MMP-9 contributes to choroidal neovascularization

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A- ABSTRACT

Age-related macular degeneration (AMD) is the primary cause of irreversible photoreceptors loss in adult patients and current therapies are limited. Increased levels of matrix metalloproteinases (MMPs) have been documented in neovascularization of severe ocular pathologies such as AMD and proliferative diabetic retinopathy. We report here that MMP-9 (gelatinase B) expression is induced and temporally regulated in the course of experimental choroidal neovascularization. We used transgenic mice expressing β-galactosidase reporter gene under the dependence of MMP-9 promoter and RT-PCR analysis on choroidal neovascular structures microdissected from serial sections by laser pressure catapulting to show that MMP-9 expression is upregulated concomitantly with the appearance of inflammatory cells in the subretinal lesion. In mice deficient in MMP-9 expression the development of choroidal neovascularization was reduced.

B- MATERIALS AND METHODS

Genetically modified mice (LacZ transgene & MMP-9-deficient)
Construction of mouse line 7700Exln-LacZ, which contains 7.7 kb of the 5'-flanking region and the first exon and intron of the MMP-9 gene linked to a β-galactosidase gene, was previously described (Munaut JBC 99). Promoter activity in these transgenic line closely parallels the activity of the endogenous MMP-9 gene during embryonic development. Homozygous 7700Exln-LacZ mice for the transgene were mated together to generate new progeny. Expression of the transgene was performed on 2% paraformaldehyde – 0.2% glutaraldehyde fixed tissues (15 to 30 min), washed three times in PBS and stained with buffered 5-bromo-4-chloro-3-indolyl-β-galactopyronoside (X-Gal) solution as described (Behringer Dev 93). As a positive control for MMP-9 expression, a thermal injury was applied on a few mice corneas (Mohan JBC 98).
Homozygous MMP-9-deficient mice (MMP-9$^{-/-}$) and the corresponding WT mice (MMP-9$^{+/+}$) of either sex were used in experiments in which neovascular membranes were quantified (Vu Cell 98). All the animals used in this study were maintained with a 12-h light/12-h dark cycle and had free access to food and water.

Murine model of laser-induced choroidal neovascularization
Animal experiments were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. Choroidal neovascularization was induced in mice by four burns (usually at the 6, 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 sec duration; 400 mW) as previously described (Campo). Animals (five or more in each group) were sacrificed at day 2,3,5,10,14 and 28 for the evaluation of the kinetic of MMP-9 expression, and at day 14 in the experiment involving MMP-9$^{-/-}$ and WT mice comparison. In the later group, before sacrifice, fluorescein angiograms (intraperitoneal injection of 0.3 ml of 1% fluorescein sodium –Ciba) were performed to evaluate the percentage of laser burns developing late phase hyperfluorescent spots (corresponding to the leakage of fluorescein from newly formed permeable capillaries). The eyes were then enucleated and either fixed in buffered 3.5% formalin solution for routine histology or embedded in Tissue TeK (Miles Laboratories, Naperville, Illinois) and frozen in liquid nitrogen for cryostat sectioning. Choroidal neovascularization was quantified as previously described (Lambert bis). Briefly, frozen serial sections were cut throughout the entire extent of each burn, and the thickest lesions (minimum of 3/lesion) used for the quantitation. Using a computer-assisted image analysis system (Olympus Micro Image version 3.0 for Windows 95/NT, Olympus Optical CO. Europe GmBH), 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, its reproducibility and correlation with fluorescein angiograms results.

Laser pressure catapulting (LPC) and RT-PCR
8 to 10 serial frozen sections were mounted directly onto a 1.35 m thin polyethylene foil (PALM, Wolfratshausen, Germany). The supporting membrane was mounted onto the glass slide using the Microbeam-MOMeNT technique (Böhm 1997). The membrane-covered slides can be stored at room temperature until needed. The Robot-Microbeam (PALM®) focused the laser (337nm) on the specimen with appropriate energy settings enabling the catapulting of the entire selected area into the microfuge cap. The entire subretinal choroidal neovascularization area and an adjacent intact chorioretinal zone (control) were microdissected separately on frozen sections (10 µm thick) at selected intervals after laser burn (day 3, 5, 10, 14 and 40). The specimens were covered with 100 µl lysis buffer and total RNA isolation was performed with the PUREscript RNA-isolation Kit (BIOzym, Landgraaf, The Netherland) according to the manufacturer's protocol. Total RNA was dissolved in a 10 µl RNA hydratation solution supplied by the manufacturer. 28S rRNA were amplified with an aliquot of 1 µl of total RNA using the GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit (Perkin Elmer) and three pairs of primers (Gibco BRL- Life Technologies) : (sense: 5'-GTTCACCCACTAATAGGGAACGTGA-3' and reverse: 5'-GGATTCTGACTTAGAGGCGTTCAGT-3'for 28S mRNA, sense: 5'-agacctgaaaacctccaacctcac-3' and reverse: 5'-tgttatgatggtcccactgaggc-3' for MMP-9, and sense: 5'-AGATCTTCTTCTTCAAGGACCGGTT-3' and reverse: 5'-GGCTGGTCAGTGGCTTGGGGTA-3' for MMP-2). 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 for MMP-2 and 28S (45 cycles for MMP-2 and 30 cycles for 28S) or by 15 sec at 94°C, 20 sec at 60°C and 15 sec at 72°C for MMP-9 (45 cycles) and terminated by 2 min at 72°C. RT-PCR products were resolved on 10% acrylamide gels and analysed using a Fluor-S Multilmager (BioRad) after staining with Gelstar (FMC BioProducts) dye. The expected size for RT-PCR products were respectively 212bp for 28S, 225bp for MMP-2 and 262bp for MMP-9.

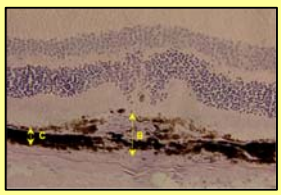
C- CONCLUSIONS

The collagenase **MMP-9** may **play a role in laser-induced choroidal neovascularization**, a model similar to the exsudative most sight-devastating form of human AMD.
Two approaches (β-galactosidase reporter gene under the dependence of MMP-9 promoter and RT-PCR on LPC products) cooperatively demonstrate that the **local expression of MMP-9 mRNA** is restricted to **day 5** after laser burn. This **coincides** perfectly with the **appearance of mononuclear cells** within the neovascular reaction and could identify the inflammatory cells as a predominant provider of MMP-9.
At the **protein level**, MMP-9 was immunohistochemically **localized in the laser-induced lesion** already at **day 3** and before local mRNA expression. Since a similar observation was done with the corneal positive control, it can be suggested that MMP-9 probably first **arised from early recrutment and degranulation of neutrophils before a transcriptional induction of its local expression.**
Angiogenesis appreciated by immunohistochemistry and quantitative histology was **significantly reduced in MMP-9-deficient animals** compared to WT controls.

These data indicate that **MMP-9 contributes to choroidal neovascularization in the murine laser-induced model.**

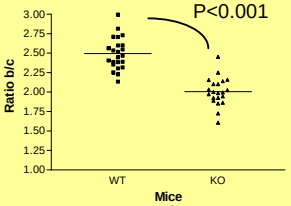
A combination of MMP-9 inhibitors with other anti-angiogenic agents may be a promising strategy.

MMP-9 KO Mice neovascularization



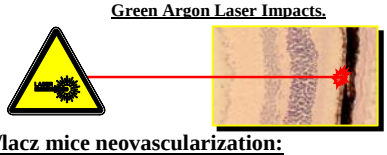
B = the thickness from the bottom of the choroid to the top of the neovascular area.
C= thickness of intact adjacent choroid.

Quantification of neovascularization.



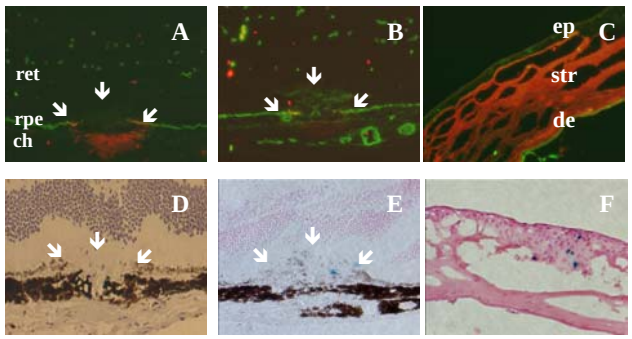
Angiogenesis is significantly reduced in MMP-9 deficient animals compared to WT.

MODEL OF CHOROIDAL VASCULARIZATION:
Green Argon Laser Impacts.



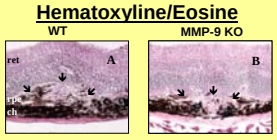
532 nm
50 µm ∅
0.05 sec
400mW

MMP-9/lacz mice neovascularization:

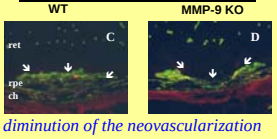


MMP-9 protein is present in an early stage (day 3 post-impact,A) around the laser burn. The β-galactosidase expression does not strictly colocalized with the MMP-9 protein. A &D : day 3; B&E day 5; C&F control in cornea.

Hematoxyline/Eosine

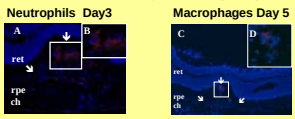


CD31(red)/Coll. IV(green)



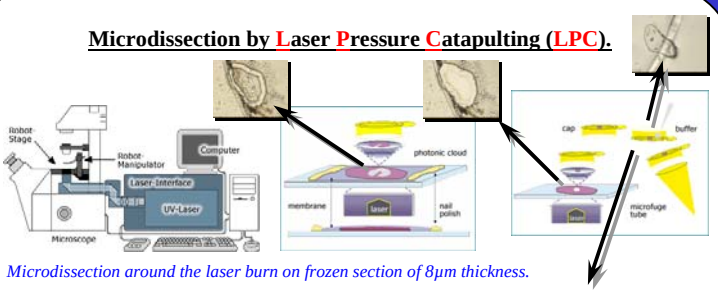
A diminution of the neovascularization Is observed in the MMP-9 KO mice.

Inflammatory cells (red)



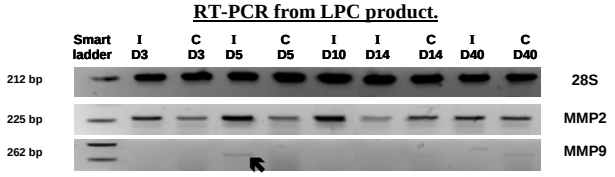
Neutrophils appear in the impact at day 3 (A,B) and desappear at day 5. But mononuclear inflammatory cells were absent in the choroid at day 3, and appeared in the impact at day 5 (C,D).

Microdissection by Laser Pressure Catapulting (LPC).



Microdissection around the laser burn on frozen section of 8µm thickness.

RT-PCR from LPC product.



MMP-2 and MMP-9 expression are differentially modulated during the progression of choroidal neovascular reaction in a murine laser-induced model. I: Impact zone; C: retinal Control zone; D : Day