MMP-2 and MMP-9 synergize in promoting choroidal neovascularization[1]

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SPECIFIC AIMS

Previous studies have shown that the production of gelatinase (MMP-2 and MMP-9) belonging to the matrix metalloproteinase family is increased in human choroidal neovascularization (CNV) occurring during the exudative most aggressive form of age-related macular degeneration (AMD). To more precisely delineate the respective roles of MMP-2 and MMP-9 in choroidal neo-angiogenesis, we investigated their expression and activities in the course of laser-induced murine choroidal neovascularization,. This model was applied to single (MMP-9 KO, MMP-2 KO) or double (MMP-2,9 KO) -deficient mice and to their corresponding wild-type (WT) controls.

PRINCIPAL FINDINGS

1. MMP-2 and MMP-9 are produced in lesions associated with choroidal neovascularization

Gelatin zymography analysis of ocular posterior segments demonstrated that both MMP-2 and MMP-9 were increasingly produced during the early stages of CNV formation, with the appearance of active forms of MMP-2. In situ zymography revealed a predominant gelatinase activity in the CNV area.

2. Different regulation of MMP-2 and MMP-9 expression

RT-PCR evaluation showed that MMP-9 expression was up-regulated during early phases of CNV formation whereas MMP-2 was constitutively expressed without any transcriptional modulation. MT1-MMP mRNA was concomitantly up-regulated, suggesting that the presence of active MMP2 forms was due to the expression and activity of its activator.

3. Severe inhibition of choroidal neovascularization in MMP-2,9 double-deficient mice

Fluorescein angiography performed before death (**Fig. 1*F***) showed a significant reduction ($P<0.001$) in the number of leaking spots (corresponding to newly formed immature microvessels with leakage of fluorescein) in MMP-2,9 double KO mice compared with MMP-2- or MMP-9-deficient mice. This was associated with a strong inhibition of neovascular progression estimated on day 14 after induction by immunostaining with anti-PECAM antibodies in combined KO mice (Fig. 1*D*) as compared with each single-deficient mouse (Fig. 1*B, C*) or WT (Fig. 1*A*). Choroidal lesion associated with neovascularization was then quantified by determining the B/C ratio between total lesion thickness (B, maximal height lesion measured from the bottom of the choroid to the top of the neovascular area) to the thickness of adjacent normal choroid (C). A significant reduction of the B/C ratio was observed in MMP-9 (33%), MMP-2 (44%), and MMP-2,9 (56%) -deficient mice vs. their corresponding WT ($P<0.001$, Fig. 1*E*).

4. Fibrinogen/fibrin accumulation in double MMP-2,9-deficient animals

Consistent with the modulation of fibrinolysis by matrix metalloproteinase system, we observed by immunohistochemical staining that fibrinogen/fibrin accumulated in double MMP-2,9-deficient animals. In contrast, similar fibrinogen/fibrin deposits were found in WT and single gene-deficient mice. These findings suggest that the absence of both gelatinases impaired fibrinolytic activity in choroidal neovascular membrane.

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[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.03-0113fje; doi: 10.1096/fj.03-0113fje

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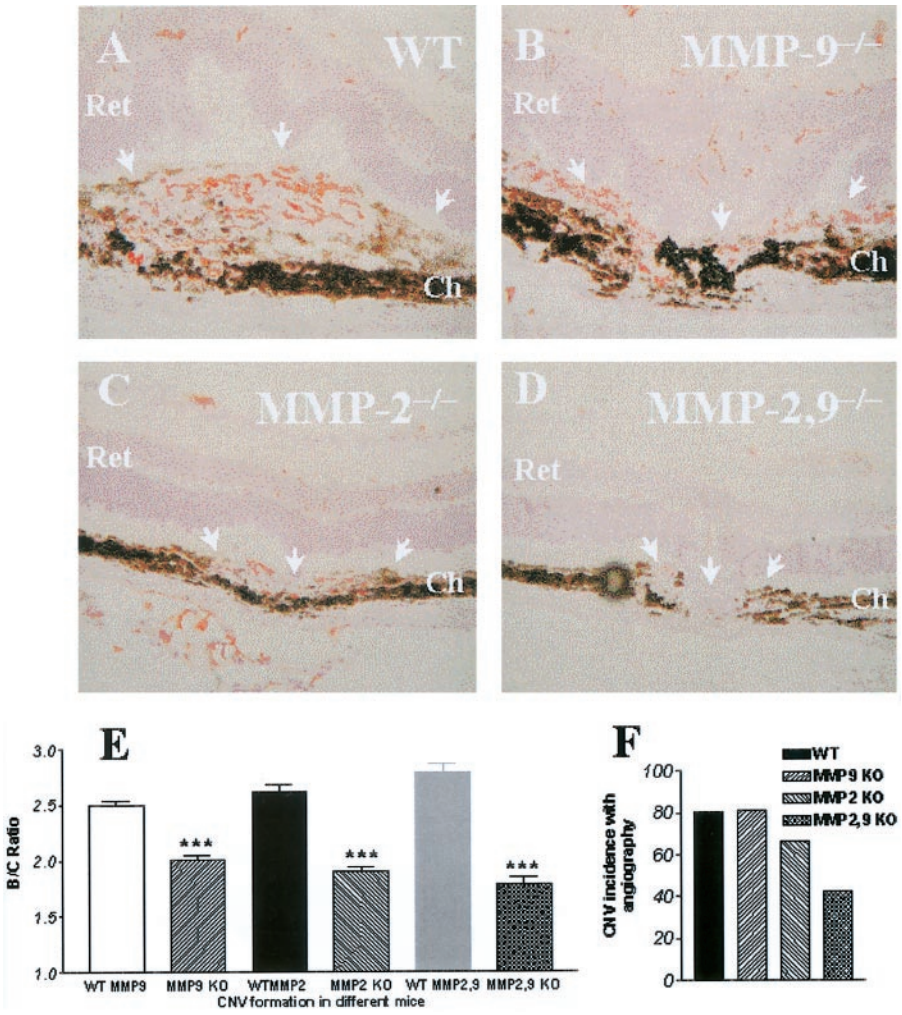

Figure 1. Absence of MMP-2 and MMP-9 prevents the development of experimental choroidal neovascularization. Hematoxylin-eosin staining of a representative area of choroidal neovascularization at the site of laser-induced trauma in control (*A*) or in mice deficient for MMP-9 (*B*), MMP-2 (*C*), and both MMP-2,9 (*D*). An almost complete absence of neovascularization is visible in mice deficient for both MMP-2 and MMP-9 when vessels were immunostained with anti-mouse PECAM antibody (immunostained in orange with AEC) compared with other conditions, thereby confirming the reduced incidence of neovascularization calculated before death by fluorescein angiography evaluation of the number of leaking laser spots (*F*). The neovascular reaction was determined with computer-assisted image analysis by evaluating the B/C ratio on day 14 after laser injury of the Bruch's membrane in single/double-deficient mice and in the corresponding WT controls (*E*). The neural retina (ret) and choroidal layer (ch) are indicated; neovascular area (arrows). ***$P < 0.001$; error bars = SE. Original magnification, 200×.

5. MMP inhibitors decrease the development of CNV

In a second approach to assess whether MMPs contribute to CNV development, we first induced endogenous overexpression of TIMP-1 or TIMP-2 by adenoviral-mediated delivery in WT mice. Both TIMP-1 and TIMP-2 overexpression significantly reduced choroidal angiogenesis ($P<0.001$) compared with WT controls injected with control viruses (AdRR5). We then evaluated the effects of broad spectrum (BB-94) or more selective MMP inhibitors (Ro 28-2653 inhibiting preferentially MMP-2, MMP-9, and MT1-MMP) on CNV development by treating WT mice with daily systemic injections. Both inhibitors significantly reduced CNV formation. However, Ro-28-2653 was significantly more efficient ($P<0.001$) than BB-94. Selective MMP inhibition treatment begun 5 days after laser induction also significantly inhibited the development of choroidal angiogenesis (40% inhibition).

CONCLUSIONS AND SIGNIFICANCE

Several lines of evidence support cooperation between MMP-2 and MMP-9 in the course of experimental choroidal neovascularization. First, both gelatinases are increasingly processed and concentrated in the region of CNV development. Second, choroidal pathological angiogenesis is almost fully prevented in MMP-2/MMP-9 double-deficient mice but only partly impaired in the single MMP-deficient mice. Third, choroidal angiogenesis was strongly inhibited in mice treated with a selective gelatinase/MT1-MMP synthetic inhibitor. In addition, we provide evidence that MMP-9 expression in the course of CNV development was transcriptionally regulated while MMP-2 could be regulated by zymogen activation as the result of an overexpression of its main activator, MT1-MMP.

Although these results support the concept that MMPs play a key role during the early phases of choroidal neovascularization, our understanding of their function in CNV is far from complete and more knowledge is needed before potential clinical applications for AMD treatment. Of interest is the finding that a synthetic inhibitor interacting preferentially with MMP-2, MMP-9, and MT1-MMP inhibited more efficiently choroidal neovascularization than a broad-spectrum synthetic inhibitor. This suggests that a broad-spectrum inhibitor might repress some beneficial MMP effects. Indeed, it appears that MMPs might have dual

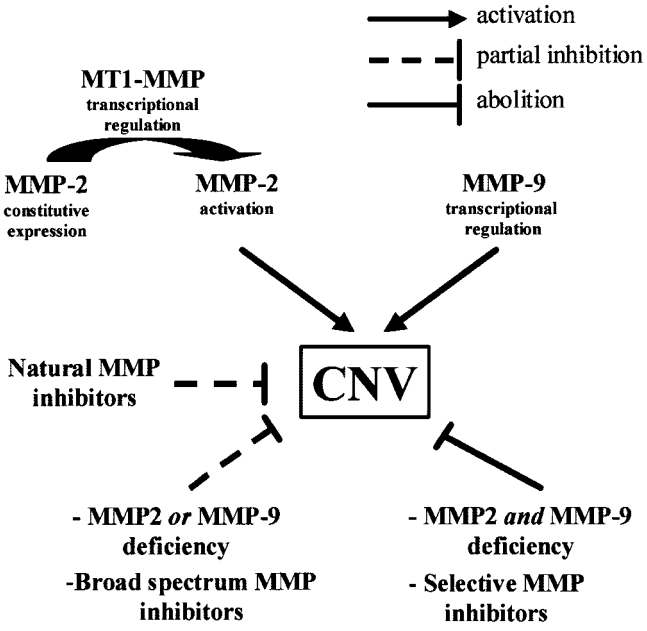

Figure 2. Schematic representation of putative functions of gelatinase, MT1-MMP, and MMP inhibitors in CNV formation. CNV development is associated with an up-regulation of MMP-9 at a transcriptional level and an activation of pro-MMP-2 by MT1-MMP. This process can be inhibited by physiological (TIMP-1 and TIMP-2) and synthetic inhibitors, impaired in single MMP-deficient mice and abolished in double gelatinase KO mice.

functions and that other members of the complex MMP family could limit the spatial extension of the neovascular disease (**Fig. 2**). Finally, our observation that selective MMP inhibition was efficient even when treatment started 5 days after laser burn suggests that MMP inhibitors might have a potential interest for neovascular regression. This is a crucial question in the clinic, since most patients affected by the exudative form of AMD present at a late stage when the neovascular membrane is already developed.

In addition, the observation of a synergy between MMP-2 and MMP-9 might be of interest for other pathological conditions associated with angiogenesis such as tumoral development. FJ