·Basic Research·

The role of MMP-9 and TIMP-3 in induction of choroidal neovascularization in a murine model

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Foundation items: National Natural Science Foundation of China (No.60378040) and Foundation of Scientific and Technical Committee of Shanghai (No.024119055)

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Received:2009-01-21 Accepted:2009-04-29

Abstract

• AIM: To examine the expression of MMP-9 and TIMP-3 mRNA during choroidal neovascularization (CNV) in a murine model and to investigate the role of them in the development of CNV.

• METHODS: CNV was induced in C57BL/6J mice by intensive diode laser (810nm) photocoagulation (120mW, 75μm, 0.1s) of the fundus whereafter eyes were enucleated at 1, 3days, 1, 2, and 4 weeks. The MMP-9 and TIMP-3 mRNA expressions were analyzed using *in situ* hybridization and image analysis system.

• RESULTS: Both expression of MMP-9 and TIMP-3 mRNA had dynamic changes. For MMP-9, the expression was 1, 2, 4 wk>3d>1d ($P<0.05$), whereas TIMP-3 mRNA, 3d, 1, 2, 4 wk>1d ($P<0.05$).

• CONCLUSION: The imbalance between the changes of MMP-9 and TIMP-3 may accelerate the degrading of extracelluar matrix, and then be involved in the pathogenesis of CNV.

• KEYWORDS: matrix metalloproteinase-9;tissue inhibitor of metalloproteinase-3;choroidal neovascularization;mice

Zhang SS, Liao HP, Zhang L, Zhu CH, Zhang Q, Wang L, Wang KS. The role of MMP-9 and TIMP-3 in induction of choroidal neovascularization in a murine model. *Int J Ophthalmol* 2009;2(2):118-122

INTRODUCTION

Choroidal neovascularization (CNV) is one of the serious complications of some macular diseases such as age-related macular degeneration and hypermyopic macular degeneration. The development of CNV can cause some pathological changes, such as exudation, hemorrhage and cicatrization, etc., resulting in severe visual disorder. The mechanism of its formation is to be elucidated.

Angiogenesis is a multi-step event in which new capillaries are formed from preexisting vessels. In the initial stages of angiogenesis, microvascular cells locally degrade the underlying basement membrane and the surrounding extracelluar matrix (ECM), and then vascular endothelial cells proliferate and migrate into the tissue to be vascularized.

Matrix metalloproteinases (MMPs) and their tissue inhibitors of metalloproteinase (TIMPs) are the important factors that affect the metabolism of ECM. In this study, we examined the expression of MMP-9 and TIMP-3 mRNA in a murine model, and to investigate the role of them in the development of CNV.

MATERIALS AND METHODS

Experimental Animal Twelve healthy adult male C57BL/6J mice (supplied by Shanghai Experimental Animals Center, Chinese Academy of Sciences) weighing 25-30g were used in this study.

Experimental CNV Induction After totally dilation of the pupils with 10g/L tropicamide and anesthesia with intraperitoneal injection of 0.3mL 2.5g/L sodium pentobarbital, diode laser (810nm) was delivered into the 20 eyes (10 mice) through slit lamp and contact lens. The laser settings were 120mW intensity, 75μm diameter, and 0.1s duration. Ten burns were delivered around the optic disk. Production of bubbles could be seen at the time of laser treatment, sometimes associated with light sounds. Occasionally hemorrhage could be seen at the sites of photocoagulation. Fundus fluorescein angiography was performed at 1 day, 3 days, 1 week, 2 weeks and 4 weeks after photocoagulation to confirm the development of CNV. The other two mice without any treatment were selected as normal control.

Tissue Processing At 1 day, 3 days, 1 week, 2 weeks and 4 weeks after photocoagulation, the animals were killed and the eyeballs were enucleated, four eyeballs at each time

Table 1 Expression of MMP-9 *vs* TIMP-3 mRNA during the development of CNV (%, $\bar{x}\pm s$)

	1 day	3 days	1 week	2 weeks	4 weeks	*F*	*P*
MMP-9	8.35±2.35	12.34±2.56	24.52±0.84	25.01±2.81	25.13±1.58	56.39	0.0001
TIMP-3	15.84±2.28	38.75±12.98	36.96±4.63	34.88±4.15	36.05±7.27	6.65	0.003

point were used. The eyes were washed with PBS (0.1% DEPC) and embedded in optimum cutting temperature (OCT) compound. After stored in the ultra cold (-80℃), serial sections of 8μm thick were taken using cryomicrotome. Sections including satisfying photocoagulation scars were used and fixed in 40g/L paraformaldehyde, and then stored at -20℃ for preparing of *in situ* hybridization.

***In Situ* Hybridization**

Reagents MMP-9, TIMP-3 *in situ* hybridization kits (Boster Biotechnology, Inc., Wuhan, China).

Oligonnucleotide probe sequence MMP-9: 5-GCTAT CCAGC TCACC GGTCT CGGGC AGGGA-3, 5-GGGAA GACGC ACAGC TCTCC CGCCG AGTTG-3, 5-TAGGT CACGT AGCCC ACTTG GTCCA CCTGG-3; TIMP-3: 5-AGGCT CCAGC TGCCC AGGAG CACGA TGAGC-3, 5-CCTGT CAGCA GGTAC TGGTA CTTGT TGACC-3, 5-AAGCA AGGCA GGTAG TAGCA GGACT TGATC-3.

Procedure Frozen sections were prepared at room temperature for 60 minutes, and digested with pepsin diluted by 30mL/L citromalic acid/DEPC solution at 37℃ for 5 minutes in order to expose the mRNA. After that, the sections were balanced with 2 ×SSC/DEPC at room temperature for 15 minutes. The sections were prehybridized at 38℃ for 4 hours and hybridized at 38℃ for 20 hours. After hybridization, the reaction was blocked with blocking reagent at 37℃ for 30 minutes, then the slides were incubated with biotinylated anti-digoxigenin (DIG) antibody at room temperature for 2 hours. The slides were incubated with streptavidin-biotin-alkaline phosphatase complex (SABC-AP) at 37℃ for 30 minutes, which followed by incubated in coloring reagent containing nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3- indolylphosphate (BCIP) at room temperature. The coloring reaction was controlled under a light microscope for 1-2 hours. These slides were counterstained with nuclear fast red staining and studied by light microscopy. As a negative control, hybridization was performed with prehybridization reagent instead of hybridization reagents. Cells with cytoplasm stained as royal blue were positive ones.

Image Analysis The analysis was performed with KS 400 image analysis software package. Slides of *in situ* hybridization were selected and the images were entered into computer. We selected corresponding areas at the sites of photocoagulation scars, measured total areas and positive expression areas, and then calculated the corresponding positive expression ratio. The gray scale of background was set as 250, and positive area was 160-180. Comparison of positive ratios between different groups was analyzed using one-way analysis of variance (ANOVA) with SAS 6.12 software package (SAS Institute Inc., Cary, NC 27513-2414 USA). Difference was considered significant at $P<0.05$.

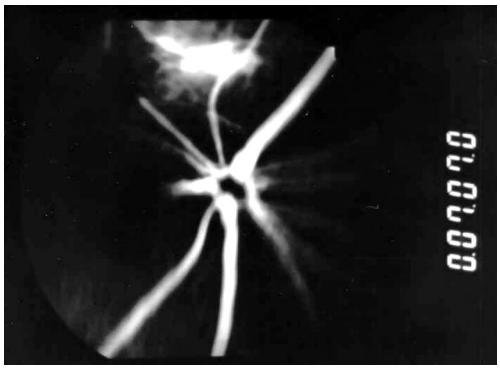

Figure 1 Photograph of FFA 1 week after photocoagulation At 1 week after laser photocoagulation, fluorescence leakage could be seen at the site of photocoagulation

RESULTS

Results of FFA At 1 day and 3 days after photocoagulation, obvious fluorescence leakage could not be detected. At 1 week, 2 weeks and 4 weeks after laser treatment, fluorescence leakage could be seen at many sites of photocoagulation scars, along with the time, the areas of fluorescence leakage expanded and its intensity increased, which proved the development of CNV (Figure 1).

Expression of MMP-9 and TIMP-3 mRNA In normal mouse, only a few expressions of MMP-9 and TIMP-3 mRNA could be seen in choroids and areas near the retinal pigment epithelium (RPE). At 1 day after laser photocoagulation, local elevation of retina and breakdown of integrity of RPE could be observed at sites of photocoagulation, wherein with a small quantity of signals of MMP-9 and TIMP-3 mRNA. At 3 days after laser photocoagulation, the pigment proliferated and became confused. The expression of MMP-9 and TIMP-3 mRNA increased with a different degree. At 1 week after laser photocoagulation, CNV developed and last for at least 4 weeks after photocoagulation. During

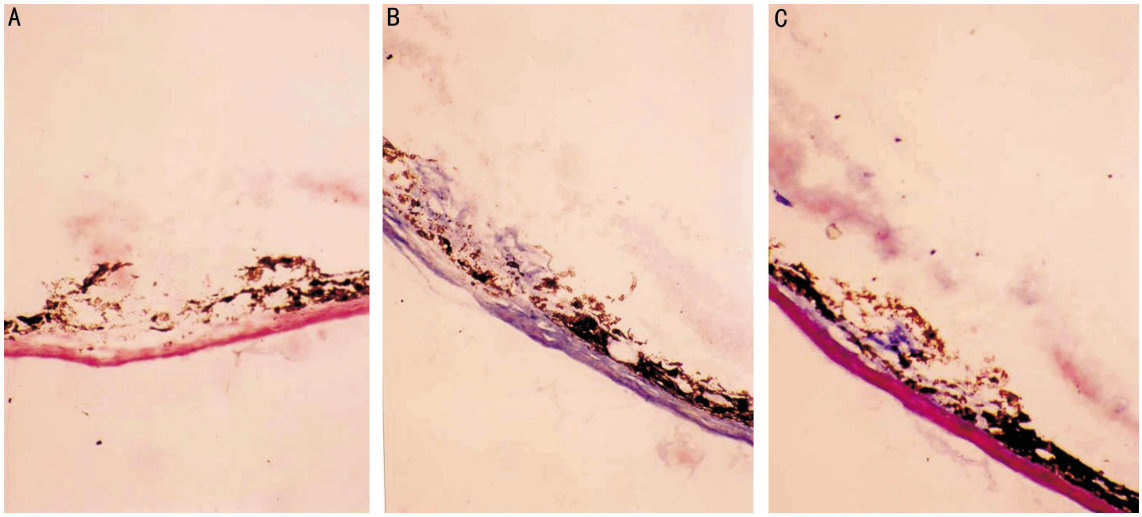

Figure 2 Photography of *in situ* hybridization in CNV tissues A: negative control; B:expression of MMP-9 mRNA (1 week after laser photocoagulation); C: expression of TIMP-3 mRNA (1 week after laser photocoagulation) original magnification×200

this period, pigment was still confused, and spindle-shaped RPE cells covering the CNV tissues could also be seen. Both expressions of MMP-9 and TIMP-3 mRNA were obviously in the areas of CNV and surrounding tissues (Figure 2).

After image analysis using the KS 400 image analysis software package, the positive ratios were calculated and showed in Table 1 and Figure 3.

As a result, both expressions of MMP-9 and TIMP-3 mRNA had a dynamic change during the development of CNV. They all increased significantly in the early stage after laser treatment, and then stabilized at a high level. For MMP-9 mRNA, the expression was 1, 2, 4wk>3d>1d (P<0.05), whereas TIMP-3 mRNA, 3d, 1, 2, 4wk>1d (P<0.05). Namely, the expression of TIMP-3 mRNA became stable at 3 days after photocoagulation, whereas MMP-9 mRNA increased constantly until 1 week after laser treatment.

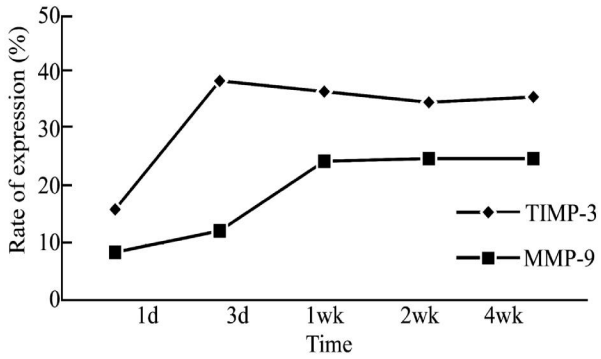

Figure 3 The changes of expression of MMP-9 and TIMP-3 mRNA during the development of CNV

DISCUSSION

MMPs are a group of extracellular enzymes that share a number of common characteristics, such as the presence of zinc and a conserved amino acid sequence at their active sites. They are a highly regulated family of at least 14 structurally related enzymes capable of degrading most, if not all, of the components of the ECM.

The MMPs are grouped into collagenases (MMP-1, 8, 13), gelatinases (MMP-2, 9), stromelysins (MMP-3, 7, 10, 11, 12), and membrane-type matrix metalloproteinase (MMP-14, 15, 16, 17). These enzymes and their inhibitors, TIMPs, are involved in maintaining the dynamic equilibrium of ECM.

Several ocular tissues secrete MMPs and TIMPs in culture, including cornea, trabecular meshwork and RPE. MMPs have also been found in aqueous humor, tears and sclera. MMP-9, the 92kDa gelatinase B, is present in human Bruch's membrane, vitreous and interphotoreceptor matrix (IPM)[1-3].

In order to investigate the possible role of MMPs in CNV, Steen *et al* [4] analyzed the mRNA expression of MMPs in subfoveal fibrovascular membranes from five patients with age-related macular degeneration. They found that MMP-9 expression was distinctly expressed by cells at the margins of the membranes and often in proximity to a thickened Bruch's membrane-like layer under the retinal pigment epithelial cells. It may be an attempt by the RPE cells to reform a Bruch's membrane-like structure around the CNV lesion in an effort to create a physical barrier that inhibits further CNV growth. The presence of MMP-9-expressing cells next to this Bruch's-like layer possibly heralds a

processing collagen IV degradation so that the CNV membrane can grow further into the subretinal space. Das *et al* [5] also observed that levels of MMPs (MMP-2 and MMP-9) in retinas were significantly increased in an animal model with ischemia-induced retinal neovascularization, namely, newborn mice exposed to the variable oxygen cycle. Neovascularization was significantly inhibited (72% reduction) with intraperitoneal administration of a synthetic MMP inhibitor, BB-94 (1mg/kg).

In this study, we found that there was some expression of MMP-9 mRNA in retina of C57BL/6J mouse during the early period after laser photocoagulation. Then the expression increased along with the time until the CNV development at 1 week after laser treatment. As a follow, it would stabilize at a high level. According to these findings, combinating with references showed above, we presume that MMP-9 may play a role in the pathogenesis of retinal neovasculization and CNV by degrading of ECM.

TIMPs are a family of MMP inhibitors thought to act as local regulators of matrix degradation by the MMPs. Recent histological studies have examined the location and expression of the TIMPs in retinal and choroidal tissue. While TIMP-1 could not be found, TIMP-2 was shown to be present in Bruch's membrane and choroid, and TIMP-3 mRNA expression was localized to the RPE and choroidal endothelial cells, and there is general consensus that TIMP-3 in Bruch's membrane is synthesized and secreted by the RPE. TIMP-3 is unique in having a strong affinity for ECM, while the other two are found predominantly in the media from cultured cells [6]. It is suggested that TIMP-3 normally functions for maintenance of the ECM in Bruch's membrane [7]. One role of TIMP-3 in Bruch's membrane may be as a potent local inhibitor of MMP activity, regulating the rate of Bruch's membrane turnover, as well as limiting choroidal neovascularization. In 2000, Takahashi *et al* [8] injected hemagglutinating virus of Japan liposomes containing hemagglutin epitope-tagged TIMP-3 gene into the subretinal space in rat eyes. Three days after transfection of TIMP-3 gene into retinal pigment epithelium cells, intense laser photocoagulation was performed and the incidence of CNV was assessed by FFA. They found that exogenous TIMP-3 mRNA expression in the choroid and retina was detected on day 3. The efficiency of TIMP-3 gene transfection into retinal pigment epithelium cells was greatest on day 7 and decreased gradually thereafter. The incidence of CNV in TIMP-3 gene-transfected eyes was markedly decreased compared with controls (15% *vs* 75%). This study shows that TIMP-3 gene can be transferred into rat retinal pigment epithelium and that TIMP-3 gene overexpression can inhibit development of experimental CNV. This method may represent a future treatment modality for human macular degeneration associated with CNV. Murata also confirmed the possibility of gene therapy (coding for TIMP) for the treatment of CNV [9].

In this study, we found that, although the expression of TIMP-3 mRNA increased after laser photocoagulation, CNV still developed. This may be due to the imbalance between MMPs and TIMPs: a few expressions for both MMP-9 and TIMP-3 mRNA could be detected at 1 day after laser photocoagulation. The expressions increased significantly, especially for the TIMP-3 at 3 days after laser. Along the time, although expression of TIMP-3 mRNA stabilized in a higher level, the expression of MMP-9 mRNA was not inhibited completely, and moreover it still increased. The imbalance between MMP-9 and TIMP-3 breakdown the acceleration inhibition balance of degradation of ECM, and the predominant former may activate the "angiogenic switch"[10]. As a result, the CNV was induced at 1 week after laser photocoagulation. Studies have indicated that an imbalance of MMPs and their inhibitors may be involves in the pathogenesis of ocular diseases such as glaucoma [11,12], corneal diseases [13,14] and proliferative retinopathy [15-17]. Then the new balance rebuilt between MMP-9 and TIMP-3 made the CNV exist for a longer period. In our previous studies [18], we have confirmed that CNV may be induced at 1 week after laser photocoagulation, and the incidences of CNV at 1 week, 2 weeks and 4 weeks were similar.

The mechanisms that trigger release of MMPs and TIMPs during CNV induced by laser photocoagulation are unclear. It is suggested by observations that cytokines such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) may induce production of MMPs and TIMPs by vascular endothelial cells, fibroblasts, and retinal pigment epithelial cells [19]. Majka *et al* [20] also found that TNFα and VEGF had a role in the regulation of extracelluar proteinase expression during retinal neovascularization. The stimulation of TNFα could enhance the production of MMPs in retinal microvascular endothelial cell. VEGF also played a role in this process through its regulation of TNFα-converting enzyme (TACE).

In summary, both MMP-9 and TIMP-3 play a role during the development of CNV in the murine model. It is the imbalance between the changes of MMP-9 and TIMP-3 that accelerates the degradation of ECM, and then is involved in the pathogenesis of CNV.

REFERENCES

1 Guo L, Hussain AA, Limb GA, Marshall J. Age-dependent variation in metalloproteinase activity of isolated human Bruch's membrane and choroid. *Invest Ophthalmol Vis Sci*1999;409(11):2676-2682

2 Plantner JJ, Smine A, Quinn TA. Matrix metalloproteinases and metalloproteinase inhibitors in human interphotoreceptor matrix and vitreous. *Curr Eye Res* 1998;17(2):132-140

3 De La Paz MA, Itoh Y, Toth CA, Nagase H. Matrix metalloproteinases and their inhibitors in human vitreous. *Invest Ophthalmol Vis Sci*1998;39(7):1256-1260

4 Steen B, Sejersen S, Berglin L, Seregard S, Kvanta A. Matrix metalloproteinases and metalloproteinase inhibitors in choroidal neovascular membranes. *Invest Ophthalmol Vis Sci*1998;39(11):2194-2200

5 Das A,McLamore A,Song W,McGuire PG.Retinal neovascularization is suppressed with a matrix metalloproteinase inhibitor. *Arch Ophthalmol*1999;117(4): 498-503

6 Leco KJ, Khokha R, Pavloff N, Hawkes SP, Edwards DR. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is an extracellular matrix-associated protein with a distinctive pattern of expression in mouse cells and tissues. *J Biol Chem*1994;269 (12):9352-9360

7 Fariss RN, Apte SS, Olsen BR, Iwata K, Milam AH. Tissue inhibitor of metalloproteinases-3 is a component of Bruch's membrane of the eye. *Am J Pathol*1997; 150(1):323-328

8 Takahashi T, Nakamura T, Hayashi A, Kamei M, Nakabayashi M, Okada AA, Tomita N, Kaneda Y, Tano Y. Inhibition of experimental choroidal neovascularization by overexpression of tissuse inhibitor of metalloproteinases-3 in retinal pigment epithelium cells. *Am J Ophthalmol*2000;130(6):774-781

9 Murata T, Cui J, Taba KE, Oh JY, Spee C, Hinton DR, Ryan SJ. The possibility of gene therapy for the treatment of choroidal neovascularization. *Am J Ophthalmol* 2000;107(7):1364-1373

10 Arbiser JL, Moses MA, Fernandez CA, Ghiso N, Cao Y, Klauber N, Frank D, Brownlee M, Flynn E, Parangi S, Byers HR, Folkman J. Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. *Proc Natl Acad Sci USA* 1997;94(3): 861-866

11 Määttä M, Tervahartiala T, Harju M, Airaksinen J, Autio-Harmainen H, Sorsa T. Matrix metalloproteinases and their tissue inhibitors in aqueous humor of patients with primary open-angle glaucoma, exfoliation syndrome, and exfoliation glaucoma. *J Glaucoma*2005;14(1):64-69

12 Rönkkö S, Rekonen P, Kaarniranta K, Puustjärvi T, Teräsvirta M, Uusitalo H. Matrix metalloproteinases and their inhibitors in the chamber angle of normal eyes and patients with primary open-angle glaucoma and exfoliation glaucoma. *Graefes Arch Clin Exp Ophthalmol*2007;245(5):697-704

13 Ikema K,Matsumoto K,Inomata Y,Komohara Y,Miyajima S,Takeya M,Tanihara H.Induction of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs correlates with outcome of acute experimental pseudomonal keratitis. *Exp Eye Res* 2006;83(6):1396-1404

14 Rohini G, Murugeswari P, Prajna NV, Lalitha P, Muthukkaruppan V. Matrix metalloproteinases (MMP-8, MMP-9) and the tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2) in patients with fungal keratitis. *Cornea* 2007;26(2):207-211

15 Webster L, Chignell AH, Limb GA. Predominance of MMP-1 and MMP-2 in epiretinal and subretinal membranes of proliferative vitreoretino-pathy. *Exp Eye Res*1999;68(1):91-98

16 Salzmann J, Limb GA, Khaw PT, Gregor ZJ, Webster L, Chignell AH, Charteris DG. Matrix metalloproteinases and their natural inhibitors in fibrovascular membranes of proliferative diabetic retinopathy. *Br J Ophthalmol* 2000;84 (10): 1091-1096

17 Majka S, McGuire P, Colombo S, Das A. The balance between proteinases and inhibitors in a murine model of proliferative retinopathy. *Invest Ophthalmol Vis Sci* 2001;42(1):210-215

18 Wang K,Wang KS,Wang L.A new model of choroidal neovascularization in mice. *Zhonghua Yan Ke Za Zhi*2002;38(12):750-752

19 Limb GA, Chignell AH, Green W, LeRoy F, Dumonde DC. Distribution of TNF α and its reactive vascular adhesion molecules in fibrovascular membranes of proliferative diabetic retinopathy. *Br J Ophthalmol*1996;80(2):168-173

20 Majka S, McGuire PG, Das A. Regulation of matrix metalloprotainase expression by TNF in a murine model of retinal neovascularization. *Invest Ophthalmol Vis Sci*2002;43(1):260-266