EXTENDED REPORT

Matrix metalloproteinases in human choroidal neovascular membranes excised following verteporfin photodynamic therapy

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Aim: To evaluate expression of proangiogenic matrix metalloproteinases (MMP) 2 and 9 at distinct intervals after verteporfin photodynamic therapy (PDT) in human choroidal neovascular membranes (CNV) secondary to age-related macular degeneration (AMD).

Methods: Retrospective review of an interventional case series of 49 patients who underwent removal of CNV. Twenty-six patients were treated with PDT 3 to 383 days prior to surgery. Twenty-three CNV without previous treatment were used as controls. CNV were stained for CD34, cytokeratin 18, endostatin, MMP-2 and MMP-9 by immunohistochemistry.

Results: CNV without previous therapy disclosed MMP-2, MMP-9 in RPE–Bruch's membrane, vessels and stroma in different intensities. Three days after PDT, MMP-9 expression was significantly weaker in stroma ($p=0.0019$). Endostatin was significantly reduced in vessels ($p<0.001$). At longer post-PDT intervals, a significant increase of MMP-9 in stroma ($p=0.037$) and of endostatin in RPE–Bruch's membrane ($p=0.02$), vessels ($p=0.005$) and stroma ($p<0.001$) were disclosed. No significant changes in MMP-2 expression were detected.

Conclusions: PDT induced an early, temporary decrease in MMP-9 and endostatin expression. At longer intervals, MMP-9 increase is possibly associated with the angiogenic process responsible for recurrence after PDT. MMP-9, however, acts as a double-edged sword by concomitant induction of endostatin, an endogenous inhibitor of angiogenesis.

Neovascular age-related macular degeneration (AMD) is the leading cause of visual loss in older people in the western world.¹ Verteporfin photodynamic therapy (PDT) (Visudyne, Ciba Vision Corp., Duluth, GA, USA) was found to be beneficial in clinical trials.^{2–3} However, a high recurrence rate following PDT outweighs its benefits.^{2–3} Recently, antiangiogenic agents have changed the philosophical approach for neovascular AMD treatment enabling visual improvement and less recurrence.

Neovascularisation is regulated finely by angiogenesis stimulators and inhibitors.⁴ Vascular endothelial growth factor (VEGF) is a major stimulator of choroidal neovascularisation (CNV).⁵ However, proteolytic activity is a prerequisite for neovascularisation and its progression through the intact Bruch's membrane in AMD. Among proteolytic enzymes, matrix metalloproteinases (MMP)-2 and MMP-9 are of particular interest since they specifically target collagen type IV⁶ which is a major component of the basement membranes of vessels, Bruch's membrane and the extracellular matrix (ECM) in CNV.⁷ Both MMP-2 and MMP-9 were elevated in isolated human Bruch's membrane–choroid complex with ageing⁸ and MMP-2 was increased in retina pigment epithelium (RPE)-associated interphotoreceptor matrix in AMD.⁹ Further studies revealed that MMP-2 and MMP-9 were upregulated in the early stages of CNV formation, and they synergise each other in CNV induction.¹⁰ Moreover, CNV development was reduced in MMP-2 or MMP-9 or MMP-2, 9 double-deficient mice.^{10–12}

MMPs are involved in different steps of angiogenesis. MMPs facilitate endothelial cell (EC) migration by releasing EC from their basement membranes, degrading perivascular ECM, and generating ECM degradation products that are chemotactic for EC. Additionally, MMPs increase bioavailability of VEGF and

other peptide growth factors through degradation of ECM proteins which sequester growth factors.¹⁰ Selective MMP inhibitors inhibiting experimental CNV¹³ were suggested as potential adjuvants to PDT.¹⁴ However, MMPs have a dual role, and also inhibit angiogenesis through generating and activating endogenous angiogenesis inhibitors such as endostatin.^{15–17}

Endostatin is a C-terminal fragment of collagen XVIII. Endostatin is bound to collagen XVIII by a protease-sensitive hinge. Some proteases, such as MMP-9, can cleave the hinge so that endostatin is released and active.^{16–19}

This clinicopathological study analysed the impact of verteporfin PDT on expression of MMP-2 and MMP-9 with regard to the time interval following PDT and a potential correlation with endostatin. Specimens from 26 patients treated with PDT were compared with 23 CNV without prior PDT.

METHODS

Subjects and treatments

We retrospectively reviewed 49 eyes of 49 consecutive patients with AMD, in which surgery for macular translocation was performed at 10 distinct surgical sites between 1997 and 2005. In 26 of these patients, macular translocation was performed after verteporfin PDT. The clinical characteristics of patients treated with PDT preoperatively are summarised in table 1.

Therapy options were discussed with patients. Each patient gave written informed consent after the experimental nature of

Abbreviations: AMD, age-related macular degeneration; CNV, choroidal neovascular membranes; EC, endothelial cell; ECM, extracellular matrix; MMP, matrix metalloproteinases; PDT, photodynamic therapy; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor

Table 1 Clinical characteristics of patients treated with verteporfin photodynamic therapy (PDT) before surgical removal of the choroidal neovascularisation membrane (CNV)

Case	Eye	Age/sex	CNV type	Number of PDT	Time to surgery from the first PDT/ last PDT
1	L	76/m	Classic	1	3 days
2	R	78/f	Classic	1	3 days
3	L	54/m	Pred. classic	2	113/3 days
4	L	84/m	Classic	1	3 days
5	R	74/f	Occult	1	21 days
6	L	83/m	Classic	1	34 days
7	L	85/f	Classic	1	37 days
8	R	73/f	Occult	3	208/138/40 days
9	L	78/f	Pred. classic	2	3 months/54 days
10	L	79/m	Classic	1	55 days
11	R	80/f	Classic	2	172/69 days
12	L	66/f	Occult	1	83 days
13	L	77/m	Min. classic	1	84 days
14	R	79/f	Pred. classic	1	88 days
15	L	70/m	Pred. classic	1	92 days
16	R	93/m	Classic	2	95 days
17	L	87/m	Pred. classic	1	108 days
18	R	71/m	Classic	1	112 days
19	L	81/m	Classic	2	213/131 days
20	R	70/f	Classic	2	151/132 days
21	L	78/f	Classic	3	344/222/146 days
22	L	77/m	Classic	3	329/245/147 days
23	R	79/f	Pred. classic	1	171 days
24	R	74/f	Haemorrhagic	1	246 days
25	L	81/f	Classic	6	824/300 days
26	L	73/f	Classic	4	677/558/467/383 days

L, left; R, right; m, male; f, female; min., minimally; pred., predominantly.

the treatment procedure and the risks and benefits of all treatment alternatives were discussed in details. The study followed the guidelines of the Declaration of Helsinki as revised in Tokyo and Venice. Study and histological analysis of specimens were approved by the local institutional review board.

Tissue preparation

Excised CNV were fixed in 3.7% formalin and embedded in paraffin. Each section was mounted on poly-L-lysine coated glass slides (Dako, Glostrup, Denmark) for immunohistochemical staining.

Immunohistology

Serial paraffin sections were de-paraffinised and rehydrated with a graded series of alcohol. Antigen retrieval was accomplished by proteolytic digestion with 0.5% protease XXIV (Sigma, St Louis, MO, USA) for cytokeratin 18 and endostatin immunostaining, and by heat treatment in citrate buffer (0.01 M, pH: 6.0) for CD34, MMP-2 and MMP-9 immunohistology.

Immunohistochemical staining with the primary antibodies specific for human CD34 (mouse, Mab, Immunotech, Hamburg, Germany), cytokeratin 18 (mouse, Mab, Progen, Heidelberg, Germany), MMP-2 and MMP-9 (mouse, Mab, Oncogene, San Diego, CA, USA) was performed using horse-radish peroxidase as previously described.²⁰ For MMP-2 and MMP-9 stainings, AEC high-sensitive substrate chromogen (Cytomation, Code K3461, Dako) was used. Hematoxylin (Chemmate, Code S2020, Dako) was used for counterstaining.

Immunohistochemical staining for endostatin was performed by the alkaline-phosphatase method as previously described²⁰ using an anti-human endostatin antibody (rabbit, polyclonal, Dianova GmbH, Hamburg, Germany).

For negative controls, the primary antibodies were substituted either by appropriate normal sera or omitted.

Analysis

Serial sections from a specimen were analysed independently by two masked observers (OT, SG) by light microscopy.

EC and RPE were labelled with CD34 and cytokeratin 18, respectively.^{21, 22}

Immunoreactivity for MMP-2, MMP-9 and endostatin were analysed separately in RPE-Bruch's membrane complex, vessels and stroma. A grading scheme indicating the degree of staining was used. 3, 2, 1, 0 were assigned to indicate intense (70–100% positive cells), moderate (40–69% positive cells), weak labelling (1–39% positive cells) and absence (0%) of any staining, respectively.

Intensity of MMP-2, MMP-9 and endostatin immunostainings of defined subgroups described in mean $\pm$ standard error ($M \pm SE$) were comparatively analysed with the Student's *t* test; $p < 0.05$ was considered significant.

Figure 1 Graphs showing the intensity of MMP-2 (A), MMP-9 (B) and endostatin (C) immunostaining in CNV without PDT, CNV extracted 3 days or more than 20 days after PDT. MMP-2, MMP-9 and endostatin immunostaining in RPE-Bruch's membrane, vessels and stromal cells were evaluated separately and semiquantitatively as intense (70–100% positive cells), moderate (40–69% positive cells), mild (1–39% positive cells) or absent.

RESULTS

The frequency of MMP-2, MMP-9 and endostatin immunostaining intensity in CNV are summarised in fig 1.

Immunohistopathologic findings in CNV without prior PDT

RPE were stained for MMP-2 and MMP-9 in all specimens. RPE displayed mostly strong ($n = 16$, 69.6%) to moderate ($n = 6$, 26.1%) MMP-2 immunostaining (fig 1A, 2A). MMP-9 staining intensity in RPE was either strong ($n = 19$, 82.6%) or moderate ($n = 4$, 17.4%) (fig 1B, 2B). RPE disclosed endostatin in 60.9% ($n = 14$) of specimens in weak ($n = 4$, 17.3%) to strong ($n = 5$, 26.3%) intensities (fig 1C, 2C).

All but one membrane were vascularised as evidenced by CD34 immunoreactivity.

Figure 2 Photomicrographs of a surgically excised CNV without previous PDT. The specimens were probed with antibodies against MMP-2 (A), MMP-9 (B) and endostatin (C) and stained with AEC (A, B) or red chromogen (C). Hematoxylin was used as counterstain. (A-C) MMP-2, MMP-9 and endostatin are expressed in RPE-Bruch's membrane complex (asterisk) and stroma (small arrow head). (B, C) Strong MMP-9 and endostatin expression in vascular structures are indicated by the arrow. (C) Endostatin negative RPE-Bruch's membrane complex is pointed out with a big arrow head. Scale bar: 50 μ m.

EC were immunonegative for MMP-2 in 59.1% (13 of 22) of membranes. In contrast, MMP-9 was not detected in EC only in 4.5% (1 of 22) of specimens. Inversely, strong MMP-2 and MMP-9 in EC was detected in 9.1% ($n = 2$) and 90.9% ($n = 20$) of samples, respectively (fig 1A,B, 2A,B). EC displayed endostatin in 86.4% ($n = 19$) and intensely in 63.6% (14 of 22) of CNV (fig 1C, 2C).

Cells within stroma expressed MMP-2 in 52.2% ($n = 12$) of specimens, being intense in only two specimens (fig 1A, 2A). In all but one CNV, stromal cells expressed MMP-9 and intensely in 78.3% ($n = 18$) of samples (fig 1B, 2B). Endostatin was present in the stroma in 73.9% (17 of 23) of specimens (fig 1C, 2C).

Angiographic and histological characterisation of the CNV treated by PDT

Angiographic and histological features in CNV differed depending on post-PDT time interval.

Figure 3 Photomicrographs of a CNV membrane (case 4, table 1), extracted 3 days after PDT. The serial sections were probed with antibodies to MMP-2 (A), MMP-9 (B) and endostatin (C). (A, B) Retina pigment epithelium (asterisk) is strongly positive for MMP-2 and MMP-9. (C) Endostatin immunoreactivity is absent in CNV. Scale bar: 50 μ m.

a) 3 days after PDT

In membranes extracted three days after PDT ($n=4$), a hypofluorescence suggesting non-perfusion of the irradiated area and CNV was seen in the early phases of angiography on the surgery day. Late phases of FA revealed hyperfluorescence and leakage at the fovea consistent with choroidal ischaemia (data not shown).

In these four membranes, RPE displayed an intense staining for MMP-2 in three specimens and for MMP-9 in all (fig 1A,B, 3A,B). Endostatin was found in the RPE-Bruch's membrane complex of only two membranes (fig 1C, 3C).

Most vessels were occluded and EC were severely damaged. EC displayed no MMP-2 but MMP-9 immunoreactivity in two samples. Stromal cells disclosed MMP-2 in one specimen weakly. Moderate-to-strong MMP-9 expression in the stroma was present in two samples (fig 1A,B, 3A,B). MMP-9 expression was significantly weaker in the stroma of these CNV ($M \pm SE: 1.00 \pm 0.70$) in comparison with CNV without previous therapy ($M \pm SE: 2.36 \pm 0.16$) ($p=0.0019$). None of these four specimens displayed endostatin either in vessels or stroma (fig 1C, 3C). Endostatin in vessels ($M \pm SE: 0 \pm 0$) was significantly weaker than in control CNV ($M \pm SE: 2.27 \pm 0.23$) ($p<0.001$).

b) Post-PDT intervals longer than 3 days

In CNV extracted at longer post-PDT intervals, FA disclosed hyperfluorescent membranes with leakage in late phases (data not shown).

CD34-positive vessels were detected in all but one membrane. Vessels were all patent and lined with EC displaying prominent nuclei. EC were immunopositive for MMP-2 in 42.9% (9 of 21) of specimens (fig 1A, 4A,B). EC expressed MMP-9 in 16 cases (76.2%), mostly intensely (15 of 21, 71.4%; fig 1B, 4C,D). Vessels displayed endostatin in 76.2% (16 of 21) of samples and in moderate-to-strong intensity in 71.2% (15 of 21) of them (fig 1C, 4E,F). Endostatin in vessels of these CNV ($M \pm SE: 2.00 \pm 0.28$) was significantly higher than in CNV extracted 3 days after PDT ($M \pm SE: 0 \pm 0$) ($p=0.005$).

All CNV disclosed MMP-2 immunoreactivity in RPE. MMP-2 was strong (10 of 22) to moderate (4 of 22) in RPE of 63.6% of specimens (fig 1A, 4B). All but one CNV displayed MMP-9 in RPE either intensely (86.4%, 19 of 22) or moderately (9.1%, 2 of 22) (fig 1B, 4D). Endostatin in the RPE-Bruch's membrane complex of 95.4% of samples ($M \pm SE: 2.00 \pm 0.19$) was significantly increased in comparison to CNV excised 3 days after PDT ($M \pm SE: 0.75 \pm 0.47$) ($p=0.02$) (fig 1C, 4F).

Figure 4 Photomicrographs of CNV membranes extracted 40, 55 and 383 days after PDT from case 8 (A, C, E), case 10 (B, D) and case 28 (F) in table 1. The sections were probed with antibodies against MMP-2 (A, B), MMP-9 (C, D) and endostatin (E, F). (A-E) Strong MMP-2, MMP-9 and endostatin expression was detected in vessels (arrows), stroma (small arrow head) and RPE cells (asterisk). MMP-2 immunonegative RPE cells are shown by the big arrow head (B). Strong endostatin expression in RPE-Bruch's membrane complex is shown by asterisk (F). Scale bar: 50 μ m.

Figure 5 The impact of verteporfin photodynamic therapy on angiogenesis in CNV. (1) 3 days after PDT, most of the vessels are occluded and endothelial cells are damaged and apoptotic but retina pigment epithelium (RPE) cells are not affected. (2) Vascular endothelial growth factor (VEGF) is significantly enhanced in RPE and VEGF predominates over angiogenesis inhibitors, pigment epithelium derived factor (PEDF) and endostatin in milieu. In addition to VEGF, strong MMP-2 and MMP-9 expression in RPE probably contributes to the re-induction of the angiogenesis cascade. (3) Corresponding to the activation and proliferation phase of angiogenesis, endothelial cells lining the patent vessels are healthy in inflammatory active CNV with high proliferative activity. Activated endothelial cells and infiltrating inflammatory cells express VEGF and proteases such as MMP-9. (4) Despite the direct angiogenesis stimulating effect of MMPs, MMP-9 cleaves endostatin from the collagen XVIII-endostatin complex in the basement membranes of vessels and Bruch's membrane and release it active. (5) Endostatin and PEDF inhibit proangiogenic factors such as MMP-2 and VEGF in the involution phase of CNV.

Stromal cells displayed MMP-2 and MMP-9 in 54.5% and in 86.4% of CNV, respectively (fig 1a,b, 4A–D). MMP-9 expression in stroma ($M \pm SE: 2.68 \pm 0.17$) was significantly increased in comparison with CNV excised three days after PDT ($M \pm SE: 1.0 \pm 0.7$) ($p=0.03$). Endostatin was absent only in two samples (9.1%) (fig 1c, 4E,F). Endostatin ($M \pm SE: 2.18 \pm 0.23$) was also significantly higher in stroma than in CNV excised three days after PDT ($M \pm SE: 0 \pm 0$) ($p<0.001$).

DISCUSSION

Photodynamic therapy is based on EC damage leading to vascular occlusion.^{23–24} However, enhanced VEGF expression²⁵ and VEGF predominance over endogenous angiogenesis inhibitors, namely pigment epithelium-derived factor (PEDF)²⁶ and endostatin,²⁷ early after PDT, possibly restarts the angiogenesis cascade. A further insight into angiogenic mechanisms involved in this rebound effect is essential to optimise the actual protocols and to establish new treatment strategies. Herein, we investigated involvement of proteolytic enzymes MMP-2 and MMP-9 which interact with the above-mentioned angiogenesis factors.

We first examined CNV membranes that were not treated with PDT. We have detected MMP-2 and MMP-9 expression in all specimens in concordance with previous reports.^{28–29} Steen *et al.* evaluated MMP-9 and MMP-2 mRNA expression in five human CNV by in situ hybridisation. MMP-2 mRNA expression was found mainly in EC but also in RPE to some extent, although MMP-9 was distinctly expressed adjacent to Bruch's membrane and RPE.²⁹ However, in our series of 23 CNV, MMP-2 was detected in RPE in all specimens and intensely in nearly 70% of them. MMP-9 expression was observed in RPE, EC and stromal cells in almost all and intensely in nearly 80% of membranes. The difference between these two studies might be due to differences in maturity, stage of angiogenesis, inflammatory activity and number of the specimens and methods of evaluation. Nevertheless, the same authors have reported MMP-2 expression in RPE and in infiltrating macrophages in experimental CNV.³⁰

In membranes extracted 3 days after PDT, RPE disclosed intense MMP-2 and MMP-9 immunoreactivity. MMPs were

reduced both in EC and in stroma; however, only MMP-9 was found to be significantly reduced in stroma compared with CNV without previous therapy. In experimental studies, infiltrating inflammatory cells were found to be major sources of MMP-9 in CNV lesions.¹² However, the density of macrophages and leucocytes was significantly decreased in these membranes excised 3 days after PDT.³¹ Furthermore, hypoxia, possibly caused by PDT through non-perfusion of choroid and CNV,^{32–34} suppresses the production and secretion of MMP-9 by monocytes.^{35–36} Therefore, significantly decreased density of inflammatory cells as well as hypoxia may contribute to a significant decrease in MMP-9 expression in these specimens. Since MMP-9 is involved in the release of endostatin,¹⁹ a significant and concomitant reduction of MMP-9 and endostatin in these specimens may be based on this interaction.

In contrast, CNV extracted at longer intervals after PDT, disclosed a significant increase in stromal expression of MMP-9 together with endostatin. Enhanced MMP-9 expression is probably due to an increase in inflammatory infiltration, activation of EC secreting proteolytic enzymes and a further increase in VEGF expression.^{17–18, 25, 31, 37–39} A concomitantly enhanced endostatin stabilises the angiogenic process^{4–15, 27–40} which otherwise would result in an uncontrolled neovascularisation. Endostatin binds to the catalytic domain of MMP-2 and blocks its activity.^{41–42} Whether enhanced expression of endostatin prevented a significant change of MMP-2 in our specimens needs to be investigated further.

MMP-2 and MMP-9 are involved in microvessel formation during early phases of angiogenesis, but also in the reabsorption of neovascularisation, involution and regression of vessels in later stages.^{43–47} MMPs play a role in vascular regression since proteolytic activity is needed for EC of the regressing vessels to detach from the ECM and for the degradation of the microvascular network and ECM scaffold around EC.^{43–48} Therefore, the dual role of MMPs in angiogenesis makes the timing of application of MMP inhibitors critical. MMP inhibitors, batimastat and marimastat, inhibited microvessel formation when applied in the beginning of angiogenesis, but stabilised microvessels and prevented vascular regression when applied after the angiogenic growth phase.⁴³ Likewise, a

selective MMP-2, MMP-9, MMP-3 and MT-MMP1 inhibitor prevented the development and growth of CNV in the early phases of angiogenesis, but was ineffective for established CNV. Hence, MMP inhibitors were suggested to be used as an adjuvant to PDT to decrease recurrence rather than as monotherapy for neovascular AMD.¹⁴ However, a similar dual role of MMPs is to be expected in the vascularisation and involution stages of CNV. Furthermore, inhibition of MMPs in longer intervals after PDT may additionally inhibit the activation of endostatin which inhibits experimental CNV^{49–51} and possibly contributes to CNV involution.

We are unaware of a previous clinicopathological correlation of the expression of MMP-2, MMP-9 and endostatin in human CNV membranes treated by PDT. Proper interpretation of this study is limited by a potential negative case selection. In summary, our recent and previous works^{20 24–27 31} reveal that EC are selectively damaged by PDT. In contrast, non-damaged RPE express VEGF intensely that predominates over PEDF and endostatin soon after PDT. At longer intervals following PDT, VEGF and MMP-9 expression increases both in the EC and stroma. This correlates with enhanced proliferation activity and activation of EC as well as with increased inflammatory infiltration. In longer intervals, however, enhanced endostatin possibly contributes to inhibition of the ongoing angiogenesis cascade. This sequence (fig 5) may be extrapolated to the pathological process of CNV formation and stresses the need for an antiangiogenic adjunctive therapy. Still, involvement of MMP-9 in endostatin activation and in vascular regression needs to be seriously considered in the strategy and timing of MMP inhibition.

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