INFLUENCE OF PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 ON CHOROIDAL NEOVASCULARIZATION

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A- ABSTRACT

The importance of plasminogen activator inhibitor 1 (PAI1) for angiogenesis was recently demonstrated in cancer. To evaluate the biological relevance of PAI1 in subretinal angiogenesis, we have induced choroidal neovascularization *in vivo* with argon laser burns into PAI1-deficient ($PAI1^{-/-}$) and wild-type (WT) mice.

The incidence of choroidal newvessels was evaluated on fluorescein angiograms. The volume of the neovascular membranes immunostained with anti-CD31 was estimated on cryosections. PAI1-deficient mice were also injected with either recombinant adenoviral vector bearing the human gene (AdCMV*PAI1*), with adenoviral vector bearing ß-galactosidase gene (AdCMV*lacZ*) or with AdRR5 (control). PAI1 expression was evaluated by RT-PCR.

A choroidal neovascular membrane with leakage on fluorescein angiograms was produced in 72% of laser impacts in WT mice but only in 21% of impacts in PAI1-deficient mice ($p<0.001$). PAI1 protein was not detected in intact chorioretinal tissue. It was present in choroidal newvessels produced in WT mice but not in $PAI1^{-/-}$. In these PAI1-deficient mice, the volume of choroidal neovascularization, when present, was reduced by 45 % ($p<0.001$). The WT pattern of choroidal neovascularization was restored when systemic and local PAI1 expression was achieved by injecting a viral vector bearing the human gene (AdCMV*PAI1*). ß-galactosidase was expressed in the RPE. Controls injected with AdRR5 demonstrated neovascular pattern similar to PA1-deficient mice.

These observations emphasize the essential role of PAI1 in the occurrence of subretinal neovascularization and contribute to explain the multifunctional aspects of PAI1 in angiogenesis.

B- MATERIALS AND METHODS

Mice
Homozygous PAI1-deficient mice ($PAI1^{-/-}$) 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. Groups were composed of 5-10 mice.

Quantitative analysis 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). Microscopic images (mag x200) of haematoxylin-stained eye section were digitalized and analysed. Frozen serial sections were cut throughout the entire extent of each burn, and the thickest lesions (minimum of 3/lesion) used for the quantitation studies. 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). Due to the small size of most lesions, that method was preferred to surface estimation for its independence in relation to oblique sections.

Immunofluorescence
Cryostat sections (5 µm in thickness) were fixed in acetone at –20 °C and in methanol at 4 °C and then incubated with the primary antibodies. Antibodies raised against PECAM (rat monoclonal antibody, PharMingen, diluted 1/20), or type IV collagen (guinea pig polyclonal antibody produced in our lab; diluted 1/100) were incubated for 1 hr at room temperature, whereas antibodies to PAI1 (rabbit polyclonal antibody produced in our laboratory, 10 µg/ml) were incubated overnight at 4 °C. The sections were washed in phosphate buffered saline (PBS) (3 x 10 min) and then 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; 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 3 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. The X-gal staining was performed with 5-bromo-4-chloro-3-indolyl-β-galactopyronoside (X-gal) as described by *Behringer et al., Development, 117, 823-833, 1993.*

Adenovirus-mediated *PAI1* gene transfer
Recombinant adenovirus bearing human *PAI1* c DNA (AdCMV*PAI1*), *Escherichia coli* β-galactosidase (AdCMV*lacZ*), control adenovirus (AdRR5) were i.v. injected 24 hrs after laser spot production (7 x 10^8 PFU). After five days, blood was sampled from the right retroorbital sinus and PAI1 antigen was measured as reported. On day 14, mice were killed and eyes were excised and processed as described above.

RT-PCR for PAI1 expression
Total RNA from eyes were extracted using RNeasyMini Kit (QIAGEN) as described by the manufacturer. PAI1 mRNA and 28S rRNA were measured in 10ng aliquots of total RNA using the GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit (Perkin Elmer) and two pairs of primers (Gibco BRL- Life Technologies) : 5'–AGGGCTTCATGCCCCACTTCTTCA-3' (sense primer) and 5'–AGTAGAGGGCATTCACCAGCACCA-3' (antisense primer) for PAI1 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 sec at 94°C, 20 sec at 68°C and 10 sec at 72°C (35 cycles for PAI1 and 19 cycles for 28S) and terminated by 2 min at 72°C. RT-PCR products were resolved on 10% acrylamide gels and analysed using a Fluor-S MultiImager (BioRad) after staining with Gelstar (FMC BioProducts) dye. The expected size is 191 bp for PAI1 and 212 bp for 28S.

C- CONCLUSIONS

By using a model of laser-induced choroidal neovascularization, we provide evidence that PAI1 plays an important role in choroidal neovascularization:

1) Angiogenesis was reduced in PAI1-deficient animals as compared to WT mice.

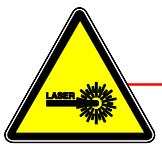
2) Restoration of PAI-1 expression in PAI-1 deficient mice by injection of recombinant adenoviruses bearing human PAI1 cDNA led to a choroidal neovascularization identical to that observed in WT animals.

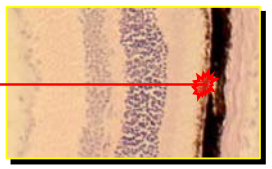
Although it has been suggested that upregulation of endogenous PAI1 could protect from retinal and choroidal neovascularization, our results, in accordance with clinical observations suggest paradoxically the opposite effect and provide evidence that **PAI1 expression is necessary for choroidal angiogenesis.**

These data confirm the essential role of PAI-1 in pathological angiogenesis. We indeed previously demonstrated that PAI1 is a key **proangiogenic molecule** during tumorigenesis and that its absence impairs tumor formation in an animal model of squamous cells carcinomas (*Bajou et al, Nature Medicine, 4, 923-928, 1998*).

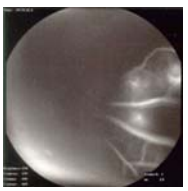
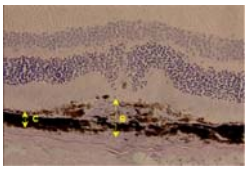
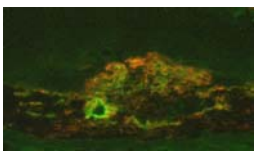
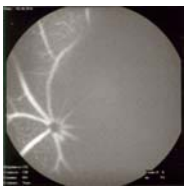
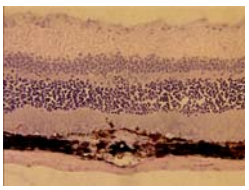
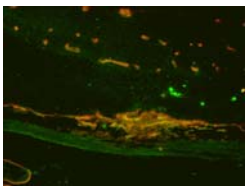
These observations identify PAI1 as potential target for therapeutic retinal anti angiogenic strategies.

MODEL OF CHOROIDAL VASCULARIZATION: Green Argon Laser Impacts.



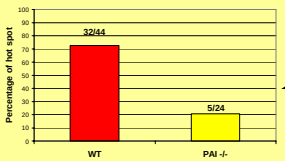


532 nm
50 µm ∅
0.05 sec
400mW

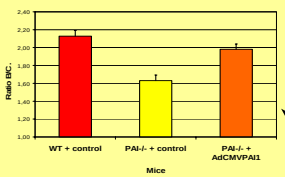
	Fluorescein angiogram.	Hematoxylin/eosine.	CD31 (green)/Coll. IV (red).
WT			
PAI -/-			

A choroidal neovascularization with fluorescein leakage was observed in 72 % of laser impacts in WT mice and in only 20 % of PAI-1 -/- mice.

Fluorescein angiogram results.

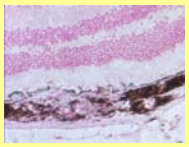


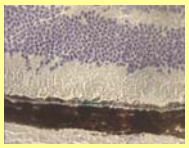
Quantification of vascularization.



B = the thickness from the bottom of the choroid to the top of the neovascular area.
C= thickness of intact adjacent choroid.

X-gal staining after injection of control AdCMV*lacZ* adenovirus.



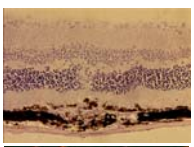


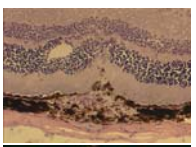
Local expression of the transgene in the retinal pigmented epithelium

Restoration of PAI-1 expression in PAI-1-/- mice by adenovirus transfer of human cDNA PAI-1 (AdCMV*PAI1*).

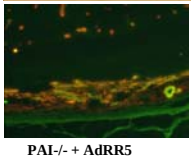
	WT + AdCMV*lacZ*	PAI-/- + AdCMV*lacZ*	PAI-/- + AdCMV*PAI1*
Mean:	52.1 ng/ml	32.9 ng/ml	7339.6 ng/ml
Range:	38.8- 65.4 ng/ml	14.4-60 ng/ml	1846.8-12608.0 ng/ml

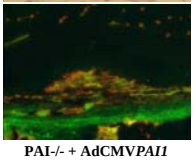
Hematoxylin/eosine :





CD31 (green)/Coll. IV (red) :





PAI-/- + AdRR5 | PAI-/- + AdCMV*PAI1*

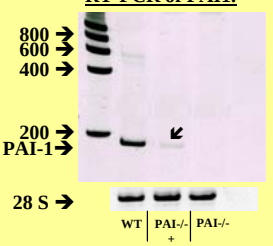
*When PAI-1 deficiency was circumvented by i.v. injection of adenoviral vector bearing human cDNA PAI-1 (*AdCMV*PAI1*), *neovascularization induced by laser was retored.*

Immunostaining of PAI1.

WT

PAI-1 is expressed in choroidal neovascular area in WT mice.

RT-PCR of PAI1.



PAI-1mRNA re-expression in PAI -/- injected with AdCMVPAI1.