Influence of plasminogen activator inhibitor type 1 on choroidal neovascularization

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ABSTRACT High levels of the plasminogen activators, but also their inhibitor, plasminogen activator inhibitor 1 (PAI-1), have been documented in neovascularization of severe ocular pathologies such as diabetic retinopathy or age-related macular degeneration (AMD). AMD is the primary cause of irreversible photoreceptors loss, and current therapies are limited. PAI-1 has recently been shown to be essential for tumoral angiogenesis. We report here that deficient PAI-1 expression in mice prevented the development of subretinal choroidal angiogenesis induced by laser photocoagulation. When systemic and local PAI-1 expression was achieved by intravenous injection of a replication-defective adenoviral vector expressing human PAI-1 cDNA, the wild-type pattern of choroidal angiogenesis was restored. These observations demonstrate the proangiogenic activity of PAI-1 not only in tumoral models, but also in choroidal experimental neovascularization sharing similarities with human AMD. They identify therefore PAI-1 as a potential target for therapeutic ocular anti-angiogenic strategies.—Lambert, V., Munaut, C., Noël, A., Frankenne, F., Bajou, K., Gerard, R., Carmeliet, P., Defresne, M. P., Foidart, J.-M., Rakic, J.-M. Influence of plasminogen activator inhibitor type 1 on choroidal neovascularization. *FASEB J.* 15, 1021–1027 (2001)

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CHOROIDAL NEOVASCULARIZATION GROWING under the retina in severe forms of age-related macular degeneration (AMD) causes irreversible photoreceptors loss and the primary source of blindness in the Western world. Insight into the molecular mechanisms associated with subretinal neovascularization is important since current therapeutic modalities are limited and concern only a small percentage of affected individuals.

Molecular signals involved in the development of choroidal neovascularization are not well defined. Alpha-v integrins show a specific expression pattern during retinal angiogenesis (1) and antibodies to this integrin or its ligands such as vitronectin inhibit neovascularization in different retinal models (2–4). Among growth factors, several lines of evidence suggest that vascular endothelial growth factor (VEGF) could be implicated in the pathogenesis of AMD since this cytokine is present in pathological specimens (5, 6) and is expressed in several models of hypoxia-related retinal neovascularization (7). However, VEGF retinal overexpression alone was not able to induce choroidal neovascularization in a transgenic mouse model (8), suggesting either a retinal specificity in the mechanisms controlling angiogenesis or a requirement for additional angiogenic molecules in AMD. In the intact choroid, polarized secretion of VEGF by retinal pigment epithelium could play an important role in the maintenance of a normal choriocapillaris (9). Recent studies suggest also a role for Fas ligand in the control of choroidal neovascularization (10).

Angiogenesis is an invasive process that requires proteolysis of the extracellular matrix, proliferation, and migration of endothelial cells with simultaneous synthesis of new matrix components. Such migratory and tissue remodeling events are regulated by different proteolytic systems including matrix metalloproteases (MMPs) and serine proteases of the plasminogen/ plasminogen activator system. The specific roles of MMPs, plasminogen activators, and their inhibitors in neovascular chorioretinopathies remain largely unexplored. In retinal pathology, a mutation in a tissue inhibitor of MMPs (TIMP3) is associated with a rare form of macular dystrophy (11), and the expression of several MMPs has been demonstrated in human choroidal neovascular membranes (12). Urokinase-type (uPA) and tissue-type (tPA) plasminogen activators are serine proteases, both able to activate the zymogen plasminogen into plasmin. Plasmin is a broadly acting enzyme that degrades extracellular matrix proteins and activates pro-MMPs and growth factors (13). Plasmino-

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gen activator inhibitor type-1 (PAI-1) is the main physiological inhibitor of uPA and tPA. It not only regulates the proteolytic activity of uPA, but also determines the level of uPA bound to its cell surface receptor (uPAR) by promoting the rapid endocytosis of the trimolecular uPA-PAI-1–uPAR complex (14). The importance of PAI-1 for tumoral angiogenesis has recently been demonstrated *in vivo* in experimental squamous cell carcinomas (15). Elevated PAI-1 levels have been correlated clinically not only with a poor prognosis in patients suffering from a variety of cancers (16), but also with various chorioretinal pathologies (17, 18).

To evaluate the biological relevance of PAI-1 in subretinal angiogenesis, we induced choroidal neovascularization *in vivo* with argon laser burns (19) into PAI-1$^{-/-}$ and wild-type (WT) mice. A choroidal neovascular membrane with leakage on fluorescein angiograms was produced at laser impacts in WT mice but not in PAI-1-deficient mice. In these PAI-1$^{-/-}$ mice, choroidal neovascularization similar to that occurring in WT mice was restored when PAI-1 expression was achieved by injecting a recombinant adenoviral vector bearing PAI-1 cDNA. These observations highlight the proangiogenic activity of PAI-1 in choroidal neoangiogenesis and identify PAI-1 as a potential therapeutic target against AMD.

MATERIALS AND METHODS

Genetically modified mice

Animal experiments were performed in compliance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Homozygous PAI-1-deficient mice (PAI-1$^{-/-}$) and the corresponding WT mice (PAI$^{+/+}$) of either sex, with a mixed genetic background of 87% C57BL/6 and 13% 129 strain, were used throughout this study (20). There were five or more mice in each group. The animals were maintained with a 12 h light/12 h dark cycle and had free access to food and water.

Laser photocoagulation and fluorescein angiograms

Choroidal neovascularization was induced in mice by laser burns as described previously (19). Briefly, mice were anesthetized with intraperitoneal (i.p.) injection of Avertin. Both pupils were dilated with 1% topical tropicamide; three burns were delivered (usually at the 9, 12, and 3 o'clock positions around the optic disc) using a green argon laser (532 nm; 50 μm diameter spot size; 0.05 s duration; 400 mW) and a cover slide as a contact lens. Fluorescein angiograms were performed 14 days later by taking serial fundus photographs (Canon) after i.p. injection of 0.3 ml of 1% fluorescein sodium (Ciba, Summit, N.J.). The percentage of burns developing late-phase hyperfluorescent spots (evaluated in comparison with the retinal normal vasculature) corresponding to the leakage of fluorescein from newly formed hyperpermeable vessels was evaluated. Animals were then killed, eyes were enucleated and either fixed in buffered 3.5% formalin solution for routine histology or embedded in Tissue TeK (Miles Laboratories, Naperville, Ill.), and frozen in liquid nitrogen for cryostat sectioning.

Quantitation of choroidal neovascularization

A quantitative morphometric assessment of thickness of choroidal new vessels was carried out using a computer-assisted image analysis system (Olympus Micro Image version 3.0 for Windows 95/NT, Olympus Optical Co. Europe GmBH). Microscopic images (working magnification of ×200) of hematoxylin-stained eye section were acquired via a video camera, digitalized, and analyzed. Frozen serial sections were cut throughout the entire extent of each burn and the thickest lesions (at least 5 sections per lesion) was used for the quantitation studies (96 sections studied). Neovascularization was estimated by the ratio (B/C) of the thickness from the bottom of the pigmented choroidal layer to the top of the neovascular membrane (B) to the thickness of the intact pigmented choroid adjacent to the lesion (C). The advantage of this method of quantification (over surface estimation) was its independence in relation to oblique sections (see example in Fig. 2c).

Immunofluorescence

Cryostat sections (5 μm in thickness) were fixed first in acetone at −20°C and then in methanol at 4°C before incubation with the primary antibodies. Antibodies raised against mouse PECAM (rat monoclonal antibody; PharMingen, San Diego, Calif.; diluted 1/20) or mouse type IV collagen (guinea pig polyclonal antibody produced in our laboratory; diluted 1/100) were incubated for 1 h at room temperature. Antibodies to mouse PAI-1 (rabbit polyclonal antibody produced in our laboratory, 10 μg/ml) were incubated overnight at 4°C. The sections were washed three times for 10 min in phosphate buffered saline (PBS) before the appropriate secondary antibodies conjugated to fluorescein-isothiocyanate (FITC) or Texas red were added. Swine anti-rabbit (Dakopat, Glostrup, Denmark; diluted 1/40) or rabbit anti-rat (Sigma, St. Louis, Mo.; diluted 1/40) were applied for 30 min. For double immunofluorescence-labeling studies, sections were first incubated with the two primary antibodies and then with FITC- and Texas red-conjugated secondary antibodies. After three washes in PBS for 10 min each and a final rinse in 10 mM Tris-HCl buffer, pH 8.8, coverslips were mounted and labeling was analyzed under an inverted microscope equipped with epifluorescence optics. Staining for β-galactosidase activity was performed with 5-bromo-4-chloro-3-indolyl-β-galactopyronoside (X-gal) as described (21). In some assays, primary anti-PAI-1 antibodies were preabsorbed for 15 min with recombinant PAI-1 (1 μg/ml, a generous gift from P. Declerck, Katholieke Universiteit Leuven, Belgium).

Adenovirus-mediated PAI-1 gene transfer

Recombinant adenovirus bearing human *PAI-1* (AdCMVPAI1), *Escherichia coli* β-galactosidase (AdCMVlacz) and control adenovirus (AdRR5) were generated as described (22). Twenty-four hours after laser spot production, mice were intravenously (i.v.) injected with 200 μl of control or recombinant adenovirus ($7×10^8$ PFU). After 5 days, blood was sampled from the right retro-orbital sinus and PAI-1 antigen was measured by ELISA as reported (22). On day 14, mice were killed and eyes were excised and processed as described above. According to regulatory constraints, the virally infected animals were permanently housed under BL3 containment and, consequently, fluorescein angiograms could not be performed.

RT-PCR for PAI-1 expression

Total RNA from eyes were extracted using RNeasy Mini Kit (Qiagen, Chatsworth, Calif.) as described by the manufac-
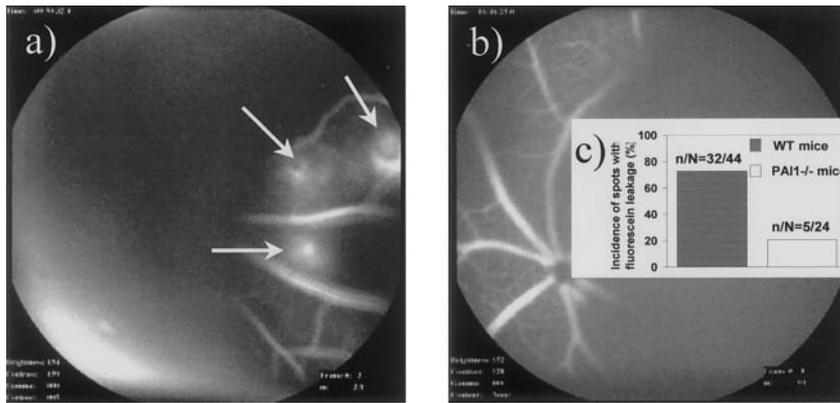
Figure 1. Fluorescein angiograms carried out 14 days after laser-induced rupture of Bruch's membrane. Fluorescein leakage (arrows) is observed from choroidal new vessels in WT (*a*) but not in PAI-1$^{-/-}$ mice showing only normal vasculature (*b*). A threefold reduction in the percentage of leaking spots was obtained in PAI-1$^{-/-}$ mice (*c*); n/N = number of leaking spots/total number of laser burns (X^2=16.8, P<0.0001).

turer. PAI-1 mRNA and 28S rRNA were measured in 10 ng aliquots of total RNA using the GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit (Perkin Elmer, Norwalk, Conn.) and two pairs of primers (Gibco BRL-Life Technologies, Grand Island, N.Y.): 5′-AGGGCTTCATGC-CCCACTTCTTCA-3′ (sense primer) and 5′-AGTAGAGGGC-ATTCACCAGCACCA-3′ (antisense primer) for PAI-1 and 5′-GTTCACCCACTAATAGGGAACGTGA-3′ (sense primer) and 5′-GGATTCTGACTTAGAGGCGTTCAGT-3′ (antisense primer) for 28S. Reverse transcription was performed at 70°C for 15 min, followed by 2 min incubation at 95°C for denaturation of RNA–DNA heteroduplexes. Amplification started by 15 s at 94°C, 20 s at 68°C, and 10 s at 72°C (35 cycles for PAI-1 and 19 cycles for 28S) and terminated by 2 min at 72°C. RT-PCR products were resolved on 10% acrylamide gels and analyzed using a Fluor-S MultiImager (Bio-Rad, Hercules, Calif.) after staining with Gelstar (FMC BioProducts, Rockland, Maine) dye. The expected size is 191 bp for PAI-1 and 212 bp for 28S.

Statistical analysis

Data were analyzed with GraphPad Prism 3.0 (San Diego, Calif.). The χ^2 test, Student's *t* test, one-way ANOVA, and Newman-Keuls post tests were used to determine whether there were significant (P<0.01) differences between WT and PAI-1$^{-/-}$ mice.

RESULTS

Choroidal neovascularization *in vivo* in PAI-1$^{-/-}$ and WT mice

To determine whether the absence of PAI-1 influences choroidal neovascularization *in vivo*, we used a murine model of retinal photocoagulation with an argon laser. The photocoagulation induced trauma at the level of outer retina, retinal pigment epithelium, and Bruch's membrane, giving rise to choroidal neovascularization under the retina similar to that observed in AMD. The damage of the Bruch's membrane was immediately identified by the ophthalmoscopical appearance of a traumatic retinal bubble at the sites of laser burns. Fluorescein angiograms performed on day 14 (**Fig. 1***a*, ***b***) showed the appearance of newly formed microvessels with significant leakage of fluorescein in 72% (32/44) of the laser-induced lesions. The incidence of

leaking spots was reduced to 21% (5/24) in PAI-1$^{-/-}$ mice (Fig. 1*c*, P<0.001). Histological analysis and immunostaining with anti-PECAM and anti-collagen type IV antibodies confirmed the presence of newly formed capillaries in lesions identified by fluorescein angiography (**Fig. 2**). WT mice showed typically large 'mushroom-like' areas of choroidal neovascularization at the site of laser-induced trauma, with migration of retinal pigmented epithelial cells along newly formed microvessels (Fig. 2*a*). The wound was usually not covered by a continuous layer of retinal pigmented epithelial cells. In PAI-1$^{-/-}$ mice, the choroidal neovascular reaction was much more restricted and consisted of only a diffuse and moderate thickening covered by confluent retinal pigment epithelium (Fig. 2*b*). In most instances, these lesions were only indirectly identified by examination of the retinal photoreceptor damage. Immunostaining with anti-type IV collagen or anti-PECAM antibodies failed to show any significant angiogenesis above the scarring tissue (Fig. 2*d*). Neovascularization was estimated by measuring, on serial sections, the maximal height of the lesion above the choroidal layer observed in neighboring intact zones. This was performed by determining the B/C ratio between total thickness of lesions ('B' from the bottom of the choroid to the top of the neovascular area) to the thickness of adjacent normal choroid ('C'). A 45% reduction of the B/C ratio was observed in PAI-deficient mice (P<0.001) as compared to WT mice.

PAI-1 is present in the neovascular area

Immunohistochemical staining demonstrated the presence of PAI-1 exclusively within choroidal neovascular membrane, but not in normal intact zones of WT mice (**Fig. 3***a*). Controls in which the primary antibody had been preabsorbed with recombinant PAI-1 were negative (Fig. 3*b*). No staining was observed at any location in PAI-1$^{-/-}$ mice (Fig. 3*c*). RT-PCR applied on the eyes (Fig. 3*d*) demonstrated, 14 days after injection with adenoviral vector carrying AdCMV*PAI1*, a weak human PAI-1 mRNA expression in PAI-1-deficient mice.

Figure 2. Histological analysis after laser treatment. Heamatoxylin-eosin staining of a representative large area of choroidal neovascularization at the site of laser-induced trauma in WT mice (*a*) and more restricted reaction in PAI-1$^{-/-}$ mice (*b*). Immunofluorescence labeling of new vessels in WT (*c*) or in PAI-1$^{-/-}$ mice (*d*) analyzed 14 days after laser photocoagulation. New vessels were detected with anti-mouse anti-collagen type IV antibody (red) and anti-mouse anti-PECAM antibody (green). R: neural retina; Ch: choroid defined by yellow arrows which could contain normal large vessels not involved in the neo angiogenic process; S: sclera; B: total thickness from the choroid to the top of the neovascular area; C: thickness of the choroid-RPE (retinal pigmented epithelium) complex. B/C: ratio of the thickness from the choroid to the top of the neovascular area, to the thickness of the choroid-RPE complex measured on this section. White arrows localize the laser impact. Original magnification 200×.
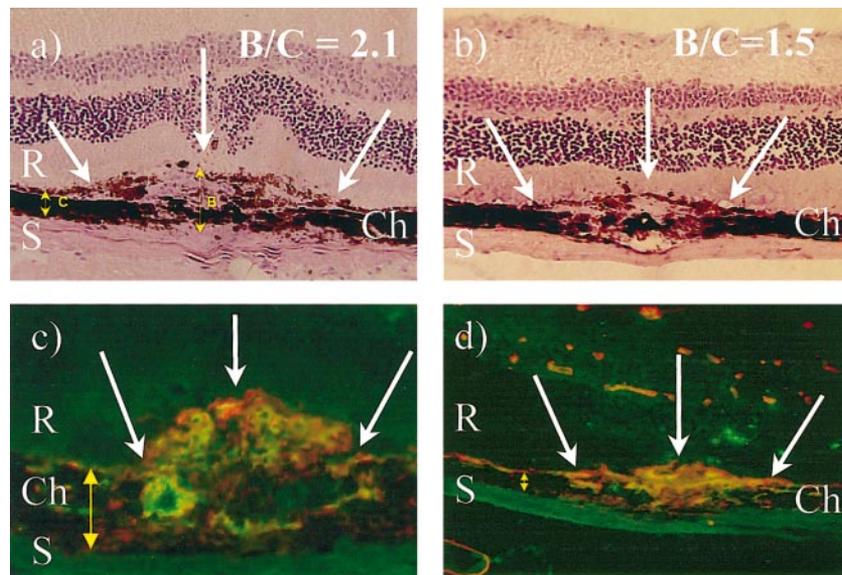
PAI-1 adenovirus injection restores choroidal angiogenesis

To further confirm the role of PAI-1 in choroidal angiogenesis *in vivo*, WT and PAI-1$^{-/-}$ mice were i.v. injected 1 day after laser burns with either a recombinant adenovirus (AdCMV*PAI1*) carrying human PAI-1 cDNA, a control virus (AdRR5), or a vector carrying LacZ cDNA (AdCMV*LacZ*). Immunostaining with anti-type IV collagen and anti-PECAM antibodies demonstrated a significant angiogenesis in PAI-1$^{-/-}$ mice injected with AdCMV*PAI1* (**Fig. 4*a***) but not in PAI-1$^{-/-}$ mice injected with a control virus (Fig. 4*b*). The injection of the virus carrying the LacZ cDNA resulted in the expression of β-galactosidase in the retinal pigmented epithelium (Fig. 4*c*).

This demonstrates that the recombinant adenoviruses were able to transduce the PAI-1 cDNA into cells in close proximity to the burn. Four days after the injection of AdCMV*PAI1*, the measured plasma levels of human PAI-1 were higher in PAI-1$^{-/-}$ mice (mean 7340 ng/ml, range 1850–12600) than the normal murine PAI-1 value in WT mice (2 ng/ml). PAI-1 was undetectable after 2 wk. Quantitation of the neovascularization on frozen sections (Fig. 4*d*) showed that the human gene expression in PAI-1-deficient mice resulted in a neovascular thickness ratio (B/C) 90% of that observed in WT animals. In contrast, neovascularization estimated by the B/C ratio in PAI-1-deficient mice injected with AdRR5 was similar to that observed in PAI-1-deficient animals.

Figure 3. Immunofluorescence labeling and RT-PCR for PAI-1. 14 days after laser treatment, PAI-1 was detected only in the neovascular area of WT mice (*a*) and not in PAI-1-deficient mice (*c*). Immunostaining disappeared in WT animals when the primary antibody was preabsorbed with recombinant PAI-1 (1 μg/ml) (*b*). White arrows localize the laser burn sites. Original magnification 200×. RT-PCR analysis (*d*) identifying the PAI-1 mRNA expressed in the eyes of WT mice (lane 2) and of PAI-1-deficient mice injected with AdCMVPAI1 (lane 3). Lane 1 represents molecular weights, lane 4 eyes of PAI−/− mice and lane 5 negative control.
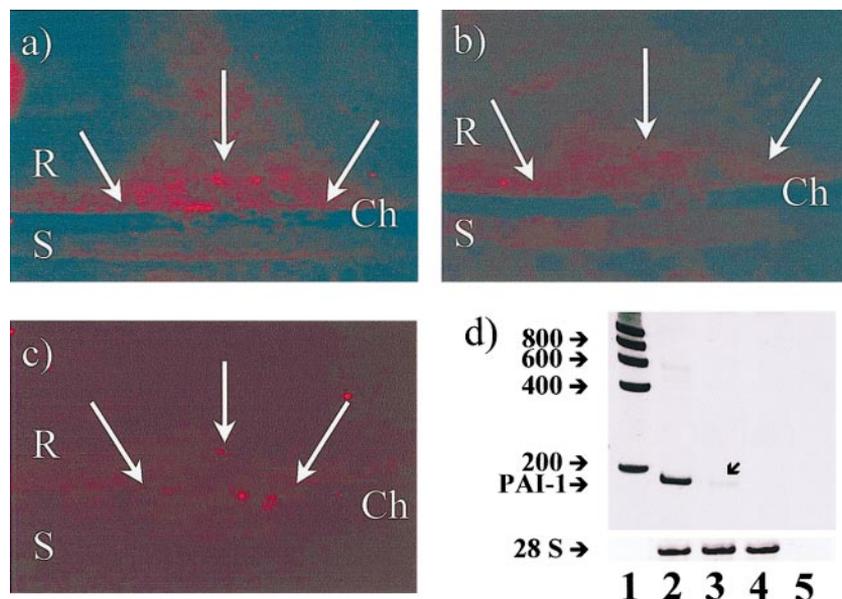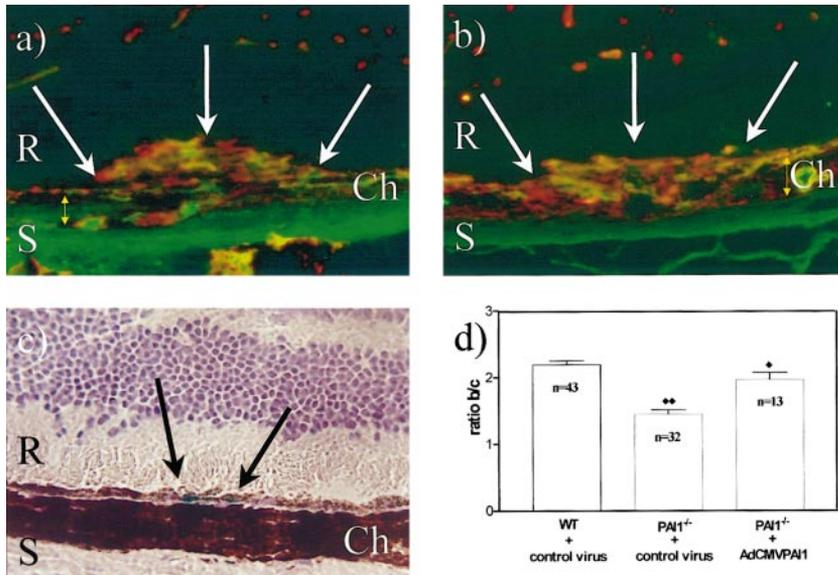
Figure 4. Injection of adenoviral vectors after laser photocoagulation. In PAI-1$^{-/-}$ mice injected with adenovirus bearing human PAI-1 cDNA (*a*), immunostaining of new vessels showed the same behavior as that found in WT mice injected with a control virus (not shown), whereas PAI-1$^{-/-}$ injected with a control virus failed to demonstrate significant neovascularization (*b*). LacZ expression (in blue) was detected in the retinal pigment epithelium (arrows) in mice injected with adenovirus bearing β-galactosidase cDNA (*c*). Quantitation of the neovascularization by the B/C ratio demonstrated a significant reduction in PAI-1−/− mice injected with a control virus (*t* test, $P<0.001$) while PAI-1$^{-/-}$ mice injected with AdCMV*PAI1* had a B/C ratio (*t* test, $P=0.06$) similar to that calculated in WT mice (*d*). *n* represents the number of different lesions analyzed. R: neural retina; Ch: choroid defined by yellow arrows that could contain normal large vessels not involved in the neo-angiogenic process; S: sclera.

DISCUSSION

Angiogenesis represents an invasive cellular process requiring the functional activity of a variety of molecules such as growth factors, extracellular matrix proteins, adhesion receptors, and proteolytic enzymes (23). MMPs and serine proteases have been implicated in the extracellular matrix remodeling associated with neo-angiogenesis. Since most MMPs are secreted as zymogens, their activation requires a limited proteolysis by plasmin. Neovascularization requires therefore a delicate balance between the activation of the serine and metalloproteases and their inhibition by specific inhibitors. The specific roles of MMPs, plasminogen activators, and inhibitors in neovascular chorioretinopathies remain largely unexplored.

Previous clinical studies detected measurable concentrations of tPA and PAI in the aqueous humor of normal eyes (24, 25). An increase in Bruch's membrane TIMP-3 (26) and interphotoreceptor matrix MMP-2 (27) has been associated with age-related macular degeneration. In the vitreous fluid of diabetic patients suffering from proliferative retinopathy, elevated concentrations of ProMMP-9 (28), tPA, and PAI-1 (29) were recently reported to be associated with high VEGF levels. As PAI-1 and TIMP-3 inhibit MMPs activation, it might have been anticipated that they reduce choroidal angiogenesis. The apparent paradox of increased tissue levels of PAI and TIMP-3 in choroidal pathology associated with neovascularization suggests that their contribution to the angiogenic ocular disorders could be different from that anticipated. We reported recently that PAI-1 is a key proangiogenic molecule during tumorigenesis and that PAI-1 deletion results in the absence of tumor formation in an animal model of squamous cells carcinoma (15).

We demonstrate here that PAI-1 plays an important role in choroidal neovascularization. In a model of laser-induced choroidal neovascularization, angiogenesis detected by fluorescein angiography and neovascular volume appreciated by immunohistochemistry and quantitative histology were reduced in PAI-1-deficient animals. Furthermore, restoration of PAI-1 expression in these mice by injection with recombinant adenoviruses bearing human PAI-1 cDNA led to a choroidal neovascularization identical to that observed in WT animals. It could be argued that the inflammatory reaction may play a role in the results involving adenoviral delivery. This is unlikely, as control and lacZ viruses were inefficient. Quantification of inflammatory cells in the lesions failed to demonstrate any significant difference (data not shown).

Although it has been suggested in a model of retinopathy of prematurity that up-regulation of endogenous PAI-1 could protect from retinal and choroidal neovascularization (30), in accordance with clinical observations our results suggest, paradoxically, the opposite effect and show that PAI-1 expression is necessary for choroidal angiogenesis.

Taken together with results observed previously in tumoral models, our observations confirm the role of PAI-1 in the development of pathological angiogenesis. The effect of PAI-1 is indeed restricted to tumoral and ocular pathological neovascularization but is not observed in placentation, embryo-development, and wound-healing reaction (T. Frandsen, unpublished observations), which are essentially normal in PAI-1-deficient mice.

Although the exact mechanism of action of PAI-1 remains to be elucidated, at least three different hypothesis can be formulated. PAI-1 could prevent excessive matrix degradation against uPA-mediated degradation, thereby providing a cell adhesion substrate for endothelial cell migration. It has been shown *in vitro* that excessive proteolysis prevents the coordinated assembly of endothelial cells into capillary shoots (31).

PAI-1 could also be considered as the molecular switch that governs uPAR- and/or integrin-mediated cell adhesion and release (32). Finally, through the inhibition of plasmin, uPA, and tPA, PAI-1 could promote angiogenesis by reducing the angiostatin generation from plasminogen (33). The use of adenovirus with mutated PAI-1 forms modulating these different pathways is one of the strategies that could give new insight into the mechanisms of PAI-1 action in angiogenesis.

Laser-induced choroidal neovascularization in mice is useful for basic investigation of choroidal angiogenesis, although it probably differs from that occurring naturally in human AMD. Our observations nevertheless emphasize the essential role of PAI-1 in the development of subretinal neovascularization and identify PAI-1 as a potential target for therapeutic retinal anti-angiogenic strategies. FJ

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