (~70%) onto vitronectin coated substrates and grown in MCDB-131 in the presence of VEGF (40ng/ml). Anti-PAI-1 antibody (12.5 μ g/ml) or normal mouse IgG was added for 16 hours and the cells were processed for immunostaining and flow cytometry. The cells were detached from the culture plate using EDTA for 30 minutes at 4° C and incubated with anti- α v/CD51 antibody (R&D Systems Inc. Minneapolis, MN) for 30 minutes. Cells were washed and incubated with an Alexa-568 conjugated secondary antibody for 30 minutes and washed again. After resuspension in 1X PBS, the cells were analyzed using a FACS Calibur system (Beckton Dickinson; Franklin Lakes, NJ) and the Cell Quest Software.

Statistical Analysis

All statistical analysis was performed using Graph-pad prism software. Data were analyzed by either Students t-test or by one way ANOVA followed by Bonferroni's Multiple Comparison test.

RESULTS

The Expression of PAI-1 is Increased in the Retinas of mice with Oxygen-Induced Retinopathy

The retinas of P7 wildtype mice, at the beginning of high oxygen exposure, have a well developed vascular bed as seen in GSA lectin-labeled whole mounts (Figure 1A). After 5 days of oxygen exposure, the central portion of the retina has undergone vaso-obliteration (Figure 1B) that is followed over the next five days by new vessel formation at the interface of the vascularized and non-vascularized portions of the retina in 100% of the animals. These new vessels grow abnormally crossing the inner limiting membrane and invade the vitreous space.²³

The level of PAI-1 mRNA was determined by real time RT-PCR during the oxygen-induced retinopathy process in these animals (Figure 2A). PAI-1 mRNA in day 12 experimental retinas was significantly less than that seen in the control retinas at this age. As PAI-1 is expressed primarily by endothelial cells, this decrease in mRNA expression may be due simply to the significant degree of vaso-obliteration and endothelial cell loss occurring in these animals at this time. Despite the difference in vascular density between control and experimental retinas, the level of PAI-1 mRNA was found to significantly increase in the experimental retinas compared to controls during the subsequent active stages of angiogenesis in this model (days 15 and 17). The increased level of PAI-1 mRNA correlated with an increase in the level of protein as determined by Western blotting analysis (Figure 2B).

PAI-1 protein in the experimental retina was localized by immunostaining and was found specifically associated with the neovascular tufts present on the surface of the retina and on vessel profiles just below the surface (Figure 3). The staining appeared to be localized primarily to the endothelial cells of the new vessels.

The Expression of Vitronectin is Increased in the Retinas during OIR Induced Angiogenesis

PAI-1 has previously been shown to mediate the attachment of cells to vitronectin in the extracellular matrix (22). A role for PAI-1 in mediating the angiogenic response in the OIR model would be supported if vitronectin were present in the retina where it could provide a suitable migratory substrate for the newly forming vessels during the angiogenic process. Retinas were examined for the expression of vitronectin during the preangiogenic and angiogenic phases in the OIR model (P12 and P15 respectively) and compared to control retinas of the same age. A significant increase in the amount of vitronectin was seen in the retinas of P15 OIR mice as compared with retinas at the beginning of the angiogenic process (P12 OIR) and was also significantly greater than that seen in the P15 control animals (Fig 4A).

The vitronectin protein was localized by immunostaining in day 15 experimental mice to the vitreoretinal interface. Staining was localized to the region of the inner limiting membrane (ILM) as well as some portions of the nerve fiber layer adjacent to the ILM (Figure 4B).

The Loss of PAI-1 Reduces the Extent of Retinal Neovascularization in Mice with Oxygen Induced Retinopathy

To determine a role for PAI-1 in retinal angiogenesis, wildtype and PAI-1^{-/-} mice were subjected to the OIR protocol and analyzed for the extent of retinal neovascularization as described.

Mice with a genetic loss of PAI-1 demonstrated a similar response to the initial high oxygen treatment as the wildtype mice. The retinas of P7 PAI-1^{-/-} mice demonstrated a dense pattern of retinal vessels that were equally susceptible to vaso-obliteration as demonstrated in the P12 PAI-1^{-/-} OIR mice when compared to the wildtype animals (Figure 5). A role for the elevated levels of PAI-1 in mediating angiogenesis in the retina was confirmed as the genetic loss of PAI-1 resulted in a significant decrease in the extent of retinal angiogenesis (Figure 6). Retinas from PAI-1^{-/-} mice had a significantly decreased number of neovascular tufts on the vitreal side of the inner limiting membrane as compared to the retinas from the wildtype mice (average of 33 nuclei vs. 62 nuclei respectively). This result demonstrates a 53% reduction in retinal angiogenesis when PAI-1 is absent.

Blocking PAI-1 Inhibits the Migration of Retinal Endothelial Cells *in vitro*

We next examined whether the inhibition of new vessel formation seen in the PAI-1^{-/-} mice might be due to a migration defect in the endothelial cells. The migration of human retinal endothelial cells was examined *in vitro* as described above. Cells plated onto vitronectin or collagen-coated migration inserts and stimulated with VEGF showed extensive migration after 18 hours. When a PAI-1 neutralizing antibody was added at the time of plating the cells showed a significant reduction in the extent of migration, and this effect was dose dependent. In addition, the effect was also found to be substrate dependent as the PAI-1 antibody had no effect when cells were migrating on a collagen substrate (Figure 7).

One potential mechanism by which PAI-1 facilitates cell migration is by regulating the surface expression of integrins.²⁵ We utilized flow cytometry to examine the surface expression of the α_v integrin in human retinal endothelial cells growing on a vitronectin

substrate in the presence and absence of anti-PAI-1 antibody. Cells exposed to the antibody for 16 hours demonstrated significantly more cell surface α_v integrin compared to cells in the absence of antibody (Figure 8). When the activity of PAI-1 was reduced with the PAI-1 antibody, 20.26 % of the cells expressed α_v integrin on the cell surface. When cells were grown in the absence of antibody only 5.37 % demonstrated surface α_v integrin. These results suggest that PAI-1 activity can regulate the interaction of endothelial cells with a vitronectin substrate by facilitating the turnover of the α_v integrin.

DISCUSSION

This study addresses the role of the proteinase inhibitor PAI-1 in the regulation of new vessel formation in a model of oxygen-induced retinal angiogenesis. The expression of PAI-1 is significantly elevated during the most active phases of new vessel growth in this model and localizes to the newly forming vessels. Animals lacking a functional PAI-1 gene, exhibited a 53% reduction in the retinal neovascular response as compared to wildtype animals. The level of the permissive migratory substrate vitronectin, to which PAI-1 has been shown to bind, was increased in the inner portion of the retina of animals undergoing angiogenesis. Vitronectin forms a suitable substrate for the migration of isolated retinal endothelial cells in response to VEGF stimulation and this migratory activity could be blocked using an anti-PAI-1 antibody. Decreasing PAI-1 function appeared to alter cell migration by retaining higher levels of α_v integrin on the cell surface that may lead to increased cell-substrate adhesion.

PAI-1 is a 50kDa protein whose primary function is the inhibition of plasminogen activator. The active form of PAI-1 is generally unstable with a short half-life but can be stabilized by interaction with components of the extracellular matrix including vitronectin.^{22,26} Although PAI-1 binds to vitronectin, it has a significantly higher affinity for the serine proteinase uPA which can be localized to the cell surface by binding to its receptor uPAR.^{27,28} The binding of PAI-1 to uPA at the cell surface leads to the internalization of the uPA:PAI-1:uPAR complex followed by degradation of uPA and PAI-1 and recycling of uPAR back to the cell surface.^{29,30}

We have utilized the oxygen-induced retinopathy model to examine the role of PAI-1 in retinal neovascularization. In this model, exposure of mice to 75% oxygen results in a significant regression of the superficial vascular network in the central portion of the retina. A condition of relative hypoxia ensues resulting in the upregulation of VEGF and other growth factors followed by the formation of abnormal preretinal neovascular tufts at the interface of the vascularized and nonvascularized retina. These new vessels are formed by the process of angiogenesis and their formation can be aided by the infiltration of bone marrow derived myeloid progenitor cells that differentiate into microglia.³¹ The hypoxia which develops in the retinal tissues of this model likely drives the expression of PAI-1 mRNA by the remaining endothelial cells and associated pericytes. In the present study we have demonstrated a significant increase in PAI-1 mRNA in the retinas during the active angiogenic period (days 15 to 17) and a corresponding increase in PAI-1 protein. The increase in PAI-1 is seen following an initial decrease in expression most likely due to the vaso-oblation and the significant delay in the development of the deep vascular plexus that occurs at this time in the model.³¹ Other studies have reported that the PAI-1 gene is responsive to hypoxia and under these conditions its activity is regulated by specific transcription factors including HIF-1 α .³²

Previous studies have examined the expression and function of PAI-1 in ocular tissues. A study using the rat model of retinal neovascularization found that treatment with high doses of recombinant PAI-1 could inhibit the formation of new vessels.³³ Decreased retinal

angiogenesis was also observed in the present study in mice lacking PAI-1 and suggests that an optimal physiological level of PAI-1 is necessary to facilitate angiogenesis in the retina. Excessively high or excessively low levels of PAI-1 would thus lead to inhibition. This hypothesis is further supported in studies of choroidal angiogenesis, where a dose-dependent modulation of choroidal neovascularization by PAI-1 was observed.^{16,17} Mice lacking PAI-1 demonstrate a deficient angiogenic response following choroidal laser photocoagulation. The response could be reversed by injection of recombinant PAI-1 protein or by treatment with adenovirus expressing human PAI-1. If PAI-1 levels were increased above the normal physiological levels found in this model the degree of neovascularization was again reduced.

A role for PAI-1 in retinal angiogenesis is strengthened by the demonstrated presence of vitronectin in the region of the experimental retinas in which the new vessels will form. Although the cell types responsible for production of the vitronectin substrate are unknown, because of its location at the vitreoretinal interface, one can speculate that vitronectin may be produced by glial cells or ganglion cells. Astrocytes in the superficial layers of the retina have been shown to form a meshwork and to secrete VEGF that facilitates the outgrowth of retinal vessels during normal development of the retinal vasculature.^{33,34} Alternatively, retinal ganglion cells may respond to the hypoxic environment by expression of vitronectin. These cells have been shown to express VEGF in an ROP model following the selective loss of astrocytes in the ischemic retina.³⁶

In this study we have demonstrated that isolated retinal vascular endothelial cells rely on PAI-1 to migrate efficiently on a vitronectin substrate in vitro. PAI-1 could function by two independent or complementary mechanisms in this situation. PAI-1 may protect the matrix from excessive degradation by regulating the production of plasmin thereby maintaining the migratory substrate.³⁷ In addition, PAI-1 may function by regulating cell-substrate interactions independent of its anti-proteolytic activity. PAI-1 has been shown to demonstrate high-affinity binding to vitronectin in a region referred to as the somatomedin B domain.^{38,39} Binding of PAI-1 in this region not only stabilizes the active form of PAI-1 but also regulates the interaction of both uPAR and integrin with vitronectin that would be expected to impact the adhesive and migratory behavior of cells.^{40–42}

During cell migration, PAI-1 may be important for the normal detachment of cells from the matrix through the internalization and recycling of cell surface receptors. Since the α_v integrin interacts with uPAR on the cell surface, the internalization of the uPA:uPAR:PAI-1 complex would be expected to decrease the overall density of α_v receptors on the cell surface and promote cell detachment and subsequent migration.^{24,42} In this study we have demonstrated that a PAI-1 blocking antibody decreased migration of retinal endothelial cells on vitronectin and increased the amount of α_v integrin on the cell surface. This data would support the hypothesis that inhibition or loss of PAI-1 would decrease overall retinal angiogenesis by increasing cell adhesion to a vitronectin-containing substrate, and identifies PAI-1 as a potential therapeutic target for retinal neovascularization.

Acknowledgments

Grant Information: Funding provided by the National Institutes of Health Grant, RO1 EY12604.

References

1. Das A, McGuire PG. Retinal and Choroidal Angiogenesis: Pathophysiology and strategies for inhibition. *Prog Retinal Eye Res.* 2003; 22:721–748.
2. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature.* 2000; 407:249–257. [PubMed: 11001068]

3. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. *Nature Medicine*. 2000; 6:389–395.
4. Ritter MR, Banin E, Moreno SK, Aguilar E, Dorrell MI, Friedlander M. Myeloid progenitors differentiate into microglia and promote vascular repair in a model of ischemic retinopathy. *J Clin Invest*. 2006; 116:3266–3276. [PubMed: 17111048]
5. Friedlander M, Dorrell MI, Ritter MR, et al. Progenitor cells and retinal angiogenesis. *Angiogenesis*. 2007; 10:89–101. [PubMed: 17372851]
6. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med*. 1994; 331:1480–1487. [PubMed: 7526212]
7. Das A, Fanslow W, Cerretti D, Warren E, Talarico N, McGuire PG. Angiopoietin/Tek Interactions Regulate MMP-9 Expression and Retinal Neovascularization. *Laboratory Investigation*. 2003; 83:1637–1645. [PubMed: 14615417]
8. Colombo ES, Menicucci G, McGuire PG, Das A. Hepatocyte growth factor/scatter factor promotes retinal angiogenesis through increased urokinase expression. *Invest Ophthalmol Vis Sci*. 2007; 48:1793–1800. [PubMed: 17389513]
9. Das A, McGuire PG, Eriqat C, et al. Human diabetic neovascular membranes contain high levels of urokinase and metalloproteinase enzymes. *Invest Ophthalmol Vis Sci*. 1999; 40:809–813. [PubMed: 10067990]
10. McGuire PG, Jones TR, Talarico N, Warren E, Das A. The urokinase/urokinase receptor system in retinal neovascularization: Inhibition by $\tilde{A}6$ suggests a new therapeutic target. *Invest Ophthalmol Vis Sci*. 2003; 44:2736–2742. [PubMed: 12766081]
11. Binder BR, Mihaly J, Prager GW. uPAR-uPA-PAI-1 interactions and signaling: A vascular biologist's view. *Thromb Haemost*. 2007; 97:336–342. [PubMed: 17334498]
12. Herz J, Clouthier DE, Hammer RE. LDL receptor-related protein internalizes and degrades uPA/PAI-1 complexes and is essential for embryo implantation. *Cell*. 1992; 71:411–421. [PubMed: 1423604]
13. Zhang JC, Sakthivel R, Kniss D, Graham CH, Strickland DK, McCrae KR. The low density lipoprotein receptor-related protein/alpha2- macroglobulin receptor regulates cell surface plasminogen activator activity on human trophoblast cells. *J Biol Chem*. 1998; 273:32273–32280. [PubMed: 9822706]
14. Balsara RD, Castellino FJ, Ploplis VA. A novel function of plasminogen activator inhibitor-1 in modulation of the AKT pathway in wild-type and plasminogen activator inhibitor-1 deficient endothelial cells. *J Biol Chem*. 2006; 281:22527–22536. [PubMed: 16785241]
15. McMahon GA, Petitclerc E, Stefansson S, et al. Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. *J Biol Chem*. 2001; 276:33964–33968. [PubMed: 11441025]
16. Lambert V, Munaut C, Carmeliet P, et al. Dose-Dependent modulation of choroidal neovascularization by plasminogen activator inhibitor type I: Implications for clinical trials. *Invest Ophthalmol Vis Sci*. 2003; 44:2791–2797. [PubMed: 12766088]
17. Lambert V, Munaut C, Noël A, et al. Influence of plasminogen activator inhibitor type 1 on choroidal neovascularization. *FASEB J*. 2001; 15:1021–1027. [PubMed: 11292663]
18. Eitzman DT, Krauss JC, Shen T, Cui J, Ginsburg D. Lack of plasminogen activator inhibitor-1 effect in a transgenic mouse model of metastatic melanoma. *Blood*. 1996; 87:4718–4722. [PubMed: 8639841]
19. Almholt K, Nielsen BS, Frandsen TL, Brunner N, Dano K, Johnsen M. Metastasis of transgenic breast cancer in plasminogen activator inhibitor-1 gene-deficient mice. *Oncogene*. 2003; 22:4389–4397. [PubMed: 12853975]
20. Stefansson S, Petitclerc E, Wong MK, McMahon GA, Brooks PC, Lawrence DA. Inhibition of angiogenesis in vivo by plasminogen activator inhibitor-1. *J Biol Chem*. 2001; 276:8135–8141. [PubMed: 11083866]
21. Maud J, Maillard C, Lecomte J, et al. Tumoral and choroidal vascularization: Differential cellular mechanisms involving plasminogen activator inhibitor type 1. *Am J Pathol*. 2007; 171:1369–1380. [PubMed: 17717143]
22. Zhou A, Huntington JA, Pannu NS, Carrell RW, Read RJ. How vitronectin binds PAI-1 to modulate fibrinolysis and cell migration. *Nature Structural Biology*. 2003; 10:541–544.

23. Smith LEH, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci.* 1994; 35:101–111. [PubMed: 7507904]
24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta C_t$ method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
25. Czekay RP, Aertgeerts K, Curriden SA, Loskutoff DJ. Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. *J Cell Biol.* 2003; 160:781–91. [PubMed: 12615913]
26. Hekman CM, Loskutoff DJ. Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. *J Biol Chem.* 1985; 260:11581–11587. [PubMed: 3930479]
27. Nielsen LS, Kellerman GM, Behrendt N, Picone R, Dano K, Blasi F. A 55,000–60,000 Mr receptor protein for urokinase-type plasminogen activator. Identification in human tumor cell lines and partial purification. *J Biol Chem.* 1988; 263:2358–2363. [PubMed: 2828365]
28. Daniel A, Selvi Palaniappan L, Stefansson S, et al. Characterization of the binding of different conformational forms of plasminogen activator inhibitor-1 to vitronectin. Implications for the regulation of pericellular proteolysis. *J Biol Chem.* 1997; 272:7676–7680. [PubMed: 9065424]
29. Estreicher A, Mulhauser J, Carpentier JL, Orci L, Vassalli JD. The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. *J Cell Biol.* 1990; 111:783–792. [PubMed: 2166055]
30. Nykjaer A, Conese M, Christensen EI, Olson D, Cremona O, Gliemann J, Blasi F. Recycling of the urokinase receptor upon internalization of the uPA;serpine complexes. *EMBO J.* 1997; 16:2610–2620. [PubMed: 9184208]
31. Ritter MR, Banin E, Moreno SK, Aguilar E, Dorrell MI, Friedlander M. Myeloid progenitors differentiate into microglia and promote vascular repair in a model of ischemic retinopathy. *J Clin Invest.* 2006; 116:3266–3276. [PubMed: 17111048]
32. Liao H, Hyman MC, Lawrence DA, Pinsky DJ. Molecular regulation of the PAI-1 gene by hypoxia: contributions of Egr-1, HIF-1 α and C/EBP α . *FASEB J.* 2007; 21:935–949. [PubMed: 17197388]
33. Penn JS, Rajaratnam VS. Inhibition of retinal neovascularization by intravitreal injection of human rPAI-1 in a rat model of retinopathy of prematurity. *Invest Ophthalmol Vis Sci.* 2003; 44:5423–5429. [PubMed: 14638747]
34. Fruttiger M. Development of the mouse retinal vasculature: Angiogenesis versus vasculogenesis. *Invest Ophthalmol Vis Sci.* 2002; 43:522–527. [PubMed: 11818400]
35. Gariano RF, Gardner TW. Retinal angiogenesis in development and disease. *Nature.* 2005; 438:960–966. [PubMed: 16355161]
36. Stone J, Chan-Ling T, Pe'er J, Itin A, Gnessin H, Keshet E. Roles of vascular endothelial growth factor and astrocyte degeneration in the genesis of retinopathy of prematurity. *Invest Ophthalmol Vis Sci.* 1996; 37:290–299. [PubMed: 8603833]
37. Bajou K, Masson V, Gerard RD, et al. The plasminogen activator inhibitor PAI-1 controls in vivo tumor vascularization by interaction with proteases, not vitronectin. Implications for antiangiogenic strategies. *J Cell Biol.* 2001; 152:777–784. [PubMed: 11266468]
38. Deng G, Royle G, Wang S, Crain K, Loskutoff DJ. Structural and functional analysis of the plasminogen activator inhibitor-1 binding motif in the somatomedin B domain of vitronectin. *J Biol Chem.* 1996; 271:12716–12723. [PubMed: 8662688]
39. Lijnen HR. Pleiotropic functions of plasminogen activator inhibitor-1. *J Thrombosis and Haemostasis.* 2004; 3:35–45.
40. Okumura Y, Kamikubo Y, Curriden SA, et al. Kinetic analysis of the interaction between vitronectin and the urokinase receptor. *J Biol Chem.* 2002; 277:9395–9404. [PubMed: 11773078]
41. Stefansson S, Lawrence DA. The serpin PAI-1 inhibits cell migration by blocking integrin $\alpha v \beta 3$ binding to vitronectin. *Nature.* 1996; 383:441–443. [PubMed: 8837777]
42. Pepper MS. Role of the matrix metalloproteinase and Plasminogen activator-plasmin systems in angiogenesis. *Arterioscler Thromb Vasc Biol.* 2001; 21:1104–1117. [PubMed: 11451738]

43. Carriero MV, Del Vecchio S, Capozzoli M, et al. Urokinase receptor interacts with $\alpha v\beta 5$ vitronectin receptor, promoting urokinase-dependent cell migration in breast cancer. *Cancer Research*. 1999; 59:5307–5314. [PubMed: 10537314]

Figure 1. Vascular density of retinal whole mounts from P7 (A) and P12 OIR (B) wildtype C57Bl6 mice. Retinas were fixed and stained with FITC-GSA lectin and photographed. Retinas from P12 mice following 5 days of high oxygen treatment display a significant loss of vessels in the central portion of the retina which over time becomes revascularized by an angiogenic process.

Figure 2.

(**A**) PAI-1 mRNA expression in control and experimental OIR mice. The level of PAI-1 mRNA increases significantly in the retina from OIR mice in comparison to control mice on postnatal days 15 and 17 following an initial decrease on day 12. Values are the mean $\pm$ sem for N=4 in each group. *Significantly different from controls. (d12C vs. d12E, $p=0.0018$; d15C vs. d15E, $p=0.0057$; d17C vs. d17E, $p=0.0103$). (**B**) Representative Western blot of PAI-1 protein in control and OIR retinas from P12 (lanes 1 and 4), P15 (lanes 2 and 5) and P17 (lanes 3 and 6) animals.

Figure 3. Representative images of PAI-1 localization in P17 OIR retina. **(A)** Section stained with anti-PAI-1 antibody demonstrating the presence of PAI-1 in neovascular tufts (arrows) on the surface of the retina. **(B)** Similar section incubated without primary antibody. V = vitreous cavity. Bar = 100 μ m.

Figure 4.

(A) Western blot analysis of vitronectin protein in P12 and P15 wild type control and OIR retinas. Duplicate samples of P12 and P15 retinal extracts are shown. (B) Representative images of vitronectin immunostaining in P15 wildtype OIR retina. Vitronectin is localized to the inner limiting membrane and extends down into the nerve fiber layer (arrows). (C) Representative section from P15 wildtype OIR retina, no primary antibody control. Bar = 100 μ m.

Figure 5. Vascular density in retinal whole mounts from P7 (A) and P12 OIR (B) PAI-1^{-/-} mice. Retinas were fixed and stained with FITC-GSA lectin and photographed. Retinas from P12 mice following 5 days of high oxygen treatment display a significant loss of vessels in the central portion of the retina which over time becomes revascularized by an angiogenic process. This response is similar to that seen in the wildtype mice (compare with Figure 1).

Figure 6.

Quantitation of retinal neovascularization in P17 wild type C57Bl6 and PAI-1^{-/-} mice. Neovascularization was quantitated by counting the number of vascular nuclei present on the vitreous side of the inner limiting membrane as described in the Materials and Methods. A 53% reduction in the angiogenic response is seen in PAI-1^{-/-} mice compared to wild type animals. Data are the mean $\pm$ sem for N= 4 animals per group. * Significantly less than wildtype animals (p= 0.0001)

Figure 7.

Role of PAI-1 in the migration of human retinal endothelial cells. Cell migration was examined using a modified Boyden chamber assay. Representative images of migratory cells on either vitronectin (**A and B**) or type I collagen (**C and D**) coated inserts. Cells were stimulated to migrate to the underside of the filter with VEGF in the absence (A and C) or presence (B and D) of anti-PAI-1 antibody. (**E**) Quantification of cell migration. Values are the mean $\pm$ sem of N= 3 wells for each condition. Differences among means were tested by ANOVA and corrected using the Bonferroni post test. Values for $p < 0.05$ were considered significant (*). No antibody vs. 6.25 mg/ml anti PAI-1, $p < 0.001$; No antibody vs. 12.5 mg/ml anti PAI-1, $p < 0.001$; No antibody vs. 37.5 mg/ml anti PAI-1, $p < 0.001$. Bar = 100µm.

Figure 8.

The effect of PAI-1 on the surface localization of α_v integrin assessed by flow cytometry. Representative histograms of human retinal endothelial cells grown either in the absence (**A**) or presence (**B**) of anti-PAI-1 neutralizing antibody and stained for α_v integrin. The gating is indicated by horizontal lines and the percentage of positively stained cells is noted. (**C**) Cells plus anti-PAI-1 antibody, non-specific staining (secondary antibody only)